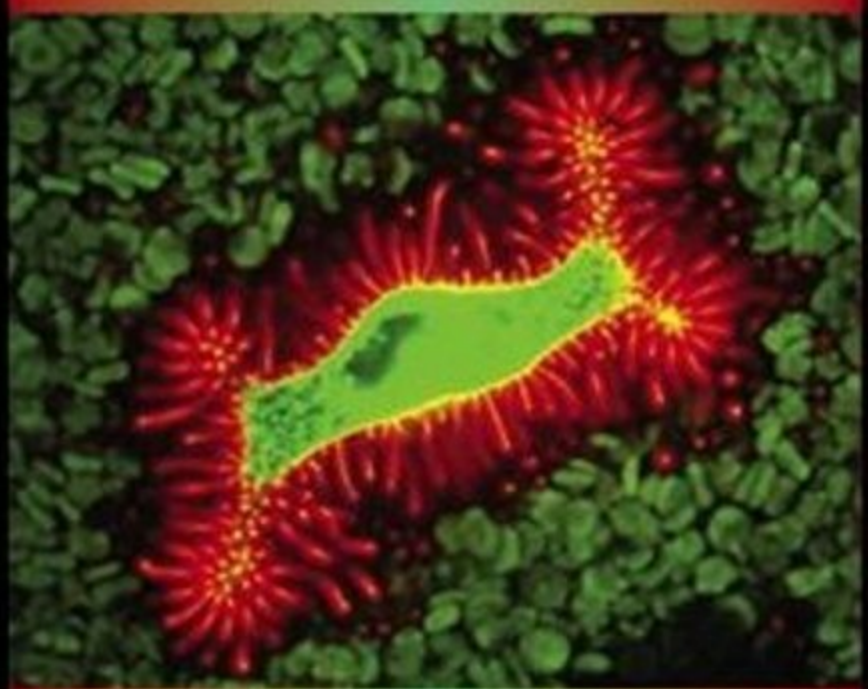
Hyaluronan in Cancer Biology



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To the memory of my father and my mother, and for Tali, Aaron & David

Preface

This volume is devoted entirely to the subject of hyaluronan (HA) and cancer. The importance of HA in malignancy is very well documented. However, the subject has been largely neglected. This volume attempts to bring attention to the critical role of HA in cancer biology, in its initiation, progression, and spread. An excellent monograph on cancer, published in 2007 (R.A. Weinberg, The Biology of Cancer, Garland Science, New York) does not cite HA a single time in the index. Reference to the HA receptor, CD44 is given once. However, that citation does not mention that CD44 is the receptor for HA.

The current volume attempts to redress such oversights, to draw attention to this important molecule to colleagues in the cancer field, and to provide students that are entering the area of HA cancer biology with a comprehensive overview.

Hyaluronan is a ubiquitous high molecular size unbranched carbohydrate polymer that is prominent in vertebrate extracellular matrix during embryogenesis, inflammation, in wound healing, whenever there is rapid tissue turnover and repair, but particularly, in neoplasia. Although HA is a simple disaccharide that repeats thousands of times, reaching a molecular mass of several million Daltons, it has a remarkable array of biological functions. This is unusual because among all the glycosaminoglycans, it is the only one that is not sulfated nor modified in any other way throughout its length.

Preparations of HA are well known in the commercial world. It is the filler used by ophthalmologists following cataract surgery, known as Healon.® Lightly cross-linked forms of HA are used as cosmetic fillers, as in Restylane,® and as a visco-supplement for synovial fluid, used by orthopedic surgeons, known as Synvisc.® It can be found in many cosmetic preparations, as a ''feel-good'' for facial creams and ointments.

Proteins associated with HA metabolism are also finding increasing commercial use. Hyaluronidases, the enzymes that degrade HA, are used to enhance sperm penetration in the process of *in vitro* fertilization (Cumulase®), as an aid in dispersing *i.v.* solutions that have accumulated subcutaneously, as in the newborn nursery, and when caustic chemotherapy agents accumulate in local tissues. They are used for enhancing

drug absorption of small and large molecules, as Hylenex® and Enhanze® respectively.

A stabilized pegylated (polyethylene glycol cross-linked) version of a hyaluronidase is now in early clinical trials, as an adjunct in cancer chemotherapy, acting to promoting drug uptake and penetration. However, as HA and its associated molecules achieve increasing commercial visibility, it remains an obscure entity in the life sciences.

The cancer community is beginning to realize the importance of HA, now that its involvement has been documented in stem cells, the stem cell niche, and particularly its involvement in cancer stem cells. It makes this volume all the more germane for enhancing our understanding of HA in the malignant process, and for highlighting how it functions as a critical tool for cancer research.

A historic overview is given in Section I (Stern, Israel/Palestine), tracing the history of HA through its several metamorphoses and baptisms, from ground substance, to acid mucopolysaccharide, to hyaluronic acid, and to hyaluronan, presumably its final incarnation.

A general context is then provided for this interesting molecule in Section II, by Toole and Slomiany (USA). Heldin and her co-workers (Sweden) outline the growth factors that modulate HA deposition, while Datta (India) describes the role of an HA-binding protein in cancer biology.

There are several receptors for HA. An overview of this area is provided in Section III. The predominant receptor for HA, CD44, is one of the most complex molecules in all biology. It has a variety of isoforms, derived from combinations of ten alternatively spliced exons. Vast numbers of post-translational modifications of CD44 increase dramatically the multiple forms in which the receptor occurs. Four chapters, by Stamenkovic (Switzerland) and Yu (USA), another by Bourguignon (USA), by Naor and his colleagues (Israel), and Waugh and colleagues (U.K.) provide overviews of CD44 in its multiple guises, and their involvement in cancer biology. Another HA receptor, Rhamm (Hmmr) interacts with CD44, and modulates its malignant involvement, as shown by McCarthy (USA) and Turley (Canada).

Hyaluronan has an extremely rapid rate of turnover, providing controls at multiple levels for its net deposition. The several enzymes that synthesize HA are the HA synthases, or HASs, that sequentially add sugars to the reducing termini. These are described by two pioneers in the field, Kimata and Itano (Japan) in Section IV.

The hyaluronidase enzymes are the endoglycosidases that degrade HA, described in Section V. Known as the HYALs, they have been controversial, since both increases and decreases in enzyme levels are associated with cancer progression. Two chapters summarize that area, by Lokeshwar and Selzer (USA), and another by Stern (Israel/Palestine). The HA polymer takes on different biological properties as it becomes cleaved, as outlined by Sugahara (Japan and USA). The hyaluronidases are presumably

involved in size-specific cleavage reactions. Binding proteins and hyaluronidase inhibitors are presumed to be involved in generating fragments, as well as maintaining polymers at a particular fragment length. But how this occurs is entirely unknown.

The stroma surrounding malignancies is highly abnormal, and is the subject of Section VI. Hyaluronan is intimately involved in the cross-talk between cancers and the host peritumor stromal. The scirrhous reaction or desmoplasia of carcinomas has long been recognized by Pathologists. The extent of that reaction, and the prominence of HA in the reaction are often utilized clinically as prognostic indicators. However, as outlined by the group headed by Raija and Markku Tammi (Finland), the dynamic reciprocity between cancers and stroma, and the role of HA therein have many subtleties. The laboratory of Seth and Ana Schor (U.K.), another husband and wife team, was among the first to document the striking similarity between fetal fibroblasts and peritumor fibroblasts. This is an association that parallels the biology of oncofetal proteins.

In Section VII, focus is placed on site-specific cancers, on prostate, malignant melanoma, and breast cancers. These are summarized by three leading laboratories in their fields, respectively, headed by Simpson (USA), Simon (Germany), and Brown (Australia).

Translational research is now being emphasized by granting agencies. The importance of HA-related molecules has finally begun to be realized in the clinic, as outlined in Section VIII. A historic overview is provided by Baumgartner and Hamilton (Austria). In Europe, hyaluronidase has a history of being used in combination chemotherapy regimens, something that has, until recently, not been permitted in the United States. Certain aggressive lymphoblastic lymphomas, resistant to chemotherapies, became sensitive when hyaluronidase was included in the protocol. Dr. Baumgartner was the first oncologist to incorporate that strategy into cancer treatment. Platt and Szoka (USA) explore various strategies for targeting cancer chemotherapies using the high-affinity binding of HA to its CD44 receptor.

And finally, in Section IX, as in much of cancer research, we have learned to expect the unexpected, an entirely unanticipated dimension has appeared. One of the hyaluronidases, HYAL-2, is a cell surface receptor for a class of animal tumor viruses, as described by Miller (USA).

Hyaluronan does not give up its secrets easily. But recent rapid progress makes this volume all the more timely. Within a few years, I predict it will not be possible to summarize the field again within a single volume.

Robert Stern
Jerusalem, Palestinian Territory
August, 2008

Foreword

Karl Meyer described a polysaccharide in the vitreous body of the eye in 1934 and gave it the name hyaluronic acid (now hyaluronan). He and his collaborators subsequently showed its presence in many other tissues, and determined its chemical structure as a linear chain of alternating units of glucuronic acid and N-acetylglucosamine linked by β(1-3) and β(1-4) linkages. Around 1950, Alexander G. Ogston and his collaborators in Oxford characterized a hyaluronan–protein complex from synovial fluid, found that it had a molecular weight in the order of millions, and extended over a large volume. Duran-Reynals described the so called ''spreading factor'' in 1928, which turned out to be hyaluronidase.

This was the background that existed when I started to work on hyaluronan in 1949 under the tutorship of Endre A. Balazs. It was at that time commonly believed that hyaluronan was an inert filling material between cells without any specific biological activities. Much work on the polymer during the 1950s and 1960s was therefore directed towards understanding the macromolecular properties of the compound and their importance for the physical state of the cell environment. However, it is notable that by about 1950, Endre Balazs had already begun studying the effects of hyaluronan on cell growth in tissue culture. Notably, he together with my classmate in medical school, Jan von Euler, investigated the connection between hyaluronan and cancer.

A breakthrough in hyaluronan research came in 1972 when Hardingham and Muir found that cartilage proteoglycans specifically bind to hyaluronan. Subsequently a number of extracellular proteins and cell surface receptors have been discovered that interact with the polymer. Suddenly hyaluronan was found to directly and specifically regulate many cellular functions.

The development of the hyaluronan field has accelerated in the last few decades. It is apparent that hyaluronan plays an important role in such fields as mitosis, embryological development, cellular motility, pathological reactions such as inflammation and many other basic functions. Of special interest in recent years has been the discovery of specific biological effects of different size fragments of hyaluronan. The number of researchers working in the field has increased rapidly, and international

conferences on the specific subject of hyaluronan are now held every third year.

In parallel with the discoveries of the basic functions of hyaluronan, the substance has become a tool in clinical medicine, much of that due to Endre Balazs. It is used for example in eye surgery, in treatment of arthrosis, and as a space filler in tissues. It is also used as a moisturizer in skin creams and has become a commercial success.

Robert Stern has been a leading scientist in the hyaluronan field in the last decades during its period of very rapid development, and I have admired his work. He has now edited a volume on hyaluronan that focuses entirely on cancer biology, in order to make researchers in the cancer field aware of the importance of this unique polymer. I sincerely hope that this will become a successful endeavor. I also wish that, had I been younger, I could have helped him in this important task.

Torvard Laurent
Uppsala, Sweden
October, 2008

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CHAPTER

1

Association Between Cancer and "Acid Mucopolysaccharides": An Old Concept Comes of Age, Finally

Robert Stern

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INTRODUCTION

The influence of hyaluronan (HA) on cancer progression has been exceedingly well described (Toole, 2002; Toole et al., 2002; Toole and Hascall, 2002; Stern, 2005). However, recognition of this important phenomenon has lagged, and inexplicably, continues to be neglected by most cancer biologists. Knowledge in this area has advanced extremely rapidly, and has taken on additional significance, now that it is documented that the major receptor for HA, CD44, is expressed on the surface of virtually all stem cells, including cancer stem cells (e.g., Al Hajj et al., 2003). This volume aims to bring attention to the field of HA and its role in cancer initiation, progression, and spread.

Assembly of these reviews is now particularly timely. It is the first volume ever to appear dedicated entirely to the role of HA in cancer biology. A recent textbook on basic oncology, widely recognized to be of superior quality, does not have a single citation in the index for HA (Weinberg, 2006). CD44 is given one citation, without mentioning that it is the predominant receptor for HA. Ironically, even the Weinberg laboratory has since then become aware of the significance of HA and CD44 in cancer progression (Godar et al., 2008).

Our purpose here is to draw attention to a critical molecule that that has been neglected, and up until now, poorly understood by most cancer scientists. The time has come, finally, to bring HA, previously known as hyaluronic acid (Balazs et al., 1986), and before that, as simply an acid mucopolysaccharide, to the attention of a wider audience.

HYALURONAN

Historical Perspective

The term "ground substance" was first applied to the amorphous material between cells by the German anatomist, Henle, in 1841 (Henle, 1841). It is a mistranslation of the German "Grundsubstanz," which would be better translated as "basic," "fundamental," or "primordial" substance. By 1852, sufficient information had accrued for the inclusion of "Grundsubstanz" in a textbook of human histology (Koellicker, 1852).

The modern era of ground substance research began in 1928 with the discovery of a "spreading factor" by Francisco Duran-Reynals. Testicular extracts stimulated rapid spread of materials injected subcutaneously on the backs of shaved rabbits, while simultaneously causing dissolution of the ground substance (Duran-Reynals, 1928; 1929; Duran-Reynals and Suner Pi, 1929; Duran-Reynals and Stewart, 1933). The active principal of these extracts was later shown to be the enzyme, hyaluronidase (Chain

and Duthrie, 1940; Hobby et al., 1941), the class of enzymes that degrade HA. Interestingly, in one of the studies by Duran-Reynals, hyaluronidase-like activity was demonstrated in extracts of human malignancies, particularly from breast cancers and malignant melanoma (Duran-Reynals et al., 1929).

"Ground substance" was subsequently renamed "acid mucopolysaccharides," a term first proposed by Karl Meyer (1938), who first described HA (Meyer and Palmer, 1934; 1936). This was the term to designate the hexosamine-containing sugar polymers that occurred in animal tissues alone, as well as when bound to proteins. Chondroitin sulfate is the major GAG of the matrix of such tissues as cartilage, tendon, and scar. However, it is now well established that HA is by far the predominant "acid mucopolysaccharide" that constitutes true "ground substance," though heparan sulfate is the most abundant GAG at the cell surface.

Overview

Hyaluronan is a high-molar-mass linear glycosaminoglycan (GAG) found intracellularly, on the surface of cells, but predominantly in the extracellular matrix (ECM) between cells. This linear polysaccharide can reach a size of 6 to 8 MDa. It is a ubiquitous polymer with the repeating disaccharide structure of (-β1,3-*N*-acetyl-D-glucosamine-β1,4-D-glucuronic acid-)$_n$. It has one carboxyl group per disaccharide repeating unit, and is therefore a polyelectrolyte with a negative charge at neutral pH. It is near perfect in chemical repeats, with no known deviations in its simple disaccharide structure with the possible exception of occasional deacetylated glucosamine residues.

Hyaluronan, at low concentrations, is ubiquitous. However, it is found in high concentrations during embryogenesis, and whenever rapid tissue turnover and repair are occurring. It occurs in particularly high concentrations in fetal tissues, in amniotic fluid, is the major constituent of fetal structures such as Wharton's jelly of the umbilical cord, but also in malignancies. Over 50% of total body HA occurs in the skin (Reed et al., 1988).

At the cellular level, a burst of HA synthesis occurs just prior to mitosis, enabling some cells to become dissociated from neighboring cells and to lose the adhesion from their surrounding ECM in preparation for division (Toole et al., 1972; Tomida et al., 1974; Mian, 1986; Brecht et al., 1986). It is during this short period within the cell cycle that normal cells most closely resemble transformed cells. The deposition of HA preceding mitosis promotes detachment, and also confers motility directly upon cells (Turley and Torrance, 1984; Turley et al., 1985), correlating possibly with the movement of metastatic tumor cells.

Cancer cells do not do unusual things, but do usual things at unusual times. The formulation can be posited that cancer cells emulate that point in the cell cycle when cells synthesize increased levels of HA, round up, detach from their substratum, and leave temporarily the social contract in order to divide. Normal cells then degrade that HA in order to reattach to the substratum and to carry on the business of being normal tissue components. Cancer cells have learned to eliminate this step, to retain their HA coat, enabling them instead, to continue to divide endlessly (Itano et al., 2002).

Hyaluronan Can Influence Cell Fate: Studies from Embryology

Classical studies in embryogenesis document that HA is ubiquitous in developmental processes and in tissue modeling. Hyaluronan is particularly prevalent when undifferentiated cells are proliferating rapidly and move from their stem cell niche to the site of organ development. This stage of cell proliferation and movement ends when cells commit to a program of differentiation. In fact, the HA environment actively inhibits differentiation, creating instead an environment that promotes proliferation (Ozzello et al., 1960). Cells must lose their HA-rich environment in order for that commitment to differentiation to occur (Toole, 1991). Such a series of events were demonstrated for limb development, as well as cornea, the neural tube, cartilage and muscle development, and branching morphogenesis of parenchymal organs (Bernfield and Banerjee, 1972; Gakunga et al., 1997). Neuroectoderm pinches off to become neural crest elements, which then wander through the vertebrate body in an HA-rich environment. Such movement ceases just as HA becomes degraded (Pratt et al., 1975).

Again, parallels can be drawn between this window of normal tissue development and the onset of tumor growth, when cancer cells move and proliferate. Normal proliferating cells shed their HA through hyaluronidase activity. In most cases, it may be the failure to remove the HA coat, or the continuous turnover and replacement that promotes, malignant cell growth and the development of cancer.

Early studies of the influence of an HA environment on cell fate were from the laboratory of Arnold Caplan (Kujawa et al., 1986b). Primitive myoblasts derived from chick embryo skeletal muscle plated on plastic will proliferate, fuse to form a syncytium, and will begin to synthesize actin and myosin, and even begin to have contractile activity. However, the same cells grown on an HA-covered dish will grow and proliferate, but will not fuse, will not express skeletal muscle actin or myosin, nor show contractile behavior.

An effect chain length was demonstrated for this phenomenon (Kujawa et al., 1986a), one of the first demonstration of size dependency for HA

polymers. Similar results have been shown for chondrogenesis; addition of small amounts of HA inhibit formation of cartilage nodules (Toole et al., 1972).

Cancer Is a Price Paid for Metazoan Evolution

Hyaluronan synthesis occurred relatively late in metazoan evolution. The primitive nematode *C. elegans* contains chondroitin, and no HA (Yamada et al., 1999; Toyoda et al., 2000; Hwang et al., 2003). It can be postulated that HA developed late in evolution, at a time when stem cells had to move from their original niche, and travel some distance to another body site for growth, proliferation, and differentiation. It is precisely this fragment of metazoan biology that may have been commandeered by malignant cells. Emergence of HA may parallel the step in evolution when malignancies first arose.

The difference between chondroitin and HA is the epimerization of one 4-hydroxyl group, resulting in an *N*-acetylglucosamine from the original *N*-acetylgalactosamine. The galactose moiety is widely utilized in immune recognition in higher organisms. The axial hydroxyl group when it is epimerized becomes covert and unavailable for recognition. The HA chain is thus able to avoid the primitive immune-like surveillance system, enabling stem cells to move through the metazoan organism without recognition. This may explain how HA became a ''stealth'' molecule (Lee and Spicer, 2000), and why it was necessary in evolution.

STROMAL–EPITHELIAL INTERACTION IN CANCER

Extracellular Matrix of Normal Cells

The ECM is a heterogeneous mixture of proteins and proteoglycans that surrounds and separates cells, supports their structure and their organization in tissues. It contains myriads of smaller molecules including growth factors, adhesion molecules and a host of other small moieties, and controls their presentation to cells. Hyaluronan and other GAGs, most of which are covalently bound to proteins, influence the behavior of malignant cells by virtue of their expansive configuration, regulate basic processes such as proliferation, recognition, modulation of adhesion, and cell–cell communication. In addition to being supportive structures, they create links intracellular and extracellular environment, are involved in transduction of key intracellular biological signals. They act as receptors, co-receptors, and catalyze profound changes that lead to the malignant phenotype.

The Stroma Around Tumors Is Highly Abnormal, but Tends to Resemble Embryonic Mesenchyme

The nature of the tumors' abilities to commandeer stromal cells to their own agenda is just now beginning to be understood. An association between the stromal elements surrounding malignant tumors, and unusual histochemical features has been noted for over a century (Kuru, 1909). These include a marked increase in the deposition of "acid mucopolysaccharides," and a hyaluronidase-sensitive metachromasia. Such observations have been routinely made by surgical pathologists over the decades during examination of cancerous tissues but without sufficient fanfare. This enriched acid mucopolysaccharide deposition in the stroma surrounding malignant tumors, was long ago predicted to be the product of the peritumor fibroblasts, rather than from the cancer cells themselves (Grossfeld et al., 1955; Ozello and Speer, 1958).

It soon became apparent that the stroma that surrounded cancers was not entirely abnormal. The peritumor stroma tended to resemble fetal or embryonic fibroblasts, more than normal adult fibroblasts, as documented in the pioneering work of Seth and Ana Schor (Schor et al., 1989; Chen et al., 1989; Gray et al., 1989; Schor and Schor, this volume). This again underlines the concept that cancers do not always do unusual things. Sometimes, they do usual things at unusual times.

Clinically, striking increases of HA in the serum of many cancer patients has also been well documented (Manley and Warren, 1987; Wilkinson et al., 1996). This suggests that the increased deposition of "acid mucopolysaccharides" or HA in cancerous tissue is not a local phenomenon, but may have had wide spread consequences.

Mechanisms for Peritumor Stromal Abnormalities

There are a number of mechanisms that can be invoked for the differences observed between normal and peritumor stroma, or how it was that malignant cells were able to influence their surrounding stroma. Some of these mechanisms could not have been conceivable when such differences were first documented. Tumor cells may commandeer a small subpopulation of stromal cells to expand, and to become the predominant population. Tumor cells may be able to recruit cells from the bone marrow, to take up residence in and around the tumor cell population. The purpose of such stromal cells is to provide growth factors, and perhaps those very growth factors that are provided in a fetal-like environment, providing an environment conducive to angiogenesis, growth and remodeling. It is likely that a combination of these two scenarios attend human malignancies in their Darwinian drive to survive, grow, and spread.

HYALURONAN IN CANCER

Malignancies Have Increased Hyaluronan

It is now widely recognized that HA is dramatically increased in most malignancies. This increase in HA correlates with tumor virulence, and is often used as a prognostic indicator. But such observations were made in number of experimental systems, long before it was appreciated in human cancers.

Among the earliest observations on HA in animal tumors was by Elvin Kabat in 1939 (Kabat, 1939), who later went on to make major contributions to immunology and to the chemistry of blood group substances. In those early studies, he demonstrated that the ''mucinous substance'' associated with Rous sarcomas in the chicken was identical to the material that had been characterized by Karl Meyer. The same material was then demonstrated to be produced in cultures of Rous sarcoma cells (Grossfeld, 1962).

Following infection of avian cells with the sarcoma virus, there is a five-fold increase in the HA-synthases, the enzymes that synthesize HA (Ishimoto et al., 1966), causing a dramatic stimulation of HA deposition. Infection with other oncogenic viruses also caused enormous increases in rates of HA production, as well as abnormal acceleration of cell growth (Hamerman et al., 1965). Treatment with tumor promoters stimulated HA synthesis as well (Ulrich and Hawkes, 1983).

The HA isolated from such transformed cells has the ability to stimulate proliferation of growth-retarded, non-transformed cells (Henrich and Hawkes, 1989). The constitutive HA synthesized by non-transformed cells does not possess this property, an ability attributed to the size difference between the two classes of HA polymers (Stern et al., 2006; Sugahara et al., 2006).

Hyaluronan was demonstrated in a number of other experimental animal model tumors, including the rat Walker carcinoma (Fiszer-Szafarz and Gallino, 1970). Another was the rabbit V_2 carcinoma (Toole et al., 1979), one of the earliest studies to demonstrate a direct relationship between HA and invasive tumor growth. Aggressiveness of other murine tumors were subsequently shown to correlate with HA content (Knudson et al., 1984). Increased levels of HA correlate with high metastatic potential in variants of mouse mammary carcinoma cells (Angello et al., 1982a; b; Kimata et al., 1983).

In human mammary cell culture systems, highly aggressive breast cancer cell lines such as MDA-MB-231 synthesize greater amounts of HA than the much less virulent cell line MCF-7. But, in addition, the HA synthesized by the breast cancer lines remains cell-associated while

normal breast epithelial cells secrete most of their HA into the medium (Chandrasekaran and Davis, 1979).

Hyaluronan is produced not only by cancer cells, but production can be induced by the tumor cells in their surrounding stromal cells. In human cancers, levels of HA often correlate inversely with prognosis (Ropponen, 1998; Auvinen et al., 2000; Anttila et al., 2000). But, as a practicing anatomic pathologist, when staining for HA in human breast cancers, it is apparent that patterns differ widely (Stern, R., unpublished). Some tumors have abundant HA within the tumor, some within the surrounding stroma, and some with pronounced HA deposition in both tumor and stroma, while some breast malignancies show little HA deposition in either tumor or stroma. In pursuing prognostic indicators, it may be important to separate such patterns. As indicated in the opening line of Tolstoy's *Anna Karenina*, "All happy families are happy in the same way, but all unhappy families are unhappy each in their own way." The same can be said of malignancies.

Mechanisms for the Increased Hyaluronan in Malignancies

Cancer cell culture systems facilitated identification of "factors" that modulated expression of HA. Among the earliest of such observations included the ability of 17-β-estradiol and of growth hormone to stimulate production of acid mucopolysaccharides in fetal fibroblasts (Ozello, 1964). Tumor cells also secrete factors that can induce increased synthesis of HA in fibroblasts (Knudson et al., 1984a; Knudson and Pauli, 1987; Asplund et al., 1993). A similar factor occurs in both fetal serum and the serum of cancer patients (Decker et al., 1989). Some of these tumor-derived factors have become defined (Suzuki et al., 1985), while others have defied explication, despite intense efforts (Decker et al., 1989). Some of these are soluble factors, while others require cell–cell contact (Knudson et al., 1984b).

Do stromal cells become abnormal by direct contact with cancer cells? Are there soluble factors that influence such a conversion, and are such putative factors similar to fetal-derived growth factors? Do such factors influence HA production in the stromal populations induced to expand, or induced to migrate from the bone marrow by tumor cells? Again, it is likely that all of these scenarios participate in cancer growth and spread.

Cancers Are Resilient in Utilizing Hyaluronan Metabolism for Their Own Promotion

Not surprisingly, examples abound demonstrating that cancer cells have commandeered every aspect of the metabolism of HA in promoting their Darwinian quest to survive. For HA, examples of its synthesis (Kimata and Itano, this volume), receptors and related signal transduction

networks (Bourguignon, this volume), fragmentation (Sugahara, this volume), degradation (Lokeshwar, this volume), and the various other strategies that cancer cells have achieved utilizing HA for their own promotion (Toole, this volume).

Anomalously, Hyaluronan Oligomers Can Inhibit Tumor Growth

An anomaly of HA cancer biology is that HA oligomers injected into cancer sites markedly inhibit tumor growth. This observation was made *in vivo* using visible skin tumors, and would be attractive for the control of tumors such as malignant melanoma (Zeng et al., 1998). *In vitro*, the HA oligomers inhibit anchorage-independent growth of several tumor cell types. They induce apoptosis and stimulate caspase-3 activity through the phosphoinositide 3-kinase/Akt cell survival pathway (Ghata et al., 2002). A possible mechanism for HA oligomers' ability to thwart tumor growth may be by competing with high molecular weight chains for HA receptors.

These observations are best understood in the context that high molecular weight HA is a reflection of intact healthy tissues, and that HA oligomers are distress signals indicating that tissue injury has occurred. The range of activities and biological functions of variably sized HA fragments have recently been reviewed (Stern et al., 2006). Some of the recent observations that hyaluronidase treatment can suppress cancer growth may well be a reflection of the HA fragments generated, rather than a direct effect of the hyaluronidase itself (Shuster et al., 2002).

The physiological effects of HA and its associated water of hydration on tumor interstitial fluid pressure, and the ability of hyaluronidase treatment to relieve such pressure adds another intriguing element to their relationship.

ABNORMALITIES IN OTHER GLYCOSAMINOGLYCANS OCCUR IN MALIGNANCY

Abnormal forms and concentrations of glycosaminoglycans other than HA have also been reported for a variety of cancers. These proteoglycans play an important role in neoplasia. One of the earliest reports described the stimulating properties of chondroitin sulfate on the growth of Ehrlich ascites tumor cells (Takeuchi, 1965).

Early studies were performed using radiolabeled [^{35}S]sulfate, comparing normal and cancerous tissues. Such experiments would not have detected changes in HA metabolism. Twelve-fold increases in the deposition of chondroitin-4 and -6 sulfate were documented in colon tumors (Iozzo et al., 1982). Histochemically, this increase occurred in the intercellular matrix of the connective tissue adjacent to the tumor. The leucine- and

chondroitin sulfate-rich proteoglycan, decorin, functions as a paradigm for the profound changes that tumor matrix can exert (Iozzo et al., 1989; Iozzo and Cohen, 1994).

Heparan sulfate (HS) proteoglycans have also been implicated in tumor pathogenesis in a widespread and convincing manner (Sanderson et al., 2004). The HS proteoglycans actually comprise a wide variety but closely related family of GAGs derived from a common precursor, but varying in their glycan sequence and composition, particularly in relation to their sulfate composition. Other families of proteoglycans rich in HS side chains are characterized by having entirely different proteoglycan core proteins. These include syndecans, glypicans, and perlecan. Each of these HS proteoglycans is associated with tumor progression or suppression, or both. The reason that proteoglycan and HA biology has not been studied more carefully by cancer biologists is precisely because of the complexity of their structure and functions. Nevertheless, the HS proteoglycans and HA constitute major targets for potential anti-cancer therapeutics.

CONCLUSIONS

Hyaluronan has long been recognized to be concentrated in the areas around cancer cells. The purpose of this volume is to bring attention to this relatively neglected area of cancer cell biology. Stromal influence on malignancies is a related concept that also has not achieved the attention it deserves. And, lastly, entirely new and unpredicted directions are identified that further widen the role of hyaluronan in cancer biology (Miller, A.D., in this volume).

Hyaluronan has long been recognized as essential to cancer biology. However, critical recognition has been lacking. Investigations of the ECM have up to now been at the periphery of cell biology. A dynamic reciprocity is becoming apparent between extra- and intracellular events, the ability of the ECM to control and orchestrate that dialogue, the ability of HA and its size-specific fragments to induce signal transduction pathways by engagement of HA-specific receptors, and the recognition that HA is prominent in the peritumor ECM, and is at the core of such interactions in malignancy.

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CHAPTER

2

Hyaluronan–CD44 Interactions and Chemoresistance in Cancer Cells

Mark G. Slomiany and Bryan P. Toole

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INTRODUCTION

The association of high levels of hyaluronan with malignant tumors has been known for some time (Knudson et al., 1989). Hyaluronan is often enriched preferentially in the stroma that surrounds tumors rather than in parenchymal regions (Bertrand et al., 1992; Koyama et al., 2007; Tammi et al., 2008; Toole et al., 1979), most likely as a result of stromal cell–tumor cell interactions (Asplund et al., 1993; Edward et al., 2005; Knudson et al., 1984). However, hyaluronan synthesis is also increased in many malignant

tumor cells themselves (Calabro et al., 2002; Kimata et al., 1983; Zhang et al., 1995) and is frequently found in direct association with tumor cells *in vivo* (Tammi et al., 2008).

Resistance of cancers to various chemotherapeutic agents, i.e. multidrug resistance, can arise in numerous ways, e.g. decreased uptake of drugs due to cell and tissue barriers, activation of repair and detoxification mechanisms, altered metabolic phenotype, increased activities of cell survival/anti-apoptotic signaling pathways, or enhanced drug efflux via cell membrane transporters of the ATP-binding cassette (ABC) family (Cheng et al., 2005; Dai and Grant, 2007; Gottesman et al., 2002; Li and Dalton, 2006; Tredan et al., 2007). A relatively new paradigm is the likely contribution of cancer stem-like cells to chemoresistance since these cells are highly enriched in ABC-family drug transporters (Dean et al., 2005).

In recent years, hyaluronan and CD44 have been shown to influence drug resistance at several of these different levels, namely, cell survival signaling pathways, drug transporter expression and activity, glycolytic phenotype, and cancer stem-like cell characteristics. These advances are discussed in this review.

HYALURONAN, CD44, AND DRUG RESISTANCE

The possibility that hyaluronan might influence drug resistance was suggested in the findings that local hyaluronidase treatment enhances the action of various chemotherapeutic agents *in vivo* (Baumgartner et al., 1998), and that hyaluronidase-induced dispersion of drug-resistant, multicellular, tumor cell spheroids reverses their drug resistance (Kerbel et al., 1996; St Croix et al., 1998). The mechanistic action of hyaluronidase on drug resistance was not understood at the time of these studies, but was usually explained in terms of possible effects on cell adhesion barriers (Kerbel et al., 1996) or drug penetration (Baumgartner et al., 1998; Desoize and Jardillier, 2000) rather than hyaluronan-specific effects on signaling pathways. Initial studies by our laboratory showed that calcium-independent aggregation of transformed cells can be due to hyaluronan-mediated, multivalent cross-bridging of receptors on adjacent cells (Underhill and Toole, 1981). This observation and the finding that hyaluronan–receptor interactions regulate cell survival signaling pathways known to be important in drug resistance (Ghatak et al., 2002) led our group and others to further investigate the possible role of hyaluronan in multidrug resistance.

Employing a drug-resistant human carcinoma cell line, we demonstrated that disruption of endogenous hyaluronan-induced signaling by treatment with small hyaluronan oligomers suppresses resistance to several drugs, including doxorubicin, taxol, vincristine, and methotrexate

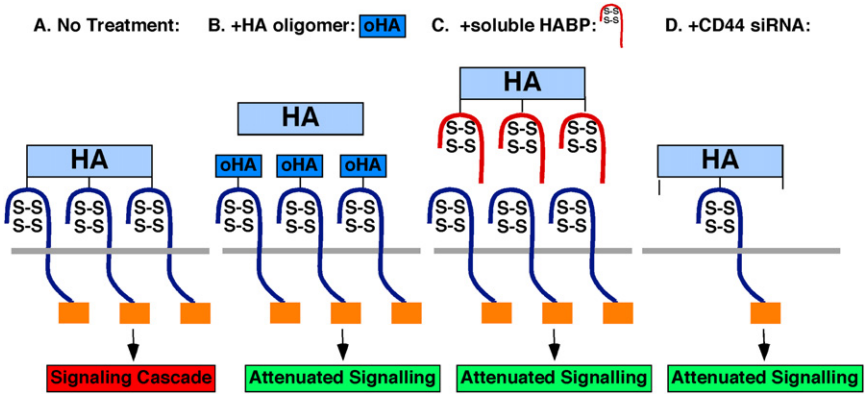

FIGURE 2.1 Antagonists of hyaluronan–CD44 interactions. Other antagonists include blocking antibodies against CD44 (Guo et al., 1994) and inhibitors of hyaluronan synthesis (Simpson et al., 2002). From Toole and Slomiany (2008). HA, hyaluronan; HABP, HA binding protein.

(Misra et al., 2003). Other antagonists of hyaluronan–CD44 signaling (Fig. 2.1) had similar effects. In addition, we showed that increased hyaluronan production, induced by over-expression of the hyaluronan synthase, HAS-2, caused increased drug resistance in the relatively chemosensitive MCF-7 breast cancer cell line. This increased resistance was reversed by treatment with hyaluronan oligomers or other antagonists of hyaluronan–CD44 signaling (Misra et al., 2003; 2005). It should be noted that the resistant cell line used in these studies was the so-called MCF-7/Adr human breast cancer cell. However, it has now been shown that this cell line is actually a drug-resistant ovarian carcinoma line, specifically OVCAR-9 (Liscovitch and Ravid, 2007). Subsequently, studies from other laboratories have similarly shown that hyaluronan promotes resistance to cisplatin, methotrexate, doxorubicin and etoposide in head and neck squamous carcinoma cells (Wang and Bourguignon, 2006; Wang et al., 2007), to cisplatin in non-small cell lung cancer cells (Ohashi et al., 2007), and to vincristine in lymphoma cells (Cordo Russo et al., 2008).

CELL-AUTONOMOUS REGULATION OF CELL SURVIVAL SIGNALING PATHWAYS BY HYALURONAN AND CD44

Elevated levels of cell survival/anti-apoptotic pathways, a common occurrence in cancer cells, are major factors contributing to drug resistance (Cheng et al., 2005; Dai and Grant, 2007). Receptor tyrosine kinases are a class of plasma membrane receptors that bind various regulatory factors, such as EGF, IGF, HGF, and PDGF, and activate several intracellular

signaling pathways, including the phosphoinositide 3-kinase/AKT cell survival pathway. Aberrant activities of these receptors, especially members of the ERBB family, have been implicated in the progression of numerous types of human cancers. Increased activity of receptor tyrosine kinases can arise from gene amplification, activating mutations or altered regulation, e.g. by cross-talk between these receptors and integrins or other receptors, or by altered autocrine and paracrine stimulation by various regulatory factors. These changes lead in turn to enhanced tumor cell growth, motility, survival, and resistance to therapies (Gschwind et al., 2004; Krause and Van Etten, 2005; Yarden and Sliwkowski, 2001).

Several reports have documented augmentation of receptor tyrosine kinases and downstream signaling pathway activities after treatment of cancer cells with exogenous hyaluronan (Bourguignon, 2008a; b). We have demonstrated that manipulation of constitutive hyaluronan production and interactions in cancer cell themselves has profound effects on these pathways. Consequently, we have shown that constitutively high levels of active, i.e. autophosphorylated, ERBB2 in carcinoma cells are dependent on endogenous hyaluronan–CD44 interaction and that experimentally increased hyaluronan production causes sustained, elevated ERBB2 phosphorylation in cells that normally exhibit low levels of ERBB2 activity (Ghatak et al., 2005). Furthermore, stimulation of hyaluronan production induces assembly of a constitutive, lipid raft-associated, signaling complex containing phosphorylated ERBB2, CD44, ezrin, phosphoinositide 3-kinase, and the chaperone molecules, HSP90 and CDC37; inhibition of endogenous hyaluronan–receptor interactions causes disassembly of this complex. Antagonists of hyaluronan interactions used in these studies included hyaluronan oligomers, soluble hyaluronan binding proteins and siRNA against CD44 (see Fig. 2.1), all of which caused disassembly of this complex and inactivation of ERBB2 (Ghatak et al., 2005). Recent work in our lab (unpublished results) shows that hyaluronan oligomers also cause rapid internalization of ERBB2 and CD44. In addition, similar influences of constitutive hyaluronan–CD44 interaction may occur with other receptor tyrosine kinases, i.e. EGFR, IGF-1R, PDGFR, and c-MET (Misra et al., 2006), and corresponding effects have been shown for downstream anti-apoptotic and proliferation pathways that are known to be regulated by these receptor kinases and to be important oncogenic pathways in several cancers. For example, increased hyaluronan production stimulates the phosphoinositide 3-kinase, MAP kinase and COX-2 pathways whereas antagonists of hyaluronan interactions suppress these pathways (Ghatak et al., 2002; Misra et al., 2003; Misra et al., 2008). Importantly, interactions between CD44 and receptor tyrosine kinases may lead to very different outcomes in normal versus cancer cells and at different pericellular hyaluronan concentrations (Li et al., 2006; Li et al., 2007c).

HYALURONAN, CD44, AND DRUG TRANSPORTERS

In addition to the oncogenic, anti-apoptotic activities of the phosphoinositide 3-kinase/AKT signaling pathway, mentioned above, this pathway also induces increased expression of ABC family multidrug transporters, such as P-glycoprotein (MDR1/ABCB1), multidrug resistance-associated protein-1 (MRP-1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2) (Lee et al., 2004; Misra et al., 2005; Mogi et al., 2003). We have demonstrated that constitutive hyaluronan–CD44 interaction regulates expression of the ABC family drug transporters, P-glycoprotein and BCRP, in carcinoma and glioma cells, respectively, most likely via receptor tyrosine kinase-mediated activation of the phosphoinositide 3-kinase/AKT pathway (Gilg et al., 2008; Misra et al., 2005).

Hyaluronan–CD44 interaction may also stabilize drug transporters at the plasma membrane. We have found that CD44 co-localizes with P-glycoprotein and BCRP in the plasma membrane of cancer cells and that treatment of these cells with an antagonist of hyaluronan interactions, viz. hyaluronan oligomers, rapidly induces internalization of CD44 and the transporters into the cell and inhibits drug efflux (Fig. 2.2) (unpublished results). Others have also shown that hyaluronan and CD44 influence transporter expression and activity, as well as malignant cell properties (Cordo Russo et al., 2008; Miletti-Gonzalez et al., 2005; Ohashi et al., 2007). In a study comparing multi-drug resistant cell lines of breast, oral, and ovarian origin that express elevated levels of P-glycoprotein with their respective P-glycoprotein-negative, drug-sensitive, parental cell lines, a positive correlation was demonstrated between the expression of CD44 and P-glycoprotein. The two proteins were found to co-immunoprecipitate, and drugs or siRNA that interfere with the function of P-glycoprotein were shown to inhibit cell motility and invasion (Miletti-Gonzalez et al., 2005), which are properties strongly related to CD44 receptor activity (Hill et al., 2006; Tzircotis et al., 2005). In a similar way, co-immunoprecipitation and co-localization of P-glycoprotein and CD44 have been demonstrated in drug resistant melanoma cells, and the two molecules were found to cooperate in promoting invasive behavior (Colone et al., 2008). These and other observations (Dean et al., 2005; Raguz et al., 2004; Yang et al., 2003) suggest a close relationship between malignant cell properties and resistance to therapy, and the likely involvement of hyaluronan and CD44 as major factors mediating this relationship.

Confocal microscopic co-localization and fluorescence resonance energy transfer studies in NIH3T3 cells have shown that P-glycoprotein is closely associated with CD44 and other components of plasma membrane lipid microdomains, commonly known as lipid rafts (Bacso et al., 2004). It was also shown in this study that P-glycoprotein is anchored to the

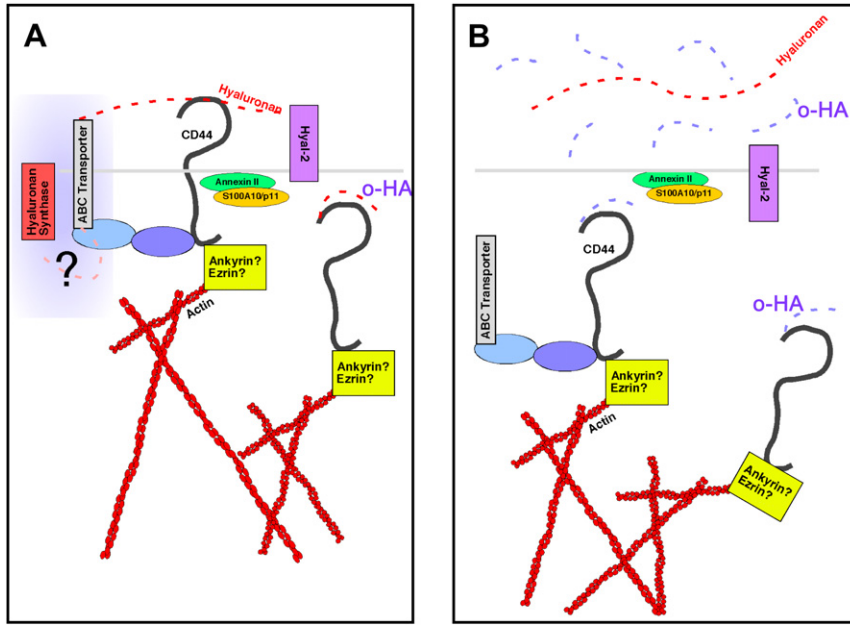

FIGURE 2.2 Internalization of drug transporters by treatment with hyaluronan oligomers. (**A**) In the absence of treatment, hyaluronan is tethered by CD44 at the plasma membrane whereby it stabilizes actin-linked CD44–transporter complexes in lipid microdomains. Hyaluronan is cleaved by hyaluronidase (most likely HYAL-2) and internalized via CD44 in an orderly manner (Knudson et al., 2002; Tammi et al., 2001). (**B**) Oligomers of hyaluronan (o-HA) stimulate CD44 internalization en masse, destabilizing transporter complexes. Inhibitors of actin assembly inhibit this process. From Toole and Slomiany (2008).

cytoskeleton. CD44 binds to the actin cytokeleton through ERM-family proteins (Tsukita et al., 1994) or ankyrin (Singleton and Bourguignon, 2004). Thus, these results suggest that CD44 resides in close molecular vicinity to P-glycoprotein and may be one of the proteins responsible for the cytoskeletal association of this transporter. Furthermore, raft localization of P-glycoprotein seems to be of functional importance since cholesterol depletion results in inhibition of transporter activity (Bacso et al., 2004). It has also been noted that drugs that interfere with P-glycoprotein can also affect localization of CD44 on the cell membrane and promote CD44 capping, and therefore might act via inhibition of actin polymerization (Miletti-Gonzalez et al., 2005). Similarly, we have seen that hyaluronan oligomer-induced internalization of CD44 and transporters is inhibited if the cells are co-treated with an inhibitor of actin polymerization, latrunculin, thus suggesting that the transporters and CD44 are anchored to actin filaments (Fig. 2.2) (unpublished results).

Hyaluronan synthesis and secretion may be directly related to drug transport since recent work suggests that hyaluronan might be secreted

through multidrug transporters in vertebrate cells (Prehm and Schumacher, 2004; Schulz et al., 2007). Studies employing a battery of inhibitors as well as siRNA to sort out possible transporters involved in hyaluronan export led to the conclusion that MRP5 is the most likely hyaluronan transporter in human fibroblasts, but other transporters are probably involved in other cells (Prehm and Schumacher, 2004). Although this evidence supports a role for drug transporters in hyaluronan secretion, other studies strongly suggest that constitutive export of hyaluronan requires only the hyaluronan synthases themselves (Weigel and Deangelis, 2007). Moreover, definitive direct evidence for hyaluronan export through ABC transporters, rather than regulation by transporter activity, is lacking. Nevertheless it is likely that such export does occur at least under certain circumstances. Our findings support a close relationship between hyaluronan and drug transporters in that treatment with hyaluronan oligomers inhibits hyaluronan production or export (Misra et al., 2008) and induces rapid internalization of the drug transporters, BCRP and P-glycoprotein (unpublished results).

HYALURONAN, EMMPRIN, AND THE GLYCOLYTIC PHENOTYPE

An almost universal property of malignant cancers is increased glycolysis – the ''Warburg effect.'' Increased glycolysis in cancer is associated with various conditions such as hypoxia, acidosis, and mitochondrial defects, which result in enhanced drug resistance and malignancy. An outcome of increased glycolysis is lactate production and extrusion across the plasma membrane via proton-coupled monocarboxylate transporters (MCTs). Lactate efflux and resultant pericellular acidification stimulate cell invasion, metastasis, and drug resistance (Gatenby and Gillies, 2004; Martinez-Zaguilan et al., 1996; Pelicano et al., 2006; Tredan et al., 2007). Two sets of observations indicate a possible relationship of hyaluronan to the glycolytic phenotype.

First, an essential partner in the activity of MCTs is emmprin (CD147) (Halestrap and Meredith, 2004), a tumor cell surface glycoprotein that also stimulates hyaluronan production (Marieb et al., 2004). Emmprin was originally identified as a factor that is expressed at high levels on the surface of malignant tumor cells and induces production of matrix metalloproteinases via cell–cell interactions (Biswas et al., 1995; Yan et al., 2005). More recent work from our lab revealed that emmprin also stimulates hyaluronan production in cancer cells and, as a consequence, induces anchorage-independent cell proliferation (Marieb et al., 2004) and drug resistance (Misra et al., 2003). Emmprin is crucial for the proper function of several monocarboxylate transporters, specifically MCT1, MCT3, and

MCT4. These MCTs require association with emmprin in the endoplasmic reticulum for trafficking to the plasma membrane and, in the absence of emmprin, are targeted for degradation (Gallagher et al., 2007; Kirk et al., 2000; Wilson et al., 2005). It has been shown that emmprin and MCT4 trafficking to the plasma membrane of breast cancer cells are mutually interdependent and that suppressed expression of MCT4 results in decreased migratory capacity in these cells, most likely due to inhibition of emmprin function (Gallagher et al., 2007).

Second, lactate stimulates hyaluronan synthesis and expression of CD44 variants in fibroblasts (Stern et al., 2002) and melanoma cells (Rudrabhatla et al., 2006), and lactate response elements are present in several hyaluronan-related genes, e.g. CD44 and the hyaluronidase, HYAL-1 (Formby and Stern, 2003).

Since emmprin–MCT interaction is required for lactate secretion and both emmprin and lactate stimulate hyaluronan production, we have examined the relationship of hyaluronan and CD44 to MCT1 and MCT4, the most commonly expressed MCTs in cancer cells. We find that CD44 interacts and co-localizes with these MCTs in the plasma membrane of breast cancer cells (Slomiany et al., 2008). Furthermore, treatment of the cells with hyaluronan oligomers leads to their internalization and to attenuation of their function, in similar fashion to our findings with RTKs and drug transporters mentioned above. Other investigators have documented a similar relationship between hyaluronan–CD44 interaction and the Na(+)-H(+) exchanger 1 (Bourguignon et al., 2004).

HYALURONAN, CANCER STEM CELLS, AND DRUG RESISTANCE

Recent evidence suggests that malignant tumors contain sub-populations of stem-like cells, termed: ''cancer stem cells,'' ''cancer progenitor cells'', or ''tumor-initiating cells,'' that are highly malignant and resistant to therapies. These cells are able to rapidly regenerate a fully grown tumor that recapitulates the heterogeneous cellular composition of the tumor of origin when implanted in small numbers in an animal host (Dalerba et al., 2007a; Hill and Perris, 2007; Vescovi et al., 2006). These cells may also comprise the metastatic sub-population of tumors (Brabletz et al., 2005; Li et al., 2007b). A striking property of these cells is their resistance to treatment with cytotoxic chemotherapeutic agents or radiation, possibly underlying the tendency of many malignant cancers to recur after treatment (Ailles and Weissman, 2007; Dean et al., 2005; Neuzil et al., 2007).

However, the cancer stem cell hypothesis remains controversial since the precise nature and origin of cancer stem-like cells have yet to be elucidated (Hill and Perris, 2007; Patrawala et al., 2005; Shipitsin et al.,

2007; Wang et al., 2008). These cells may comprise the metastatic sub-population of tumors (Brabletz et al., 2005; Li et al., 2007b) or, stated differently, may merely reflect heterogeneity within tumors with respect to various oncogenic and metastatic properties (Shipitsin et al., 2007). Nevertheless, the presence of highly malignant, therapy-resistant sub-populations within human tumors is well-established and our increased understanding of the properties of these cells is likely to yield more effective therapeutic strategies.

The hyaluronan receptor, CD44, is one of the most common markers used for isolation of cancer stem-like cells from carcinomas (Al-Hajj et al., 2003; Dalerba et al., 2007b; Li et al., 2007a; Prince et al., 2007). Recent studies indicate that CD44 is functionally important in leukemia stem cells (Jin et al., 2006; Krause et al., 2006). Other studies point to a possible role for another hyaluronan binding protein, Rhamm, in myeloma progenitors (Crainie et al., 1999; Maxwell et al., 2004); hyaluronan synthases are also altered in myeloma progenitors (Adamia et al., 2005; Calabro et al., 2002). Moreover, hyaluronan may have an important role in normal stem cell behavior (Avigdor et al., 2004; Matrosova et al., 2004; Nilsson et al., 2003; Pilarski et al., 1999). However, virtually nothing has been published on the potential role of hyaluronan–CD44 interactions in cancer stem-like cells.

Recently we have begun to examine the effects of perturbing hyaluronan interactions with hyaluronan oligomers on the malignant and therapy-resistant properties of stem-like cells isolated from cancer cell lines and from patient-derived tumors. These oligomers most likely displace constitutively bound hyaluronan polymer from their receptors, resulting in attenuation of hyaluronan-induced signaling (Ghatak et al., 2002; Ghatak et al., 2005; Misra et al., 2006). We find that hyaluronan oligomers inhibit the growth of a very aggressive stem-like sub-population of C6 glioma cells in a novel spinal cord engraftment model that replicates invasive behaviors of human gliomas in the central nervous system (Gilg et al., 2008). Furthermore, the oligomers cause increased apoptosis and decreased proliferation in these tumors. The C6 stem-like cells show elevated activation of EGFR and AKT, expression of the BCRP drug transporter and resistance to treatment with methotrexate, when compared with the C6 parental cells; these parameters were also reduced by treatment with the hyaluronan oligomers (Gilg et al., 2008), indicating the potential importance of hyaluronan in the properties of these cells.

Cancer stem-like cell sub-populations can be enriched using Hoechst dye exclusion (the ''side-population'': due to efflux by drug transporters, especially BCRP), cell surface markers, or spheroid formation. However, the efficacy of these different methods varies among different cancer types, and in each case the enriched preparations are heterogeneous. Depending on the method of separation and the cancer type, these cell preparations express various subsets of markers and exhibit varying degrees of

malignancy and resistance to drug treatment. For example, in a study of lung cancer the side-population cells were found to exhibit stem cell-like properties such as multidrug resistance, high telomerase activity, and tumor-repopulating capacity, and consistently expressed high levels of ABC transporters, in comparison to non-side population cells (Ho et al., 2007). However, CD44 and other commonly used cancer stem cell markers were present in both side-population and non-side population cells, pointing to lack of exclusivity of the markers to the side-population in these lung cancer cells (Ho et al., 2007). In another study it was found that the side-populations from various carcinoma cells were enriched in stem-like and malignant properties, yet cancer cells lacking BCRP expression were similar in tumorigenicity to BCRP-positive cells (Patrawala et al., 2005). A common marker for identification of cancer stem-like cells is CD133 (Mizrak et al., 2008; Neuzil et al., 2007) but it has been shown recently that CD133-negative cells can also express stem-like properties (Beier et al., 2007; Wang et al., 2008). Thus, a challenge to understanding the relationship of multidrug resistance to cancer stem-like cells will be isolation of homogenous populations that can be compared and analyzed with respect to transporter expression and function, anti-apoptotic signaling pathways and their specific relationships to hyaluronan and CD44.

Despite these caveats, it is reasonable to expect that antagonists of hyaluronan–CD44 interaction, e.g. small hyaluronan oligomers, may be useful in therapeutic strategies aimed at preventing tumor recurrence from therapy-resistant sub-populations within malignant cancers.

CONCLUSIONS

The findings discussed in this review imply that hyaluronan–CD44 interactions stabilize several types of cell surface complexes that lead to numerous downstream effects, including multidrug resistance (summarized in Fig. 2.3). It is not yet clear whether these effects are due to unique involvements of specific sub-fractions of CD44 variants, to general interactions with membrane compartments such as lipid rafts, or to global effects of a hyaluronan-based pericellular matrix that influences mechanocellular signaling interactions. With respect to the latter, it should be pointed out that constitutive cell surface hyaluronan not only tethers the pericellular matrix to the cell surface but also forms complexes with numerous other factors that may play a role in stabilizing membrane-bound complexes (Evanko et al., 2007). For example, PDGF induces formation of a pericellular matrix around smooth muscle cells that contains aggregates of hyaluronan, versican, and link protein (Evanko et al., 2001) and that is required for proliferation and migration (Evanko et al., 1999). Other components of these matrices may be TSG-6, inter-alpha trypsin

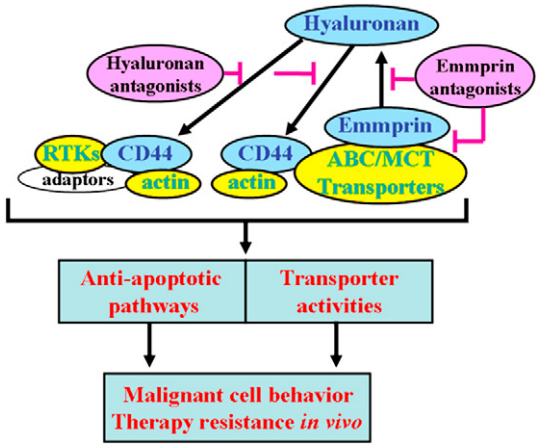

FIGURE 2.3 HA, CD44, and emmprin interactions. A scheme showing possible interactions of hyaluronan, CD44, and emmprin with receptor tyrosine kinases (RTKs), ATP-binding cassette (ABC) multidrug transporters, and monocarboxylate (lactate) transporters (MCT). The potential effects of antagonists of hyaluronan–CD44 and emmprin interactions are also shown. From Toole and Slomiany (2008).

inhibitor, tenascin, and thrombospondin-1; also, versican can be replaced by other hyaluronan binding proteoglycans such as aggrecan, neurocan, or brevican in different tissues (Evanko et al., 2007). Perturbation of pericellular matrices, either with hyaluronan oligomers or by other means, may lead to destabilization and internalization of matrix components themselves as well as associated membrane complexes. For example, protease-generated fragments of aggrecan that are complexed with hyaluronan, but not intact aggrecan–hyaluronan complexes, are internalized via hyaluronan–CD44 interaction (Embry Flory et al., 2006). Further investigation of the relationships of pericellular matrix composition and perturbation to plasma membrane-bound complex formation and stability is needed to clarify these issues.

These interactions of pericellular hyaluronan with the plasma membrane and with other pericellular macromolecules may serve both structural and signaling functions that influence malignancy and resistance to therapies. Irrespective of the mechanisms involved, it is clear that antagonists of hyaluronan, CD44, and emmprin-induced events (Fig. 2.3) are promising candidates for therapeutic strategies aimed at preventing tumor refractoriness or recurrence due to drug-resistant sub-populations within malignant cancers.

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CHAPTER

3

Growth Factor Regulation of Hyaluronan Deposition in Malignancies

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INTRODUCTION

The link between the stromal microenvironment and the promotion of cancer was first described in 1889 by Stephen Paget (Paget, 1889), who predicted that the interactions between tumor cells (the "seed," including secreted growth factors and cell surface proteins) and the host microenvironment ("the soil") determine the metastatic outcome. In recent years it has become accepted that the microenvironment of local host tissue provides tumor cells with a scaffold that promotes their attachment and

serves as reservoir for regulatory signals and thereby actively participates in tumor progression and metastasis (Schor and Schor, 2001).

In this review we focus on the extracellular molecule hyaluronan, the signals that regulate its synthesis and deposition as well as its role in cellular communication. Hyaluronan is a polysaccharide containing thousands of disaccharide repeats of glucuronic acid and *N*-acetylglucosamine residues. It is abundantly found in free form or decorated by proteoglycans in the extracellular and pericellular matrices of mammals (Heldin and Pertoft, 1993; Laurent and Fraser, 1992), as well as in the surface coats of some bacteria (Weigel, 2004) and *Chlorella* virus infected algae (DeAngelis, 2001). Hyaluronan in the pericellular matrix interacts with the cell by sustained binding to its own membrane-associated synthase or to hyaluronan receptors and with other matrix molecules; these interactions influence intracellular signaling and thereby cellular functions such as cell migration, growth and differentiation (Heldin and Pertoft, 1993; Knudson and Knudson, 1993). Importantly, intracellular and nuclear hyaluronan has also been demonstrated in both normal and tumor cells (Evanko and Wight, 2001; Li et al., 2007b). Because of its remarkable physicochemical properties and hygroscopic nature, hyaluronan has important physiological properties, including tissue organization and tissue hydration. Thus, an accumulation of hyaluronan is a common feature of remodeling tissues, for example during embryonic development, followed by its clearance. However, an aberrant increase in the amount of hyaluronan of a more polydisperse character, with a preponderance of lower molecular mass forms, is seen during inflammation and tumor progression.

Both high and low molecular mass hyaluronan can function as signaling molecules through their interactions with cell surface receptors, e.g. CD44 and extracellular matrix proteins, e.g., versican (Toole, 1990; Turley et al., 2002; Wu et al., 2005). CD44 is an adhesion receptor that is found in different splice variants on immune cells and stromal cells in a low hyaluronan binding state (Aruffo et al., 1990). However, external stimuli by cytokines can induce the transition of CD44 to its high hyaluronan binding state. Active CD44 with high hyaluronan binding capacity is found on activated leukocytes and tumor cells (Cichy and Pure, 2003; Ponta et al., 2003). The ability of CD44 to bind hyaluronan is tightly controlled. High Mw hyaluronan facilitates CD44 oligomerization whereas hyaluronan fragments bind to monomeric CD44 molecules. West and colleagues were first to demonstrate that hyaluronan oligomers are angiogenic (West et al., 1985). Subsequently, a number of laboratories, including ours, revealed that hyaluronan fragments is an important initiation factor in fibrotic tissue remodeling by the induction of collagen genes (Li et al., 2000), chemokine genes (McKee et al., 1996; Teder et al., 2002), but also an angiogenic factor, by the induction of distinct and/or common sets of genes with the known angiogenic factor fibroblast growth factor-2 (FGF-2) (Takahashi et al., 2005).

Both FGF-2 and hyaluronan oligosaccharides promote tubulogenesis in a process dependent on the co-ordinated induction of ornithine decarboxylase (Odc) and ornithine decarboxylase antizyme inhibitor (Oazi) genes. Among the genes induced selectively by hyaluronan oligosaccharides was the chemokine *CXCL1/Gro1* gene (the human homolog is IL-8); the endothelial cell differentiation was CD44-mediated leading to activation of chemokine receptor 2 which is involved in endothelial cell retraction, a common phenomenon observed during angiogenesis (Takahashi et al., 2005). Thus, hyaluronan oligomers have an important function during the inflammatory and angiogenic responses in injuries and malignancies through a sustained production of chemokines.

EXPRESSION OF HYALURONAN SYNTHASES AND HYALURONIDASES

Vertebrate, bacterial and plant hyaluronan molecules have identical chemical structure. There exist three related yet distinct hyaluronan synthase (*HAS*) genes encoding the mammalian HAS-1, HAS-2, and HAS-3 isoforms (Weigel and DeAngelis, 2007). Notably, HAS-2 is required for embryonic development, but not HAS-1 and HAS-3 (Camenisch et al., 2000); moreover the *HAS-2* gene is under tighter regulatory control than the other *HAS* genes (Nairn et al., 2007). The expression patterns of the *HAS* genes was found to vary between normal mesenchymal cells, and between normal and their transformed counterparts. In general, the expression was higher in sub-confluent than in confluent cultures (Jacobson et al., 2000; Li et al., 2007b). Each one of the *HAS* genes encodes plasma membrane proteins that are independently active, with multiple transmembrane and membrane-associated domains; the majority of the protein is inside the cell and possesses consensus sequences for phosphorylation by protein kinases (Shyjan et al., 1996; Spicer et al., 1996; Spicer et al., 1997). Recently, studies have demonstrated that HAS activities can be regulated through extracellular signal-regulated kinase (ERK) (Bourguignon et al., 2007). Each of the three HAS proteins synthesizes hyaluronan chains of high molecular mass, *in situ* ($\geq 4 \times 10^6$ Da). However, *in vitro*, the HAS-2 isoform synthesizes hyaluronan chains of high molecular mass ($\geq 4 \times 10^6$ Da), whereas HAS-3 produce polydisperse hyaluronan (average molecular mass of 0.8×10^6 Da), and HAS-1 even smaller hyaluronan chains (average molecular mass of 0.1×10^6 Da). Furthermore, the HAS-3 protein was catalytically more active than HAS-2 which in turn was more active than HAS-1. It is possible that different cytoplasmic proteins specifically interact with each HAS protein and may have accessory or regulatory roles in hyaluronan biosynthesis (Brinck and Heldin, 1999). The nature of these proteins have not yet been identified.

The newly synthesized and growing hyaluronan chain is extruded through the plasma membrane while the synthesis is in progress, contributing to the assembly of pericellular matrices by remaining attached to its own membrane-associated synthase before being released into the extracellular matrix (Heldin and Pertoft, 1993). The transfer process of newly synthesized hyaluronan is not yet known. It has been proposed that hyaluronan is transported through a pore-like passage and/or uses the multidrug resistance system (Prehm and Schumacher, 2004; Tlapak-Simmons et al., 1999). Hyaluronan overexpression amplifies MDR1 multidrug transporter expression and increases doxorubicin resistance in breast cancer cells MCF-7 (Misra et al., 2005; Misra et al., 2003); further studies are necessary to elucidate the inter-relationship between hyaluronan synthesis/export and multidrug transporters.

The turnover rate of hyaluronan in mammals is high; its intravenous $t_{\{1/2\}}$ is about 5 min and in epidermis it is less than 24 h (Fraser et al., 1981; Tammi and Tammi, 1998). Hyaluronidases, the enzymes involved in hyaluronan degradation also exist in several isoforms (*HYAL-1*, *HYAL-2*, *HYAL-3*, *HYAL-4*, and *PH-20*) and are localized in lysozomes or are glycosylphosphatidyl-inositol linked to the plasma membrane. HYAL-1 and HYAL-2 proteins are widely expressed in tissues and act in a concerted manner to degrade hyaluronan chains (Csoka et al., 2001; Lepperdinger et al., 2001).

HYALURONAN SIGNALING PROMOTES THE MALIGNANT PHENOTYPE OF TUMOR CELLS

Studies on human cancers from different origins and various malignancy grades have demonstrated a positive correlation between tumor aggressiveness and stromal hyaluronan expression (Auvinen et al., 2000; Boregowda et al., 2006). The aberrant amounts of hyaluronan in the desmoplastic stroma can be produced by the tumor cells themselves or by the stromal cells commandeered by the tumor cells (Asplund et al., 1993; Toole, 2004). Notably, a differential expression of *HAS* genes is seen during tumor progression. For example, aggressive breast cancer cells and ovarian cancer express higher levels of *HAS-2* than *HAS-3* compared to non-aggressive ones (Bourguignon et al., 2007; Li et al., 2007b), whereas metastatic prostate and colon cancer express higher levels of *HAS-3* than *HAS-2* (Bullard et al., 2003; Simpson et al., 2001). *HAS-1* was expressed only at low levels in these tumors. An important question awaiting an answer is whether there are functional differences between tumor cell-produced and stromal fibroblast- and/or mesothelial cell-synthesized hyaluronan for tumor progression. It is also important to elucidate which regulatory factors modulate the expressions and activities of HAS and HYAL proteins.

Earlier studies in our laboratory demonstrated marked differences in hyaluronan synthesis and expression of CD44 between non-aggressive and aggressive breast cancer cells. Metastatic breast carcinoma cells were found to express high levels of CD44 with high hyaluronan binding capacity and to synthesize hyaluronan. In contrast, breast cancer cell lines which have a non-invasive character synthesized much lower amounts of hyaluronan and did not express CD44 (Heldin et al., 1996). Importantly, CD44 exhibiting high hyaluronan binding capacity was expressed on malignant mesotheliomas but not on normal mesothelial cells, suggesting an up-regulation of hyaluronan–CD44 interaction upon transformation (Asplund and Heldin, 1994). In view of these observations it is possible that tumor cell invasiveness could be related to tumor cell surface CD44–matrix hyaluronan interaction or tumor cell-presented hyaluronan interaction with soluble CD44 or CD44 expressed by endothelial cells (Hill et al., 2005). However, blocking hyaluronan–CD44 interaction does not lead to complete inhibition of tumor cell migration/invasion, suggesting that also other mechanisms are involved (Fig. 3.1). Using two-photon fluorescence correlation microscopy, hyaluronan molecules were demonstrated to form continuous cage-like structures partitioning the space of melanoma tumor matrix into aqueous and viscous compartments (Alexandrakis et al., 2004), thereby facilitating cell migration.

Several approaches have been used to elucidate the importance of hyaluronan for tumor progression; manipulation of tumor cell-produced

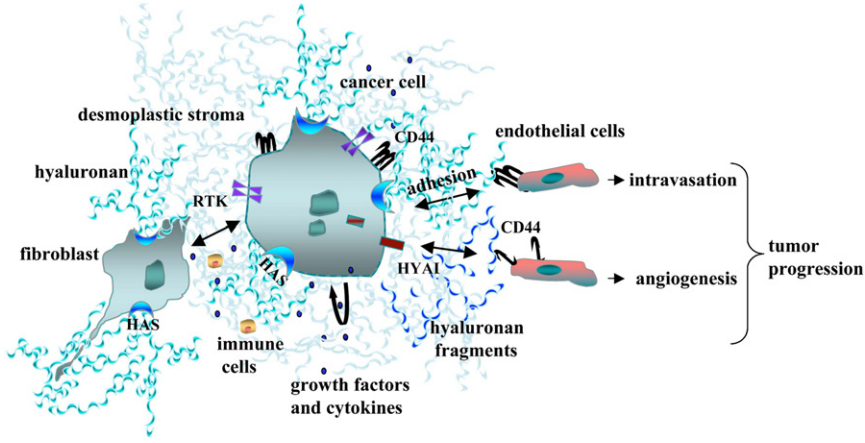

FIGURE 3.1 Tumor–host cross-talk in tumor progression. Growth factors and cytokines released by tumor cells, immune cells, and "activated" stromal cells trigger signaling events that increase the deposition of extracellular matrix macromolecules and activate tumor cell-expressed CD44 and RTK. Hyaluronan molecules form biological networks that bridges CD44 expressed by tumor and endothelial cells facilitating intravasation. Hyaluronan fragments, produced for example by the action of HYAL, bind to CD44 on endothelial cells and promote angiogenesis.

hyaluronan by overexpression of *HAS* or *HYAL* transcripts, overexpression of soluble CD44, administration of hyaluronan fragments, and treatment with antibodies that prevent hyaluronan–CD44 interactions (Toole, 2002; Toole, 2004). The impact of hyaluronan for the malignant phenotype of colon carcinomas was studied by overexpressing *HAS-2* and *HYAL-1* both *in vitro* and *in vivo*. The analysis revealed that *HAS-2* gene overexpression leads to a faster development of transplantable tumors in syngeneic rats, compared to mock-transfectants. In contrast, *HYAL-1* overexpression suppressed the growth rate of tumor cells (Jacobson et al., 2002). Similarly, inhibition of hyaluronan synthesis in prostate cancer cells impaired their growth (Liu et al., 2001; Simpson et al., 2001). Importantly, administration of hyaluronidase to mice bearing human breast cancer xenografts reduced tumor volume, hyaluronan content, and CD44 isoforms in the cancerous growth, supporting the hypothesis that loss of tumor cell-produced hyaluronan interactions is crucial for the maintenance of the malignant phenotype (Shuster et al., 2002).

Hyaluronan is abundant in highly aggressive breast cancer cells and has been shown to be a prognostic factor for patient survival of clinical breast carcinomas (Auvinen et al., 2000). *HAS-2* overexpression correlates with the promotion of the malignant phenotype of colon cancer and mesotheliomas (Jacobson et al., 2002; Li and Heldin, 2001). We therefore investigated the importance of HAS-2-synthesized hyaluronan for the malignant properties of breast cancer cells, by investigating the consequences of suppressing HAS-2 protein using specific siRNAs (Li et al., 2007b). Silencing of the *HAS-2* gene caused an about 50% reduction of the invasive and malignant phenotype of Hs578T breast cancer cells. This strong reduction of the aggressive characteristics of breast cancer cells suggests that the amount of synthesized hyaluronan influences their invasive phenotype. Similarly, antisense-mediated suppression of HAS-2 in breast cancer cells also decreased their aggressive phenotype (Udabage et al., 2005). Interestingly, addition of exogenous hyaluronan could not rescue the aggressive phenotype of breast cancer cells, suggesting that hyaluronan synthesized by neighboring fibroblasts *in vivo* is functionally not equivalent to endogenously tumor cell-produced hyaluronan. Importantly, in a mouse mammary model of spontaneous breast cancer, endogenous hyaluronan production promoted tumor epithelial–mesenchymal transition and elicited cell survival signals (Koyama et al., 2007). In addition, the aggressive phenotype could be promoted by elevated HAS activity and expression of CD44 receptors resulting in retention of hyaluronan on the surface of the neoplastic cells, facilitating their binding to the bone marrow endothelium (Draffin et al., 2004; Simpson et al., 2001). However, the involvement of hyaluronan in tumor progression is complex. Tumor cells often exhibit elevated levels of HYALs (Li et al., 2007b; Lokeshwar et al., 1999), leading to the production of angiogenic hyaluronan fragments. These observations

demonstrate a cooperativity between HAS and HYAL activities, as well as CD44 hyaluronan receptors in the maintenance of the aggressive character of breast cancer cells (Fig. 3.1).

Interestingly, hyaluronan synthesized by mammary and colon carcinomas can, through interactions with tumor cell-expressed CD44, promote activation of several receptor tyrosine kinases (RTK), including ErbB2, and thereby promote cell survival and drug resistance. Perturbating the interaction between endogenously synthesized hyaluronan and CD44, Bryan Toole and his colleagues demonstrated the necessity of hyaluronan for the malignant properties of some cancer cells; addition of hyaluronan oligosaccharides, suppressed the tyrosine kinase activities of RTKs resulting in suppression of the phosphoinositol-3-kinase/Akt survival pathway (Ghatak et al., 2002; Ghatak et al., 2005; Misra et al., 2006).

The general concept emerging from these studies is that increased hyaluronan synthesis promotes tumorigenesis and plays an important role in the local aggressive spread of tumor cells. Thus, suppression of hyaluronan synthesis and/or prevention of its binding to cell surface receptors may provide a therapeutic opportunity to suppress tumor invasion.

REGULATION OF HYALURONAN LEVELS PRODUCED BY TUMOR CELLS

The levels of hyaluronan and differences in the size of hyaluronan molecules seen in rapidly remodeling tissues, e.g. tumor tissues, are due to the concerted action of HAS and HYAL enzymes that most likely are targets of local environmental cell specific factors. During tumor development and progression, besides the epithelial malignant cells and stromal cells (fibroblasts, mesothelial cells, and endothelial cells), a large number of inflammatory cells are also present; chronic inflammation goads premalignant cells to become malignant through the influx of innate immune cells that release cytokines and chemokines, promoting the growth and invasion of tumors (Mantovani, 2005). Tumor cells themselves release a variety of growth factors, for example TGF-β and PDGF, that may function in autocrine stimulation of tumor cells or in paracrine mechanisms involving stromal cells. Importantly, these growth signals can ''activate'' stromal cells to produce and release growth factors and cytokines, resulting in modulation of the matrix macromolecular structure (desmoplasia). Additionally, these signals can activate adhesive receptors (CD44, integrins) so that they can transmit signals for cell survival and metastasis (Hanahan and Weinberg, 2000; Hill et al., 2005). During inflammation in malignancies a possible co-existence of reactive oxygen species and overexpression of HYALs results in the accumulation of hyaluronan fragments in tissues.

Very little is known about the hyaluronan-stimulatory activities in various carcinomas. Mesothelioma cells produce PDGF-BB- and bFGF-like factors that most likely stimulate hyaluronan synthesis by the neighboring mesothelial cells and fibroblasts, creating a matrix that supports the colonization of malignant cells (Teder et al., 1996). However, it is possible that these factors also in an autocrine manner stimulate mesotheliomas to synthesize hyaluronan and thereby acquire a more malignant phenotype than the non-hyaluronan producing mesotheliomas (Li and Heldin, 2001). Furthermore, the phosphoglycoprotein osteopontin is implicated in breast cancer progression and metastasis probably through its interaction with CD44 resulting in the induction of HAS-2 expression and hyaluronan synthesis (Cook et al., 2006). Additionally, heregulin (HRG) activates members of the epidermal growth factor receptor family of tyrosine kinases leading to ERK activation and subsequent phosphorylation and activation of HAS-1, HAS-2 and HAS-3, affecting ovarian cancer progression (Bourguignon et al., 2007). Notably, the HRG-ErbB2-ERK signaling caused a decrease in the size of hyaluronan from about 400 kDa to about 80 kDa. Whether this reduction in the size of hyaluronan is due to HRG-mediated activation of HYAL in ovarian cancer cells, remains to be elucidated. Notably, HYAL activity was upregulated in TGFβ-stimulated human dermal fibroblasts cultures (Li et al., 2007a). Furthermore, oncostatin M, TGFβ and phorbol 12-myristate 13-acetate (PMA) induce the hyaluronan binding capacity of CD44 in lung tumor cells (Cichy and Pure, 2000; Teder et al., 1995). Because the molecular mechanisms underlying malignancy-induced hyaluronan production are not well understood, further studies on the regulatory mechanisms modulating the activities of HASs, HYALs, and CD44 hyaluronan binding capacity in tumors are necessary.

REGULATION OF HYALURONAN SYNTHESIS BY PERITUMORAL STROMA CELLS

The emphasis in this section is on the regulation of hyaluronan synthesis by stromal mesothelial cells and fibroblasts, particularly in response to PDGF-BB and TGFβ released by cancer cells, endothelial cells, and immune cells during the malignant progression. A large body of studies revealed that *HAS-2* is the most abundantly expressed among the three HAS isoforms in mesothelial cells (Jacobson et al., 2000), corneal keratocytes (Guo et al., 2007), chondrocytes (Recklies et al., 2001), synovial cells (Stuhlmeier and Pollaschek, 2004), as well as in dermal, oral and lung fibroblasts (Li et al., 2007a; Li et al., 2000; Meran et al., 2007). *HAS-3* is also found in appreciable amounts in these cells, whereas *HAS-1* is hardly detected. However, the importance of each HAS isoform in the overall hyaluronan synthesis and assembly of the matrix surrounding cells is not known.

PDGF-BB had a potent stimulatory effect on hyaluronan synthesis through the induction of *HAS-2* in mesothelial, foreskin or dermal fibroblasts, and smooth muscle cell cultures (Heldin et al., 1992; Jacobson et al., 2000; Li et al., 2007a; Suzuki et al., 1995; van den Boom et al., 2006). Interestingly, PDGF-BB-mediated proliferation of human dermal fibroblasts and smooth muscle cells is promoted by the binding of hyaluronan to CD44 (Li et al., 2007a; van den Boom et al., 2006). Importantly, TGFβ stimulation suppresses *HAS-2* in mesothelial cells (Jacobson et al., 2000) and orbital fibroblasts from patients with Graves' ophthalmopathy (Wang et al., 2005), but potently activates *HAS-1* in synovial fibroblasts (Oguchi and Ishiguro, 2004; Recklies et al., 2001). The stimulatory effects of PDGF-BB and TGFβ were partly dependent on protein synthesis since the stimulations were partly inhibited by cycloheximide. Similarly, the protein kinase C stimulator PMA, powerfully induced hyaluronan synthesis probably via regulatory phosphorylation of a HAS isoform (Suzuki et al., 1995). Regulatory phosphorylation of each one of the three HAS isoforms has been demonstrated in a recently published study (Bourguignon et al., 2007). Combinations of PDGF-BB and TGFβ additively stimulated hyaluronan production in foreskin cultures (Suzuki et al., 1995). In contrast, in dermal fibroblast cultures TGFβ reduced the PDGF-BB-mediated hyaluronan production, probably because of activation of HYALs activity (Li et al., 2007a). These observations demonstrate that different cell types respond differentially to hyaluronan-modulating factors. More recently the downstream signaling pathways, through which PDGF-BB stimulates hyaluronan synthesis in human dermal fibroblasts were investigated. Using specific inhibitors for the major PDGF-BB-induced intracellular signaling pathways revealed that ERK MAPK and PI3K pathways are crucial for PDGF-BB-dependent *HAS-2* transcriptional activity and hyaluronan synthesis. Similarly, inhibition of NF-kB action completely suppressed hyaluronan production. The fact that the *HAS-2* promoter has putative transcription factor binding sites for CREB, NF-kB, and STAT (Monslow et al., 2003; Saavalainen et al., 2005), which are downstream of PDGF β-receptor signaling, is consistent with an important role of these signaling pathways in hyaluronan production (Fig. 3.2A).

The importance of high amounts of hyaluronan for PDGF-BB-mediated stromal fibroblast growth and migration were recently studied. The analysis revealed that hyaluronan-stimulated CD44 suppresses the activation state of the PDGF β-receptor, in PDGF-BB-stimulated human dermal fibroblasts, by the activation of a CD44-associated tyrosine phosphatase to the receptor, decreasing PDGF-BB-mediated fibroblast migration. Additionally, hyaluronan binding to CD44 is important for the mitogenic PDGF-BB response (Li et al., 2007a; Li et al., 2006) (Fig. 3.2B). Thus, dermal fibroblast CD44 binding to exogenous hyaluronan negatively regulates PDGF β-receptor-mediated migration, but positively

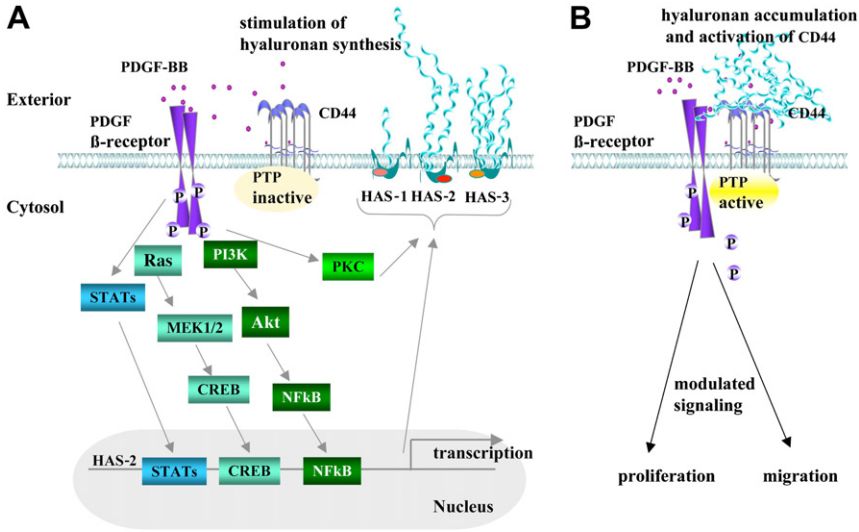

FIGURE 3.2 PDGF β-receptor-mediated hyaluronan production and its interaction with hyaluronan-activated CD44. (**A**) MEK1/2 and PI3K signaling pathways are important in mediating PDGF-BB-induced hyaluronan synthesis. Furthermore, activation of PKC is involved in the enhancement of HAS isoforms activities. (**B**) The interaction between hyaluronan-activated CD44 and PDGF β-receptor activates a CD44-associated PTP and modulates PDGF signaling, leading to cell migration and growth.

regulates its mitogenic response. Further studies are needed in order to elucidate the physiological importance of these observations during normal and abnormal tissue remodeling.

FUTURE PERSPECTIVES

Several observations support the notion that hyaluronan has an important role in tumorigenesis. Future studies should aim at unraveling the molecular mechanisms responsible for the synthesis and degradation of hyaluronan. Moreover, the mechanisms behind the expression and induction of CD44 from its low to high hyaluronan binding state as well as the functional importance of the interaction between RTK and CD44, remains to be elucidated.

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CHAPTER

4

Hyaluronan Binding Protein 1 (HABP1/p32/gC1qR): A New Perspective in Tumor Development

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Tumor development is a multistage disease that can be divided into tumor initiation, promotion, malignant conversion and progression. The role of the extracellular matrix (ECM) in tumor development is reflected in several reports that document interactions with cell surface receptors regulating the process of inflammation, wound repair, and tissue organization (Wall et al., 2003). The ECM not only acts as a barrier for growth, invasion and metastasis of tumors, it also profoundly affects the behavior of malignant cells in later stages of progression. One of the major ECM components, hyaluronan (HA), is a negatively charged, high molecular weight component that has unique capability for retaining large volumes of water (Lee et al., 2000). This molecule is extruded from the cells through the plasma membrane into extracellular space, facilitating cell motility, thereby decreasing cell–cell contacts and impeding intracellular communication (Toole and Hascall, 2002). Recent developments show that HA is critical for anchorage-independent growth in culture, and can be considered as an indicator of tumorigenicity *in vivo.* The level of HA becomes crucial in tissue organization as its elevation in malignant tumor promotes anchorage-independent cell proliferation (Laurent et al., 1996).

These diverse functions of HA are regulated by interactions with a family of proteins having specific affinity towards HA, termed hyaladherins (Toole, 1990). Overexpression of HAS in fibrosarcoma cells stimulates both tumor growth *in vivo* and anchorage-independent growth in soft agar (Kosaki et al., 1999). Likewise, perturbation of endogenous HA interactions either by overexpression of soluble CD44 (Peterson et al., 2000) or by addition of HA oligomers (Auvinen et al., 2000) inhibit anchorage-independent growth.

HA interactions directly influence various intracellular signaling pathways important for cell behavior, through its binding to CD44, leading to internalization and degradation of HA. In this respect, it is significant that some tumor cells exhibit elevated levels of hyaluronidase and the ability to internalize and degrade HA. Thus, penetration of HA rich stroma or production of angiogenic breakdown products of HA may also promote tumor progression (Liu et al., 1996; Yu and Stamenkovic, 1999).

HA-receptor for HA-mediated motility (RHAMM) interactions has also been implicated in tumor cell behavior *in vitro* and *in vivo*. RHAMM is involved in the Ras and extracellular signal-regulated kinase signaling pathways and associates with the cytoskeleton (Turley et al., 2002).

HA–RHAMM interactions induce transient phosphorylation of p^{125}_{FAK} in concert with turnover of focal adhesions in Ras-transformed cells, thus leading to initiation of locomotion. Suppression of this interaction inhibits both cellular locomotion and proliferation *in vitro* and leads to inhibition of tumor growth *in vivo*, whereas overexpression leads to enhanced tumor growth and metastasis. A detailed discussion on the role of both CD44 and RHAMM has been made in this book. However, in addition to CD44 and RHAMM, there is at least another member of hyaladherin family, HABP1 that needs to be discussed in respect to cellular processes.

HYALURONAN BINDING PROTEIN 1 (HABP1)

Purification, Cloning and Characterization

HA binding protein 1 (HABP1) was first isolated from rat kidney by HA sepharose affinity chromatography and was shown to bind to HA with high specificity with Kd of 2.38×10^{-6}/Mol-disaccharide-equivalents but not with any other GAGs (Gupta et al., 1991). The antibodies raised against the purified protein were used to immunoscreen λgt11 cDNA expression library of human skin fibroblast, and a cDNA clone encoding HABP1 was isolated and characterized (Deb and Datta, 1996). The internal polypeptide sequence (83 residues) of the purified HABP1 was found to be identical to the predicted protein sequence derived from HABP1 cDNA, thus confirming the authenticity of the clone. Interestingly, the cDNA sequence of HABP1 shows complete identity with p32, a protein co-purified with the human pre-mRNA splicing factor SF2 (Krainer et al., 1991) and gC1qR, receptor for the globular head of complement subcomponent 1q (Ghebrehiwet et al., 1994).

The open reading frame of HABP1/p32/gC1qR encodes a pro-protein of 282 amino acid residues (Das et al., 1997), which after posttranslational cleavage of the first 73 amino acid residues, form the mature protein of 209 amino acid residues. The mature protein corresponds to 23.7 kDa, but migrates at 34 kDa on SDS-PAGE due to a high ratio of polar to hydrophobic amino acid residues. The mature protein is preceded by a 60 residue-long hydrophobic stretch containing five cysteines, which in turn is preceded by a 13 residues long leader peptide, which contains the mitochondrial targeting signal sequence. The precise role of these 60 residues immediately preceding the mature protein has not yet been determined. However, it has been predicted to play a role in cellular translocation. The mature protein has a calculated pI of 4.15 suggesting its acidic nature. There are 28 glutamic acid, 20 aspartic acid, 16 lysine, 5 histidine, and 4 arginine residues present in the 209 residue long mature protein. In contrast, the first 73 residue long stretch of the pro-protein does not have any glutamic acid, aspartic acid,

lysine or histidine residues, but does have 11 arginine residues. The mature protein has only one cysteine at residue 186.

Specific Features of HABP1 Primary Structure

The computer analysis of the open reading frame (ORF) of HABP1 predicts it to contain three potential *N*-glycosylation sites spread all over the polypeptide chain. The residues involved are ^{109}*WELELN*GTEA*^{118}, ^{131}*VTFNIN*NSIPPTFD*^{144}, and ^{218}*EWKDTN*YTLNT*^{228}. In addition it is found to have one potential putative non-classical dibasic HA binding motif spanning from ^{119}KLVRKVAG**E**K^{128}, one potential tyrosine sulfation site (^{185}DCH**Y***PEDEV^{193}), one protein kinase C phosphorylation site (^{202}DIFS*IREVS^{210}), and five casein kinase II phosphorylation sites involving the residues ^{74}LHT*DGDKAFVD^{84}, ^{200}ESDIFS*IREV^{209}, ^{208}EVSFQS*TGESEWKD^{221}, ^{246}RGVDNT*FADELVEL^{259}, and ^{256}LVEL-ST*ALEHQEYI^{269} in its sequence. HABP1 shows a potential proline directed ^{160}PELTSTP^{166} sequence, which acts as the substrate phosphorylation site for protein kinases like ERK (Majumdar et al., 2002a). The presence of basic amino acid rich nuclear localization sequence ^{94}RKIQKHK^{100} and ^{118}AKLVRK^{123} also raises the possibility of the protein to be a nuclear protein. The amino acid sequence alignment studies confirm that it does not have any homology with other HA binding proteins (Deb and Datta, 1996).

HA Binding Motif in HABP1/p32/gC1qR

HABP1/p32 is the first HA binding protein belonging to a non-link module superfamily, whose three dimensional structure was solved by X-ray crystallography (Yang et al., 1994). The HA binding motif (^{119}KLVRKVAGEK^{128}) (Deb and Datta, 1996) is found to be in the loop region between the β_2 and β_3 sheet, solvent exposed and independent in topological arrangement. Structure-based alignment of p32/HABP1 sequence from human, mouse, *C. elegans* and *S. cerevisiae* shows identical HA binding motifs in human and mouse, whereas basic residues like Arg-122, Lys-123 and acidic residue Glu-127 are conserved in all four species. In contrast to the canonical definition of HA binding motif (Yang et al., 1994), HABP1 shows conservation of an acidic amino acid residue (E^{127}) in its HA binding motif. It has been shown that E^{127} is involved in the salt bridge formation with R^{246} allowing the clustering of positive charge along the putative HA-binding motif of required spacing critical for HA binding capacity for most of the non-link module hyaladherins. In addition, it has been shown that the whole three-dimensional structure is important for the HA–HABP1 interactions (Jiang et al., 1999). HABP1 is suggested to exist in a molten globule-like state in the absence of salt or at very low salt

conditions, which seem to be responsible for its molecular chaperone-like activities. Presence of counter ions in the molecular environment reduces the electrostatic repulsion by screening the charges, and hence affecting the three dimensional structure and thermodynamic stability of HABP1, due to the existence of more compact structure under acidic pH or at high ionic strength at neutral pH (Jha et al., 2003). Among all the tested ligands, only HA–HABP1 interaction is found to be tightly regulated with change in pH and ion concentration suggesting this interaction to be highly significant in terms of the biological functions. However, it is also observed that the N- and C-termini truncated variants of HABP1 with truncated α-helices do not undergo oligomerization, but their binding affinities for hyaluronan remains comparable to that of HABP1 (Sengupta et al., 2004).

Three-Dimensional Structure of HABP1/p32/gC1qR

The crystal structure of HABP1 determined by Jiang et al. (1999) at 2.25 Å resolution reveals that HABP1 adopts a novel fold with seven consecutive anti-parallel β-strands flanked by one N-terminal and two C-terminal α-helices. These monomers form a doughnut-shaped quaternary structure with an unusually asymmetric charge distribution on the surface. The doughnut-shaped trimer has an outer diameter of approximately 75 Å, an average inner diameter of approximately 20 Å, and a thickness of approximately 30 Å. All three subunits have similar conformations. Monomeric HABP1 does not have any obviously distinct domains. Each monomer consists of seven consecutive β-strands, designated from β1 through 07, which form a highly twisted anti-parallel β-sheet with β1 nearly perpendicular to β7. The β-strands are flanked by one N-terminal and one C-terminal α-helices. All the helices are located on the same side of the β-sheet. Helix αB lies parallel to the β-sheet, with helix axis perpendicular to the direction of individual strands.

Helix αB and the N-terminal portion of the helix αC make extensive hydrophobic contacts with the β-sheet, which appears to be essential for the stability of the structure. The N-terminal helix αA does not contact the β-sheet within the same monomer, but forms an anti-parallel coiled coil with the C-terminal portion of αC. This coiled coil region is important for homo-oligomerization.

Three HABP1 monomers form a doughnut-shaped quaternary structure. The channel has a diameter of about 20 Å. But the loops connecting β6 and β7 partially cover the channel, reducing the diameter of the opening to about 10 Å. The channel wall is formed by β-sheets from all the three subunits. But this β wall is distinct from all other β structures such as β-sheet and β-barrels. Because of high degree of twisting of β-strands, adjacent monomers do not form contiguous sheets. The coiled coil region

of αA and αB forms extensive intermolecular contacts: αA packs with antiparallel αB of an adjacent monomer and C-terminal region of αC packs against the back of the β-sheet. Most of these interactions are hydrophobic in nature, except for the intermolecular electrostatic pairing of Arg-246 and Asp-79. In summary, the overall structure of the trimer can be visualized as if the β-sheets form a hyperboloid-shaped spool with the α-helices wrapping around it.

HABP1 is a very acidic protein with a pI of 4.15. However, the charge distribution on the protein surface is very asymmetric. Both the sides of the doughnut and the inside of the channel have a high concentration of negatively charged residues. In contrast, the opposite side of the doughnut is much less negatively charged. This polarity in charge distribution clearly suggests asymmetric functional roles for the two sides of this protein.

Chromosomal Localization and Genomic Organization of HABP1

By using fluorescence *in situ* hybridization (FISH), chromosomal localization of gC1qR/HABP1 is assigned to human chromosome 17p13.3, showing 99.5% similarity, from base 928 to base 1163, with STS WI-9243, a STS flanking marker of human chromosome 17p12-p13 (Majumdar and Datta, 2003). The cDNA sequence of HABP1 shows complete identity with p32 (Genbank accession no. M69039), a protein co-purified with the human pre-mRNA splicing factor SF2 (Krainer et al., 1991) and gC1qR (Genbank accession no. X75913), receptor for the globular head of complement subcomponent 1q (Ghebrehiwet et al., 1994). The gene has been named as HABP1 by HUGO nomenclature Committee of GDB (Accession no. 9786126) and referred as the synonym C1QBP/p32/HABP1 in human genome.

The genomic organization of HABP1/gC1qR gene including its 5′- and 3′-flanking regions spans about 7.8-kilobase pair (kb). From the first codon of the initiation methionine to the stop codon, the gene spans 6055 bp. There are six exons and five introns in the C1qBP gene. The size of the exons range from 94 bp (exon 3) to 232 bp (exon 1), and that of the introns range from 128 bp (intron 5) to 3156 bp (intron 2). A poly (A) signal is located at 369 bp from the stop codon (Majumdar et al., 2002b; Lim et al., 1998).

The first pseudogene for HABP1 was reported in the gene poor region of chromosome 21 and was flanked by six (three on each side) pseudogenes (Hattori et al., 2000). The complete sequencing of the human genome paved way for the search for more such elements in the human genome. Genomic sequence search with the HABP1 cDNA further revealed the existence of more HABP1 pseudogenes, extending to chromosomes 15, 11, and 4, each varying in length and similarity to the parental cDNA sequence. All these pseudogene sequences lack a 5′ promoter sequence and

possess multiple mutations, with the insertion of premature stop codons in all three reading frames, confirming their identity as processed pseudogenes (Majumdar et al., 2002b). The gene encoding hyaluronan binding protein 1 (HABP1) and its homologs have been reported across eukaryotes, from yeast to human. Further, we reported not only the presence of HABP1 pseudogene in other animal species, but also the presence of a homologous sequence in *Methanosarcina barkeri*, an ancient life form. This sequence has 44.8% homology to the human HABP1 cDNA and 45.3% homology with the HABP1 pseudogene in human chromosome 21. The presence of this HABP1 cDNA-like fragment in *M. barkeri* might enable us to shed light on the evolution of the HABP1 gene and whether it was present in a common ancestral organism before the lineages separated (Sengupta et al., 2004).

Subcellular Localization

The complete cDNA sequence of HABP1, corresponding to the pro-protein, shows the presence of only a mitochondrial localization signal (Dedio et al., 1998). However, immunofluorescence and subcellular fractionation studies have detected this protein or its homologues not only in the mitochondrial matrix in yeast (Seytter et al., 1998) and mammals (Muta et al., 1997) but also on the cell surface (Gupta et al., 1991), cytoplasm (Dedio et al., 1998), and nucleus (Simos and Georgatos, 1994) of cultured cell lines.

Primary sequence of HABP1 has demonstrated a distinct mitochondrial localization signal at the N-terminal part of the pro-protein. This signal comprises of an amphipathic helix with basic residues (Muta et al., 1997). Transient expression system in PLC cells has demonstrated that the cDNA encoding full length protein localizes to the mitochondria, while the cDNA encoding the mature form of HABP1 targets it to the cytosol (Muta et al., 1997). This clearly shows that the mitochondrial localization signal exists only in the first 73 amino acids. Furthermore, the yeast homologue of HABP1 (Yeast Mam33p), is located in the mitochondrial matrix and plays an important role in the oxidative phosphorylation. The acidic surface of HABP1 is rich in aspartic and glutamic acid residues. This enables it to serve as a high capacity divalent cation storage protein and modulate the mitochondrial matrix cation concentration. Another interesting feature of HABP1 is its high negative charge density and asymmetric charge distribution, which suggests its possible mode of association with the inner mitochondrial membrane. The negatively charged side of HABP1 may potentially bind to the inner mitochondrial membrane in the presence of divalent metal ions.

Immunolocalization studies using tagged HABP1 have shown that the protein is localized mainly in the nucleus and cytosol (Van Leeuwen and O'Hare, 2001). Interaction of HABP1 with viral proteins also targets it to the nucleus (Matthews and Russell, 1998). These diverse subcellular localizations of HABP1, coupled with its various interacting proteins, led to the proposition that it could be a component of the trafficking pathway

connecting the nucleus, mitochondria, and cytoplasm and the export pathway to the cell surface (Van Leeuwen and O'Hare, 2001). Though this evolutionary conserved protein is said to be ubiquitous in nature, it is not expressed equally all over the cell, as it shows differential localization in different cell lines under various physiological conditions. It belongs to a class of protein that is initially targeted to mitochondria, but found subsequently at highly specific locations (Soltys et al., 2000).

Though enough evidence exists for mitochondrial matrix localization of HABP1, the explicit function of the protein in mitochondria and other subcellular sites is still unclear.

EVIDENCE FOR HABP1 TO BE INVOLVED IN TUMOR DEVELOPMENT

It is evident that HABP1 is present in the human fibroblast and binds specifically to HA, so it would be interesting to study the physiological significance of HABP1.

HABP1 as an Adhesive Protein

Hyaluronan binding protein, initially referred to as hyaluronectin, the term first coined by Delpech et al. (1993), was reported on the cell surface in normal fibroblasts, tumor cell lines, and sperm using indirect immunofluorescence (Gupta and Datta, 1991), while its secretory nature was established on its detection in the serum free medium of macrophage tumor cell line. A differential expression of HABP1 on the cell surface of the sub-populations of AK-5 cells, a transplantable histiocytic tumor cell line, was observed. Cell fractions responsible for developing both ascites and solid tumors were found to contain higher amounts of HABP1 than fractions which are capable of producing only ascites, suggesting its involvement in solid tumor formation. HABP1 coating on the plates allowed more cells to attach, which could be specifically blocked by antibody against HABP1, indicating its possible role in cell attachment (Gupta and Datta, 1991). The pre-treatment of AK-5 cells with HABP1 antibody abolished their capacity to grow as solid tumors, but the cells retained their capacity to grow as ascites tumor, further confirming the adhesive property of HABP1 (Gupta and Datta, 1991). However, in our consecutive papers, we made an observation that according to amino acid compositions and other properties (D'Souza and Datta, 1986), this protein is different from other HA binding proteins described by Delpech et al. (1993), such as hyaluronectin. Therefore, in later reports we refrained from using the term hyaluronectin and referred to it as hyaluronic acid or

hyaluronan binding protein. Finally, after its molecular cloning from human fibroblast (Deb and Datta, 1996) and reporting its localization on human chromosome 17p12-p13 (Majumdar and Datta, 1998), we referred it as to hyaluronan binding protein 1 as accepted by HUGO and we followed this nomenclature subsequently.

Earlier the above observations led us to propose that the adhesive interaction of this protein may be crucial for HABP1 in cellular signaling, anchoring the tumor cells to the endothelium during the initial phases of tumor formation. The presence of ^{247}RGVD^{250} instead of RGD alone by sequence analysis also supported these observations.

HABP1 and Its Proposed Roles in Signal Transduction

Among the proposed ligands interacting with HABP1, HA is the only ligand which falls under the category of non-protein in nature and can interact at physiological conditions (Jha et al., 2003). A few physiological roles had been proposed for HABP1 in macrophage cell adhesion: signal transduction, mammalian reproduction, and pathological infection on the basis of *in vitro* and *in vivo* experiments.

Signal Transduction

Cell surface receptors are known to recognize various extracellular matrix components as their ligands and play a crucial role as signal transducers. HABP1 was reported to strongly bind to HA & was present on the cell surface too.

This protein was observed to be highly phosphorylated in transformed fibroblasts compared with normal fibroblasts. Phosphorylation was enhanced in the presence of its ligand, i.e. hyaluronan, but not in the presence of other glycosaminoglycans. The regulation of the cellular and cell surface phosphorylation of HA binding protein by HA, PMA, and calyculin-A was demonstrated in different cell lines. Hyaluronan enhanced the phosphorylation of PLC-γ in association with increased formation of IP_3, both of which are specifically blocked by pretreatment of the cells with purified anti-hyaluronan binding protein antibodies (Gupta and Datta, 1991).

The role of HABP1 in hyaluronic acid-induced cellular signaling in lymphocytes has been examined. The binding of ^{125}I-HA to lymphocytes *in vivo* was found to be inhibited by pre-incubation of the cells with anti-34 kDa HA binding protein antibodies, thus confirming 34 kDa HA binding protein as the specific HA-receptor in lymphocytes. The HA-induced cell aggregation, tyrosine phosphorylation and cytoskeletal protein phosphorylation demonstrates early cellular signaling events in lymphocytes. Further, during mitogen-induced lymphocyte signaling, *in vivo* phosphorylation by HA and the inhibition of cellular aggregation and

IP_3 formation by anti-HA binding protein antibodies revealed that 34 kDa HA binding protein is one of the potential mediators in HA-induced signal transduction (Gupta and Datta, 1991). The role of hyaluronan binding protein 1 (HABP1) in cell signaling was further investigated and *in vitro* kinase assay demonstrated that it is a substrate for MAP kinase (Majumdar et al., 2002a). Phosphorylation of endogenous HABP1 was also observed following treatment of J774 cells with PMA. HABP1 was co-immunoprecipitated with activated ERK, confirming their physical interaction in the cellular context. Upon PMA stimulation of normal rat fibroblast (F111) and transformed (HeLa) cells, the HABP1 level in the cytoplasm gradually decreased with a parallel increase in the nucleus. In HeLa cells, within 6 h of PMA treatment, HABP1 was completely translocated to the nucleus, which was prevented by PD98059, a selective inhibitor of ERK. We also observed that the nuclear translocation of HABP1 is concurrent with that of ERK, suggesting that ERK activation is a requirement for the translocation of HABP1. It is thus established for the first time that HABP1 is a substrate for ERK and is an integral part of the MAP kinase cascade implying its universal role in signaling.

Upregulation of HABP1 in Apoptosis Induction

The MAP kinase cascade and cell cycle regulation pathway are closely related since the activation of the former and its downstream signaling determines whether the cell will follow normal growth and division or undergo apoptosis. Thus in continuation, the upregulation of HABP1 expression was reported in HeLa cells at 18 and 24 h of cisplatin treatment that induces apoptosis in HeLa cell (Fig. 4.1). Quantification of HABP1 expression by flow cytometry confirmed a two-fold increase in total intracellular HABP1 expression at 24 h of cisplatin treatment. Under the same conditions the HABP1 transcript level measured by semi-quantitative RT PCR showed 2.5-fold increase ascertaining transcriptional regulation of HABP1 during cisplatin-induced apoptosis. Further, in normal HeLa cells, though a small amount of HABP1 can be detected in nucleus, but with apoptosis induction, the protein is mainly concentrated around the nuclear periphery at 18 h of cisplatin treatment. It is present both in the nucleus as well as in the cytosol at 24 h of treatment, suggesting its nuclear translocation during apoptosis. To substantiate our findings, prior to the cisplatin treatment, the expression of HABP1 was reduced by short interference of RNA (siRNA) mediated knockdown. Significant reduction in apoptotic cell population was detected in cisplatin-treated HeLa cells with disrupted HABP1 conferring resistance to cisplatin-induced apoptosis (Guo et al., 1999). Like cisplatin, TNF-α, an inducer of apoptosis upregulates HABP1/gC1qR in bone marrow cells (Guo et al., 1999).

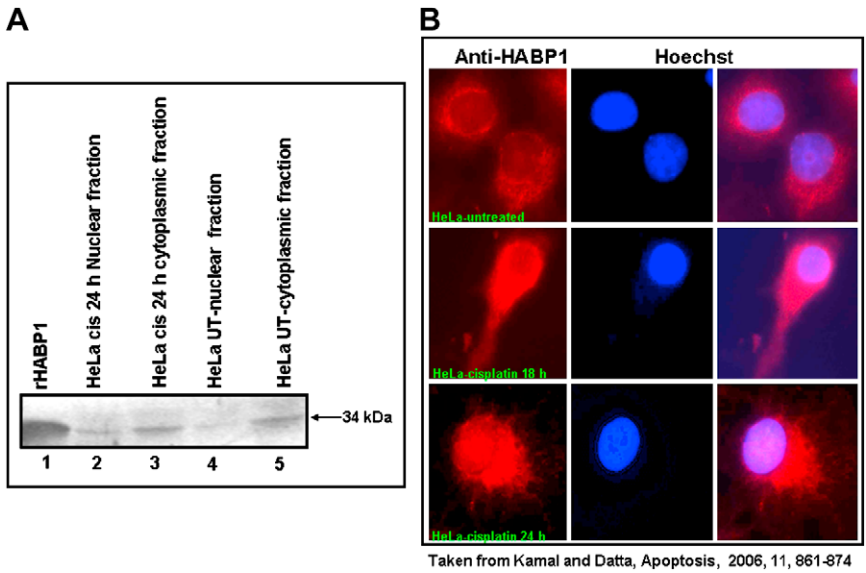

FIGURE 4.1 Translocation of HABP1 from cytosol to nucleus during cisplatin-induced apoptosis. (**A**) Immunodetection of isolated nuclear and cytoplasmic fractions of HeLa untreated and cisplatin-treated cells with anti-HABP1 antibody. Densitometry analysis shows a two-fold increase in nuclear levels of HABP1 in cisplatin treated HeLa cells at 24 h of treatment (Lane 2) in comparison to nuclear levels in untreated control (Lane 4). However, the cytoplasmic levels do not show any considerable change in cisplatin treated (Lane 5) and untreated HeLa cells (Lane 3). (**B**) Immunofluorescence analysis of HeLa untreated and cisplatin treated cells at 100× magnification. HABP1 is present predominantly in the cytoplasm with very little amounts in the nucleus of untreated HeLa cells. At 18 h of cisplatin treatment, HABP1 is concentrated around the nuclear periphery and by 24 h of cisplatin treatment, i.e. which marks apoptosis induction, HABP1 is present in the whole nucleus as well as in the cytoplasm. (See Page 1 in Color Section at the back of the book).

ECTOPIC EXPRESSION OF HABP1 INDUCES APOPTOSIS, AUTOPHAGIC VACUOLES, AND MITOCHONDRIAL DYSFUNCTION

Being an endogenous MAP kinase substrate, HABP1 may have wider implication in maintaining the delicate balance between cell cycle progression and apoptosis as the other proteins linked with MAP kinase pathway are associated with such functions.

We demonstrate that HABP1, when overexpressed in normal rat skin fibroblasts, remained in the cytosol, primarily concentrated around the nuclear periphery. However, HABP1 overexpressing cells showed extensive vacuolation and reduced growth rate, which was restored by frequent

medium replenishment. Further investigation revealed that there is an induction of Bax expression in HABP1 overexpressing cells which failed to enter into the S-phase (Meenakshi et al., 2003) as detected by FACS analysis, and this finally undergoes apoptosis observed by TUNEL assay.

Another interesting feature identified in the stable transfectant expressing HABP1 is the appearance of autophagic vacuoles. Characterization of cytoplasmic vacuoles revealed that most of these vacuoles were autophagic in nature, resembling those generated under stress conditions (Sengupta et al., 2004). The induction of autophagic vacuoles as a defense mechanism against apoptotic cell death in cancer cells by dietary chemopreventive agents is a common practice.

The ectopic expression of HABP1 in fibroblasts has provided us with a model system to examine its functional characterization. We further demonstrated (Chowdhury et al., 2008) that though HABP1 accumulation started in mitochondria from 48 h of growth, induction of apoptosis with the release of cytochrome c and apoptosome complex formation occurred only after 60 h. This mitochondrial dysfunction was due to a gradual increase in reactive oxygen species (ROS) generation in HABP1 overexpressing cells. Along with ROS generation, increased Ca^{2+} influx in mitochondria leading to a drop in membrane potential was evident. Interestingly, upon expression of HABP1, the respiratory chain complex I was shown to be significantly inhibited, followed by defective mitochondrial ultrastructure. The reduction in oxidant generation and drop in apoptotic cell population accomplished by disruption of HABP1 expression, corroborated the fact that excess ROS generation in HABP1 overexpressing cells which lead to apoptosis, was due to mitochondrial HABP1 accumulation.

Differential Expression of Hyaluronic Acid Binding Protein 1 (HABP1)/P32/C1QBP During Progression of Epidermal Carcinoma

Keeping in mind that HA level is critical for development of carcinoma, and its significant role in cellular signaling, the expression profile of HABP1 was investigated from initiation to progression of epidermal carcinoma in mice, induced by benzo[α] pyrene (B[α]P) exposure (Ghosh et al., 2004). During tumor initiation, HABP1 accumulated in inflammatory subsquamous tissue and with progression, the protein was also seen to overexpress in papillomatic and acanthotic tissue (Fig. 4.2). With the onset of metastasis, HABP1 overexpression was confined to metastatic islands, while it disappeared gradually from the surrounding mass. Such expression profiles in metastasized tissue were supported by decreased levels of HABP1, both at protein and transcript levels. These observations taken together suggest that the changes in HABP1 level coincide with specific

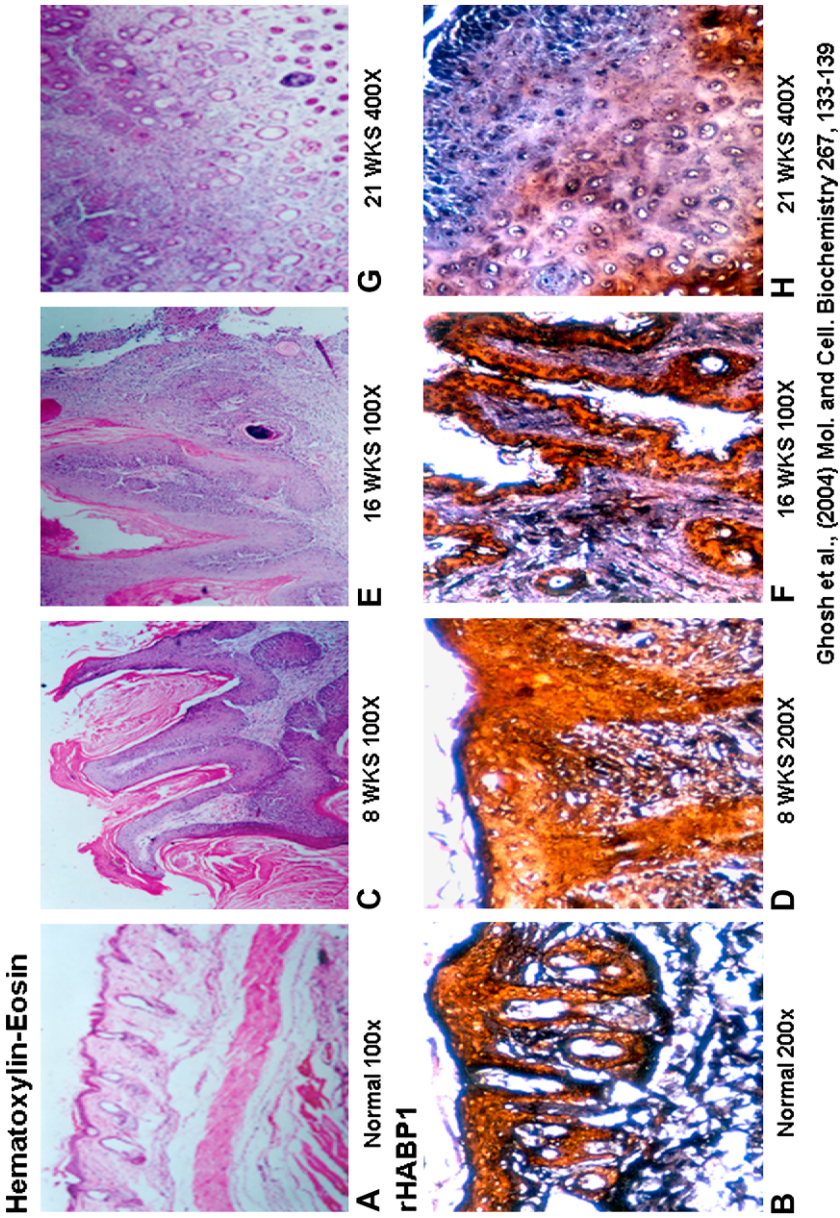
Hematoxylin-Eosin
rHABP1
A Normal 100x
B Normal 200x
C 8 WKS 100X
D 8 WKS 200X
E 16 WKS 100X
F 16 WKS 100X
G 21 WKS 400X
H 21 WKS 400X
Ghosh et al., (2004) Mol. and Cell. Biochemistry 267, 133-139

FIGURE 4.2 Immunohistochemical analysis. Immunohistochemistry of papilloma sections with anti-HABP1 antibody (anti-HABP1) [b, d, f, h] compared with hematoxilin eosin (he) stained [a, c, e, g] stages of metastasis. Normal and the B(a)P treated mice and their papillomatic tissues were collected after 8 weeks, 16 weeks, 21 weeks, each group being comprised of six mice. The normal skin tissue displaying reticulopapillodermal appendages into fatty subcutaneous tissue are shown in (**A**). With the initiation of lesions, varying degrees of dysplasia were observed in squamous cells at about 8 weeks (**C**). The hypoplastic hair follicles and sebaceous glands of early lesions gradually became hyperplastic, giving rise to advanced hyperkeratosis and acanthosis in 16-week lesions (**E**). The 21-week biopsy plates (**G**) demonstrated the proliferating clumped cells deep down, with pleomorphism and atypia in rapidly dividing nuclei, forming proliferating metastatic islands. Normal skin tissue was examined which showed expression throughout the epidermis squamous tissues, as well as in the reticulopapillodermal appendages (**B**). At 8 weeks of carcinogenesis, advanced chronic inflammation was observed infiltrating into the submucosa with HABP1 accumulation (**D**). The infiltrating proliferating squamous cells, with irregular hyperkeratosis extending towards the skin had cyst-like appendages of acanthosis and papillomatosis of the epidermis, and the accumulation of HABP1 (**F**). Examination of the epidermal carcinoma at 21 weeks with anti-HABP1 antibody revealed the accumulation of HABP1 in all proliferating squamous epidermal tissues, embedded papillary dermal cells, and compact islands of proliferating metastasis cells (**H**). As a control when sections were probed with pre-immune serum, no detectable DAB precipitation was observed (data not shown). (See Page 2 in Color Section at the back of the book).

stages of tumor progression, that lead to disruption of its interaction with HA, implying a role in the regulation of tumor metastasis.

With this in view, accumulation of HABP1 in proliferating compact metastatic islands and its downregulation in the surrounding tissues, suggest the disruption of HA–HABP1 interactions. This leads to modification of tissue organization and promotion of invasion. HABP1/gC1qR is cleaved by membrane-type1 matrix metalloproteinase (MT1-MMP1) which has a crucial role in tumor invasion by degrading and remodeling ECM (Seiki and Yana, 2003). The cleavage site of MTI-MMP resides specifically in HABP1 at Gly^{79} Gln^{80} lying within the structurally disordered connecting β3 and β4 strands of HABP1 (Jiang et al., 1999). This reduction in the level of HABP1 in the surrounding mass of metastatic tissue can be attributed to its high sensitivity to MTI-MMP degradation (Ghosh et al., 2004).

Using the combinatory Ig library with phage display, overexpression of gC1qR in a variety of adenocarcinomas was observed in thyroid, colon, pancreatic, gastric, esophageal, and lung adenocarcinomas (Rubinstein et al., 2004).

ASSOCIATION OF ALLELIC LOSS AT 17P13.3 CHROMOSOME WITH CANCER INITIATION AND PROMOTION

With the advent of the human genome project, as the hidden scripts of DNA are revealed, the mysteries behind various diseases are gradually

becoming clear. Population genetics aims to discover genetic variants (or polymorphisms) that influence traits by assessing correlations between genetic and phenotypic variation. The most abundant source of genetic variation in the human genome comprises single nucleotide polymorphisms (SNPs). Associating SNPs with human disease phenotypes has great potential for direct clinical application by providing new and more accurate genetic markers for diagnostic and prognostic purposes and, possibly, novel therapeutic targets.

The gene encoding HABP1/p32/gC1qR is reported to be localized on chromosome 17p13.3. Literature reviews showed that multiple chromosome 17 loci may be involved in cancer initiation and promotion. Loss of heterozygosity (LOH) studies have established that chromosome 17 may be a hotspot for chromosomal aberrations in various cancers like ovarian cancer, colorectal cancer, breast cancer, esophageal squamous cell carcinoma, and others. 17p13.3 is one such allele that gets lost in these carcinomas. Phillips et al. (1996) examined sporadic epithelial tumors for LOH at different loci on chromosome 17p. They found 80% of informative tumors had allelic loss in 17p13.3. Huang et al. (2000) showed that loss of this locus is associated with esophageal squamous cell carcinoma while Haga et al. (2001) reported its possible involvement in breast cancer. Therefore, it is hypothesized that HABP1 may be involved in the cancer initiation and promotion. Thus, it is now important to screen the SNPs in HABP1 to find out its association with various carcinomas as we already know that HABP1 is expressed differentially during the various stages of tumor induction and it is shown to be involved in several cellular processes that are linked with tumor initiation and promotion.

Here, it is pertinent to mention the recent report (Fogal et al., 2008), that not only supports our hypothesis but elucidates the specific role of this protein as a molecular target in tumor cells and tumor stroma. p32/gC1qR/HABP1 was shown as a cellular receptor for LYP-1, a tumor homing peptide that binds to tumor associated lymphatic vessels having antitumor activity. Corroborating our findings, they too consider the significant elevation of HABP1 cell surface level as a new marker of tumor cells.

CONCLUSION

HABP1 is a ubiquitously present glycoprotein having specific affinity towards HA, originally purified from rat tissue. Having immunological cross-reactivity with human tissue, it was used to identify the human homologue and shown to be located at human chromosome 17p13.3. Genomic organization and sequence analysis confirm its multifunctionality; it is identical with SF2/p32, the protein co-purified with

splicing factor on the receptor of globular C1q and shown as a synonym in the human genome.

Experimental evidence suggests the probable involvement of HABP1 in tumor development. It is shown to be an endogenous substrate of MAP kinase, and is involved in cell cycle regulation and apoptosis induction. Upon ectopic expression of HABP1 there is generation of excess ROS in the fibroblasts, creating intrinsic mitochondrial dysfunction along with induction of autophagic vacuoles and ultimately apoptosis. Finally, differential expression of HABP1 and loss of HABP1 in skin papilloma suggest its involvement in tumor initiation and promotion. Linkage analysis with different cancer patient samples also suggests the loss of heterozygosity of human chromosome 17p13.3, where HABP1 is located.

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CHAPTER

5

CD44 Meets Merlin and Ezrin: Their Interplay Mediates the Pro-Tumor Activity of CD44 and Tumor-Suppressing Effect of Merlin

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CD44 PROMOTES TUMOR INVASION AND METASTASIS

Long before the CD44 gene was identified, the hyaluronan (HA)-enriched extracellular matrix had been shown to support and promote cell migration during embryogenesis, inflammation, and tumorigenesis (Lesley et al., 1993; Sherman et al., 1994; Kincade et al., 1997; Stamenkovic, 2000). Moreover, HA production is often increased at the sites of tumor invasion and metastasis, and the binding affinity of CD44 for HA is often increased in proliferating and migrating cells (for a review, see Toole, 2004; Fig. 5.1A,B). As the primary cell-surface receptor for HA (Aruffo et al., 1990), CD44 binds to HA through its link protein module (Stamenkovic et al., 1989; Banerji et al., 2007). Many of CD44's functions are mediated through its binding to HA, including its ability to promote tumor invasion and angiogenesis by localizing matrix metalloproteinase-9 (MMP-9) on the tumor cell surface and by activating latent TGF-beta through its associated MMP-9 (Yu and Stamenkovic, 1999; 2000).

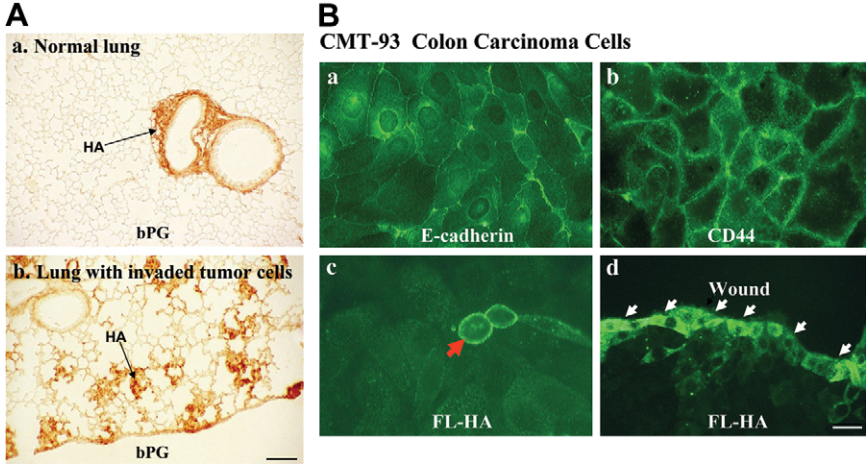

FIGURE 5.1 HA is upregulated at the sites of tumor invasion and metastasis, and the binding affinity of CD44 to HA is tightly regulated. **A**, HA production in normal mouse lung (A-a) and at the sites of infiltration by metastatic TA3 mammary carcinoma cells (A-b). Mouse lung sections, with or without tail-vein injection of TA3 mammary carcinoma cells 72 hours earlier, were used to detect HA production *in situ* with a biotinylated HA-binding proteoglycan (bPG, Yu et al., 1997) probe. **B**, Binding of fluorescein-labeled HA (FL-HA) to CMT-93 colon carcinoma cells (Xu and Yu, 2003) is enhanced in proliferating (B-c) and migrating (B-d) cells. CMT-93 cells express a high level of E-cadherin (B-a) and CD44 (B-b). A arrow in B-c indicates the proliferating CMT-93 cells and the white arrows in B-d show the migrating tumor cells at the edge of a newly formed wound, which was formed by a single cross of the monolayer of CMT-93 cells with a sterile yellow pipette tip. (See Page 3 in Color Section at the back of the book).

A recent study has further confirmed the functional relationship between CD44 and activation of TGF-beta signaling. This study demonstrated that CD44+ breast carcinoma cells are enriched in stem-cell markers and display a poor clinical outcome and an activated TGF-beta signaling pathway (Shipitsin et al., 2007). An interaction between CD44 and HA has also been found to promote chemoresistance of non-small cell lung carcinoma cells (Ohashi et al., 2007). Furthermore, studies have shown that CD44 expression is higher in tumors than in the corresponding normal tissues (Masumura and Tarin, 1992; Notterman et al., 2001; Sun et al., 2006 – data derived from www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE4290) and that highlevels of CD44 expression correlate with poor prognosis in non-Hodgkin's lymphoma (Pals et al., 1989; Jalkanen et al., 1991).

Several lines of evidence support the notion that CD44 promotes tumor progression: for example, overexpression of CD44 promotes tumor growth and metastasis in several experimental tumor models (Sy et al., 1991; 1992; Bartolazzi et al., 1994); and the v6-containing CD44 isoform confers metastatic behavior on rat pancreatic carcinoma cells (Gunthert et al., 1991). In addition, there is a good correlation between increased expression of CD44 and enhanced tumor invasiveness by some tumor types. For example, a CD44 proteoglycan can promote the motility and invasiveness of a melanoma cell line (Faassen et al., 1992), the expression of CD44 isoforms is correlated with the invasive capacity of various human breast carcinoma cell lines (Culty et al., 1994), increased CD44 expression is required for AP-1-mediated tumor invasion (Lamb et al., 1997), and CD44 promotes breast cancer invasion and metastasis to the liver (Ouhtit et al., 2007). Moreover, CD44 plays an essential role in promoting invasiveness and survival of metastatic breast carcinoma cells (Yu and Stamenokovic, 1999; 2000; Yu et al., 1997) and disruption of the CD44 gene has been reported to prevent the metastasis of osteosarcomas (Weber et al., 2002).

In contrast to these results, several other reports have suggested a very different role for CD44 in influencing the growth and metastasis of certain types of cancer. In particular, they have indicated that the expression of CD44 is repressed in neuroblastoma cells (Shtivelman and Bishop, 1991) and it is not required for the growth and metastasis of MDAY-D2 lymphosarcoma cells (Driessens et al., 1995). In addition, CD44 has been implicated in tumor suppression in prostate cancers (Kallakury et al., 1996; Gao et al., 1997; Lou et al., 1999); however, a recent study has shown that highly purified CD44+ prostate carcinoma cells are enriched in prostate cancer stem/initiating cells that display a high level of tumorigenicity and metastatic capacity (Patrawala et al., 2006). Furthermore, CD44+CD24+ESA+ pancreatic cancer cells (Li et al., 2007) and the CD44+ population of human head and neck squamous cell carcinoma cells (HNSCCs) (Prince et al., 2007) have been described as displaying the stem cell properties and CD44+CD24–/low breast cancer-initiating cells are more resistant to radiotherapy (Phillips et al., 2006) and

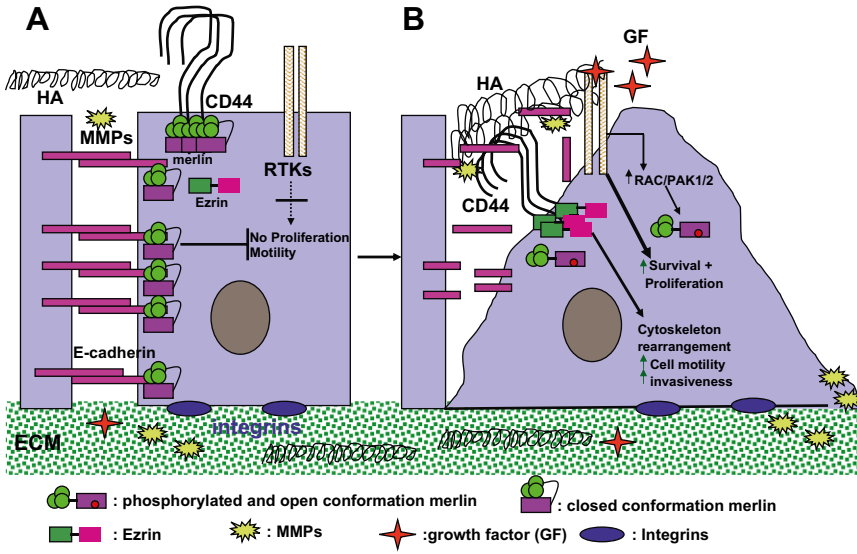

FIGURE 5.2 A working model of CD44 signaling through merlin and the ERM proteins. A detailed description of this model is given in the text.

display higher tumorigenicity than do other carcinoma cell subtypes (Liu et al., 2007). Moreover, an essential role has been suggested for CD44 in engraftment of leukemia stem/initiating cell in the bone marrow, an event that is essential to leukemia development (Williams and Cancelas, 2006). Together, these results point to an essential role for CD44 in the formation and/or maintenance of cancer stem cells.

The apparent discrepancies in the results of these studies most likely reflect the fact that CD44 plays different roles in different types of tumors or at different stages of tumor development, in part, because of differences in the availability of downstream CD44 effectors in the cellular microenvironment. In particular, recent studies have shown that the interaction of CD44 with the ERM proteins and/or merlin can lead to different functional outcomes (Figs. 5.2 and 5.3), and these differences may help explain the apparent inconsistencies in the observed effects of CD44.

CD44-MEDIATED SIGNALS AND THE DOWNSTREAM EFFECTORS OF CD44

CD44 has been linked to numerous signaling pathways: The CD44–HA interaction activates c-Src and focal adhesion kinase (FAK, Turley et al., 2002); CD44 serves as a co-receptor for c-Met (Matzke et al., 2007), and it modulates signals by members of the ErbB family of receptor tyrosine

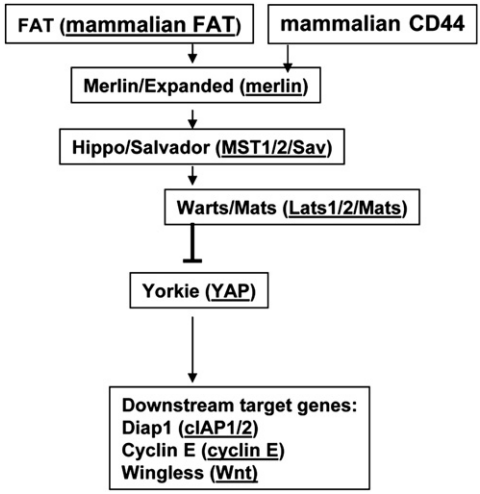

FIGURE 5.3 Merlin-Hpo–Wts–Yki signaling pathway in *Drosophila*. The mammalian homologs/orthologs of the components of the *Drosophila* mer-Hippo (Hpo)–Warts (Wts)–Yorkie (Yki) pathway are underlined.

kinases (RTKs) and of the receptors of transforming growth factor (TGF)-beta (Turley et al., 2002; Bourguignon et al., 2002; 2007); it signals through its own COOH-terminal cytoplasmic proteolytic cleavage fragment after shedding its extracellular domain (Itoh and Seiki, 2006); it promotes cell migration by activating Rac1 (Murai et al., 2004); and finally, it signals through its downstream effectors, the best characterized of which are the ERM proteins and merlin (Okada et al., 2007; Edgar, 2007; McClatchey and Giovanni, 2005; Herrlich et al., 2000), which will be the focus of this discussion.

The ERM proteins and merlin serve as cross-linkers between cortical actin filaments and the plasma membrane and regulate actin-cytoskeleton organization and cell motility (Okada et al., 2007; Edgar, 2007; McClatchey and Giovanni, 2005; Hughes and Fehon, 2007). CD44 is among several transmembrane proteins that bind to the ERM proteins and merlin (Tsukita et al., 1994; 1997; Hirao et al., 1996; Sainio et al., 1997; Morrison et al., 2001). Several basic residues in the cytoplasmic tail of CD44 are essential for the interaction between CD44 and the ERM proteins (Yonemura et al., 1998; Legg and Isacke, 1998). Merlin is the product of the neurofibromatosis type 2 (NF2) gene. Mutations in merlin or a loss of its expression are responsible for the NF2 disease, which is characterized by the development of schwannomas, meningiomas, and ependymomas (Baser et al., 2003; McClatchey and Giovanni, 2005; Edgar, 2007; Okada et al., 2007). Two major merlin isoforms, I and II, are products of alternative splicing near the 3′ end of the NF2 gene. Only merlin isoform I has been shown to exhibit tumor-suppressing activity (Sherman et al., 1997).

MERLIN ACTS AS A TUMOR SUPPRESSOR, AND THE MUTANT FORMS OF THIS PROTEIN PROMOTE TUMORIGENESIS

Like the ERM proteins, merlin regulates cytoskeletal remodeling and cell motility (McClatchey and Giovanni, 2005; Edgar, 2007; Okada et al., 2007). Merlin and the ERM proteins display a similar subcellular localization that includes areas of dynamic cytoskeleton remodeling, such as microspikes and membrane ruffles (Arpin et al., 1994; Bretscher et al., 2002). It has been well established that mutations and deletions of merlin cause NF2 and that loss of heterozygosity (LOH) of merlin is associated with sporadic schwannomas, ependymomas, meningiomas, and malignant mesotheliomas (Gutmann et al., 1997; Baser et al., 2003). Genetic analysis of NF2 patients has demonstrated that the deletion mutations in the NH_2-terminal the band **f**our-point-one/**e**zrin/**r**adixin/**m**oesin (FERM) domain occur frequently and are associated with the early tumor onset and poor prognosis (Ruttledge et al., 1996; Koga et al., 1998).

Overexpression of several merlin mutants causes overproliferation of *Drosophila* wing epithelial cells by interfering with the activity of endogenous wild-type (wt) merlin (LaJeunesse, et al., 1998). Transgenic mice expressing a naturally occurring merlin mutant or having a conditional homozygous merlin knockout in Schwann cells develop schwannomas that resemble those associated with NF2 (Giovanni et al., 1999; 2000). Loss of merlin is embryonically lethal in both the mouse and the fruit fly, suggesting broader and essential roles for this protein during embryonic development (Fehon et al., 1997; McClatchey et al., 1997). In addition, mice with a heterozygous merlin knockout (NF2+/−) genotype develop metastatic osteosarcomas, fibrosarcomas, and hepatocellular carcinomas and nearly all of these tumors have lost the wt NF2 allele (McClatchey et al., 1998). Together, these results suggest that merlin serves as a tumor suppressor in a wide array of cells and that loss of merlin may play an important role in tumor progression.

THE FUNCTION OF MERLIN IS REGULATED BY ITS POSTTRANSLATIONAL MODIFICATIONS

The domain organization of merlin is similar to that of the ERM proteins. The highest sequence homology between merlin and the ERM proteins is in the conserved tri-lobe NH_2-terminal FERM domain. In addition to the NH_2-terminal domain (head), merlin has a central alpha-helical region and an extended COOH-terminal tail (Pearson et al., 2000; Shimizu et al., 2002). Like the ERM proteins, merlin can form head-to-tail intra- and intermolecular associations. The conserved residues in the COOH-terminus of merlin and

the ERM proteins constitute the C-ERM association domains (C-ERMADs), which mediate the interaction with their corresponding NH_2-termini.

Head-to-tail self-association, which generates the closed conformation form of merlin, is required for the protein's tumor-suppressing activity (Sherman et al., 1997). Phosphorylation of merlin at the COOH-terminus abolishes this head-to-tail self-association, producing an open conformation and inactivating the tumor-suppressing activity of the molecule (Sherman et al., 1997; Shaw et al., 2001; Kissil et al., 2003). p21-activated kinase 1 (PAK1) and 2 (PAK2), as well as cAMP-dependent protein kinase A (PKA), phosphorylate merlin at Ser518 (Shaw et al., 2001; Kissil et al., 2003; Thaxton et al., 2007), whereas AKT phosphorylates merlin at Thr 230 and Ser 315 (Tang et al., 2007). Phosphorylation of merlin at S518 produces the open conformation (Shaw et al., 2001; Kissil et al., 2003; Thaxton et al., 2007), whereas AKT-mediated phosphorylation at Thr 230 and Ser 315 promotes merlin degradation through ubiquitination (Tang et al., 2007). Conversely, the myosin phosphatase MYPT1–PP1δ dephosphorylates Ser518, resulting in merlin activation (Jin et al., 2006). In addition, merlin is cleaved by calpain, a calcium-dependent cysteine protease, in schwannomas and meningiomas (Kimura et al., 2000), suggesting that merlin can be inactivated and downregulated by calpain-mediated proteolytic cleavage.

MERLIN COUNTERACTS THE EFFECT OF EZRIN AND INHIBITS THE CD44–HA INTERACTION AND CD44 FUNCTION

Studies have shown that merlin interacts with several transmembrane proteins, including CD44 (Tsukita et al., 1994; 1997; Hirao et al., 1996; Bai et al., 2007), NHE-RF/EBP50 (Murthy et al., 1998), β1 integrin (Obremski et al., 1998), and FAT, a large protocadherin (Willecke et al., 2006). Merlin also associates with spectrin (Scoles et al., 1998) and tubulin (Xu et al., 2004). Like the ERM proteins, merlin interacts with membrane-associated binding partners, including CD44, through its NH_2-terminal domain (Tsukita et al., 1994; 1997; Hirao et al., 1996; Bai et al., 2007). The ERM proteins play an important role in organizing the actin-cytoskeleton, which is essential for cell adhesion and migration. In particular, ezrin is involved in promoting tumor metastasis, most likely through its interaction with CD44 (Yu et al., 2004; Khanna et al., 2004). Unlike the ERM proteins, merlin lacks the conventional COOH-terminal actin-binding site, but it contains an unconventional NH_2-terminal actin-binding site (Xu et al., 1998) and interacts indirectly with the actin-cytoskeleton through an actin-binding protein, βII-spectrin (Scoles et al., 1998). The association of merlin with the actin-cytoskeleton likely underlies its effects on the actin-cytoskeletal organization and on cell spreading and motility.

CD44 plays an important role in promoting tumor growth and metastasis, chiefly as a result of its ability to bind HA (for reviews, see Lesley et al., 1993; Sherman et al., 1994; Kincade et al., 1997; Stamenkovic, 2000; Toole, 2004), an interaction that is tightly regulated. Studies have shown that E-cadherin negatively regulates the interaction between CD44 and HA as well CD44 function (Xu and Yu, 2003), and overproduction of HA promotes anchorage-independent growth of CD44-positive breast epithelial cells by inducing an epithelial–mesenchymal transition (Zoltan-Jones et al., 2003), presumably as a result of downregulating E-cadherin-mediated cell–cell adhesion. Furthermore, merlin plays an important role in mediating contact inhibition of cell growth through its interaction with CD44 (Morrison et al., 2001), and loss of merlin destabilizes the cadherin-containing cell–cell junctions, leading to a loss of contact inhibition and elevation of extracellular signal-regulated kinase (ERK) and c-Jun-NH2-kinase (JNK) activity (Shaw et al., 2001; Lallemand et al., 2003; Okada et al., 2005). We have recently shown that increased expression of wt merlin in Tr6BC1 schwannoma cells inhibits the binding of HA to CD44 (Bai et al., 2007). Furthermore, we found that overexpression of merlin inhibits the subcutaneous growth of Tr6BC1 cells in immunocompromised Rag1 mice. In contrast, knocking down the expression of endogenous merlin with shRNAs or overexpression of a merlin deletion mutant that is incapable of inhibiting the CD44–HA interaction can promote subcutaneous growth of Tr6BC1 cells in Rag1 mice (Bai et al., 2007). We have also shown that increased expression of merlin not only inhibits the CD44–HA interaction but also reduces the interaction between CD44 and ezrin and that between CD44 and the actin-cytoskeleton. Together, these results indicate that inhibition of the CD44–HA interaction contributes to the tumor suppressor activity of merlin, and they suggest that the various downstream effectors of CD44, the ERM proteins or merlin, play different or even opposing roles in organizing cortical actin and in tumorigenesis, presumably by activating different signaling pathways (Bai et al., 2007; Figs. 5.2 and 5.3).

MERLIN IS INVOLVED IN SEVERAL IMPORTANT SIGNAL TRANSDUCTION PATHWAYS

Tight control of cell proliferation, death, and motility is critical for maintaining normal tissue homeostasis, and loss of this control is a hallmark of cancer. CD44 acts upstream of merlin, and inhibition of its interaction with HA is one mechanism that underlies the tumor-suppressing function of merlin (Bai et al., 2007). Merlin is uniquely localized for sensing and coordinating extracellular and intracellular signaling pathways. Studies have shown that increased expression of merlin not only inhibits cell proliferation and promotes apoptosis, but also impairs cell–cell and

cell–matrix adhesion, cell spreading, and cell motility (Koga et al., 1998; Xu and Gutmann, 1998). The exact mechanism by which merlin exerts its tumor-suppressing function has not been fully established, but recent findings, including observations that place merlin in the Hippo pathway in *Drosophila* have shed some light on this issue.

Merlin has been shown to reverse the Ras-induced malignancy (Tikoo et al., 1994; Kim et al., 2002), suggesting that it negatively regulates the Ras-signaling pathway. The Rho family of small GTPases (Rho, CDC42, and Rac) are essential downstream effectors of the Ras signaling pathway: overexpression of activated Rac causes transformation of fibroblasts, and Rac activity is required for Ras-induced transformation (Qiu et al., 1995). Merlin has been shown to act downstream of Rac (Morrison et al., 2007), and its overexpression inhibits Rac-mediated anchorage-independent growth and transformation (Shaw et al., 2001). Merlin is thought to mediate contact inhibition of growth by promoting the formation and stabilization of adherens junctions (Lallemand et al., 2003) and to inhibit mitogenesis in confluent cells by blocking recruitment of Rac to the plasma membrane (Okada et al., 2005). In addition, expression of active Rac1 induces PAK-1-dependent phosphorylation of merlin at residue S518, causing merlin to adopt an inactive, open conformation (Kissil et al., 2003). PAKs (PAK1–4) are a group of serine/threonine kinases and the immediate downstream effectors of the Rac signaling pathway. Through a feedback loop, merlin inhibits PAK1 activity by binding to the Cdc42/Rac binding domain (PBD) of PAK1 and inhibiting recruitment of PAK1 to focal adhesions (Kissil et al., 2003). Therefore, loss of merlin results in increased activity of PAK1.

Genetic analyses in *Drosophila* have indicated a role for merlin in promoting endocytosis of a number of signaling receptors, including Notch, the epidermal growth factor receptor (EGFR), patched, and smoothened, and of adhesion receptors, E-cadherin and Fat (Maitra et al., 2006). Studies in mammalian cells have consistently indicated that merlin accumulates at and stabilizes the adherens junctions and that upon establishment of contact inhibition, merlin inhibits internalization of EGFR by sequestering it into an insoluble membrane compartment, thereby inhibiting EGFR signaling (Lallemand et al., 2003; Curto et al., 2007).

MERLIN ACTS UPSTREAM OF THE HIPPO SIGNALING PATHWAY IN *DROSOPHILA*

In *Drosophila*, merlin (*mer*) is one of two FERM domain-containing proteins that negatively regulate cell growth and proliferation, the other protein being, expanded (*ex*). *mer* is the functional homolog of human merlin, but whether a functional homolog of *ex* exists in mammals is not yet clear (Pellock et al., 2007). Inactivation mutants of *mer* or *ex*

display reduced apoptosis and increased cell proliferation in *Drosophila* (McCartney et al., 2000; Hamaratoglu et al., 2006). Analysis of *mer* mutants has indicated that merlin signaling occurs upstream of the Hippo (Hpo)/Salvador (Sav)/Warts (Wts)/Mob1 (Hippo) signaling pathway that regulates cell proliferation and survival (McCartney et al., 2000; Hamaratoglu et al., 2006; Curto et al., 2007; Pellock et al., 2007). Hpo and Wts are Thr/Ser kinases of the sterile 20 (Ste20) and the nuclear Dbf2-related (NDR) family, respectively. In *Drosophila*, Hpo phosphorylates and activates Wts, which in turn phosphorylates and inhibits Yorkie (Yki; Huang et al., 2005), leading to lose of its pro-proliferative and anti-apoptotic activity. Conversely, inactivation of the Hpo/Wts pathway results in upregulation and activation of Yki, which in turn upregulates *Drosophila* inhibitor of apoptosis protein 1 (DIAP1) and cyclin E, resulting in increased proliferation and cell survival (Huang et al., 2005; Pellock et al., 2007).

In *Drosophila*, studies have shown that FAT (Willecke et al., 2006; Hamaratoglu et al., 2006), merlin, and Hpo signals (McCartney et al., 2000; Huang et al., 2005; Hamaratoglu et al., 2006; Curto et al., 2007; Pellock et al., 2007) all converge in the activation of Wts and inactivation of Yki through which they influence the expression of a common set of downstream target genes that play essential roles in cell proliferation, survival, and motility, including cyclin E, DIAP1, and wingless (wg) (Cho et al., 2006; Fig. 5.3). The mammalian homologs/orthologs of Hpo, Wts, Yki, DIAP, and wg are mammalian sterile twenty-like (MST) kinase 1 and 2 (MST1/2) (Creasy and Chernoff, 1995), Lats1 and 2 (Chan et al., 2005; Takahashi et al., 2005; Aylon et al., 2006), yes-associated protein (YAP) (Overholtzer et al., 2006; Zender et al., 2006), cellular inhibitor of apoptosis1/2 (cIAP1/2), and the Wnt family proteins (Fodde and Brabletz, 2007; Fig. 5.3), respectively. Like MST1 and MST2, the Lats1/2 genes encode serine/threonine kinases and display anti-tumor activity (St John et al., 1999; Li et al., 2003). Loss of Lats1 causes a predisposition to soft-tissue sarcoma and ovarian tumor development in mice (St John et al., 1999). In addition, mouse embryonic fibroblasts derived from the Lats2-null mice show a growth advantage and display a profound defect in contact inhibition of growth (McPherson et al., 2004). Furthermore, overexpression of Lats1 induces G2/M arrest and subsequent apoptosis (Xia et al., 2002), and Lats2 suppresses RasV12-induced transformation of NIH 3T3 cells (Li et al., 2003).

In contrast, YAP, which encodes the mammalian ortholog of *Yki*, is amplified in human cancers and displays oncogenic activity (Overholtzer et al., 2006; Zender et al., 2006). As a transcriptional co-activator, YAP promotes cell proliferation and inhibits apoptosis by upregulating the expression of cyclin E and cIAP1/2, respectively (Yagi et al., 1999; Vassilev et al., 2001). Like *Yki*, YAP rescues the pupal lethality caused by overexpression of Hpo or Wts in *Drosophila* (Huang et al., 2005). Similarly,

human homologs of Hpo and Wts can rescue their corresponding *Drosophila* mutants (Tao et al., 1999; Wu et al., 2003; Lai et al., 2005). However, it is not yet know whether the entire Hpo–Wts–Yki signaling pathway is conserved in mammalian cells, and the potential relationships between merlin and MST1/2, Lats1/2, YAP, and cIAP1/2 have not been fully established in mammalian cells. A very recent study showed that merlin is a potent inhibitor of high-grade human glioma and that increased expression of merlin correlates with activation of MST1/2-Lats2 signaling pathway and inhibition of Wnt signals (Lau et al., 2008).

A MODEL FOR CD44 SIGNALING THROUGH MERLIN AND THE ERM PROTEINS

Observations in mammalian cells and *Drosophila* point toward a model of CD44 signaling through merlin and the ERM proteins (Fig. 5.2). When there is no contact inhibition, and signaling from the activated receptor tyrosine kinases (RTKs) has occurred (Fig. 5.2B), PAK1 and PAK2 are activated, and these molecules in turn phosphorylate and inactivate merlin. Under these circumstances, CD44 predominantly interacts with ezrin and displays a high binding affinity for HA. The HA–CD44 interaction allows the cells to recruit MMPs and to activate latent TGF-beta, which stimulates motility and invasiveness. The cell-surface bound MMPs may also contribute to the shedding or cleavage of E-cadherin, which leads to further loss of adherens junction integrity and a loss of cell–cell adhesion. In addition, the HA–CD44 interaction enhances RTK-derived mitogenic and survival signals, further promoting the activation of RAC and PAK1/2 and the deactivation of merlin (Fig. 5.2B).

In contrast, once the cells have established contact inhibition through the homophilic E-cadherin interactions and experienced a decrease in the RTK-derived activation signals (Fig. 5.2A), merlin adopts an unphosphorylated closed conformation and displays tumor suppressor activity. Merlin is recruited to the plasma membrane and interacts with CD44 and E-cadherin, stabilizing the homophilic E-cadherin interaction. At this point, the CD44 pool that predominantly interacts with merlin can no longer bind HA, and therefore cell–cell adhesion is enhanced further. Merlin thus stabilizes cell–cell adhesion complexes and renders the cells stationary. Under these conditions (Fig. 5.2A), CD44 cannot function to enhance RTK-derived signals to effectively promote cell survival and proliferation. On the other hand, CD44-associated merlin can activate MST1/2 and Lats1/2, which in turn inactivate YAP. Loss of YAP activity can lead to a reduction in the level of cycle E, cIAP1/2, and Wnt signals, which causes a reduction in cell proliferation and motility and enhanced sensitivity to apoptosis (Figs. 5.2 and 5.3). Thus, the data accumulated to

date suggest that merlin serves as a key point of contact between the extracellular and intracellular signaling networks, and that its function is exerted through its interaction with cell-surface adhesion receptors including, and perhaps predominantly, CD44.

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CHAPTER

6

Hyaluronan-Mediated CD44 Interaction with Receptor and Non-Receptor Kinases Promotes Oncogenic Signaling, Cytoskeleton Activation and Tumor Progression

Lilly Y.W. Bourguignon

OUTLINE

Hyaluronan (HA) Metabolism in Cancer Progression 89
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HYALURONAN (HA) METABOLISM IN CANCER PROGRESSION

Hyaluronan (HA) is a non-sulfated, unbranched glycosaminoglycan consisting of the repeating disaccharide units, D-glucuronic acid and *N*-acetyl-D-glucosamine and found in the extracellular matrix (ECM) of

most mammalian tissues (Toole, 1991; Laurent and Fraser, 1992). HA is often overexpressed at tumor cell attachment sites and appears to play an important role in promoting tumor cell-specific behaviors (Lee and Spicer, 2000; Toole, 2001; Turley et al., 2002). The biosynthesis of HA is regulated by three mammalian HA synthase isozymes, HA synthase1 (HAS-1), HA synthase 2 (HAS-2), and HA synthase 3 (HAS-3) (Weigel et al., 1997; Itano and Kimata, 1996; Itano and Kimata, 1998; Spicer and Nguyen, 1999). Although the three HAS genes are located on different chromosomes, they share a great deal of sequence homology (Spicer and McDonald, 1998; Shyjan et al., 1996; Watanabe and Yamaguchi, 1996). HAS-1 synthesizes a low level of a large-size HA (~$1 \times 10^6 - 1 \times 10^7$ Da) (Spicer and McDonald, 1998; Shyjan et al., 1996). HAS-2 produces relatively high amounts of large-size HA (~$1 \times 10^6 - 1 \times 10^7$ Da) and is definitely involved in cell proliferation, angiogenesis as well as embryonic and cardiac development (Watanabe and Yamaguchi, 1996). HAS-3, which synthesizes a small-size HA (~1×10^5 Da), is one of the most biologically active HAS molecules and is known to contribute to the malignant phenotype in many different cell types (Spicer and McDonald, 1998; Spicer and Nguyen, 1999). Recent studies indicate that the expression of HAS-1 restores the metastatic potential of mouse mammary carcinoma mutant cells that have low HA production and low metastatic capability (Itano et al., 1999). Furthermore, overexpression of HAS-2 and HAS-3 stimulates both tumorigenicity and tumor progression (Kosaki et al., 1999; Liu et al., 2001). In ovarian cancer patients, all three HAS molecules are detected in ovarian carcinoma tissues (Yabushita et al., 2004). In particular, HAS-1 expression in ovarian cancer patients appears to be closely associated with tumor angiogenesis and disease progression; and is also an important predictor of patient survival (Yabushita et al., 2004). Thus, dysregulation of HAS expression and activities often results in abnormal production of HA and directly contributes to aberrant cellular processes such as transformation and metastasis (Zhang et al., 1995).

HA can also be digested into various smaller-sized molecules by hyaluronidases (Stern and Jedrzejas, 2006). Somatic hyaluronidases (acid-active enzymes) have been found to be significantly increased in breast tumor metastases as compared to primary tumors (Bertrand et al., 1997). Currently, at least six hyaluronidase-like gene sequence products [e.g. HYAL-1, HYAL-2, HYAL-3, HYAL-4, *PHYAL*-1, PH-20 (or Spam1)] have been reported. HYAL-1 is considered to be a candidate of tumor suppressor gene product (Csoka et al., 1998; Frost et al., 2000; Lerman and Minna, 2000), whereas HYAL-2 appears to function primarily (but not exclusively) as an oncogene (Lepperdinger et al., 1998; Strobl et al., 1998; Lepperdinger et al., 2001). Both HYAL-1 and HYAL-2 genes are found tightly clustered on chromosome 3p21.3 and are the major hyaluronidases in human somatic tissues (Linebaugh et al., 1999; Nicoll et al., 2002).

HYAL-3 has been detected in chondrocytes (Flannery et al., 1998). The level of HYAL-3 expression increases when fibroblasts undergo chondrocyte differentiation (Nicoll et al., 2002). HYAL-4 based on preliminary evidence appears to be a chondroitinase and *PHYAL*-1 (a pseudogene) is not commonly translated in humans (Csoka et al., 1999). PH-20 (or Spam1), the neutral-active hyaluronidase is detected primarily in testes and in breast tumors (Cherr et al., 2001; Beech et al., 2002). Low pH environment promotes tumor cell-specific behaviors including activation of ECM-degrading enzymes such as hylauronidases for tumor cell invasion (Chambers et al., 1997; Duffy, 1992; Mignatti and Rifkin, 1993). Among all mammalian hylauronidases, HYAL-2 appears to be present in virtually all tissues (Lepperdinger et al., 1998; Strobl et al., 1998). HYAL-2 belongs to a family of endo-*N*-acetylhexosaminidases that hydrolyze HA and to a lesser extent chondroitin sulfates (Lepperdinger et al., 1998; 2001; Strobl et al., 1998). It is a cell surface enzyme via glycosylphosphatidyl-inositol (GPI) linkage to the plasma membrane (Lepperdinger et al., 2001; Rai et al., 2001; Bourguignon et al., 2004). The enzymatic activity of HYAL-2 is upregulated in acidic compartments derived from invaginated plasma membrane microdomains such as lipid rafts (Lepperdinger et al., 2001; Rai et al., 2001). Activated HYAL-2 hydrolyzes high molecular mass HA into intermediate sized fragments of approximately 20 kDa corresponding to about 50 disaccharide units (Lepperdinger et al., 1998). Interestingly, fragments of such size stimulate inflammatory cytokine synthesis (Nobel, 2002). Overexpression of HYAL-2 in murine astrocytoma cells accelerates intracerebral but not subcutaneous tumor formation and invasion (Novak et al., 1999). HYAL-2 also functions as a receptor for the jaagsiekte sheep retroviruses (Rai et al., 2001). In this system, HYAL-2 negatively regulates receptor tyrosine kinase (Danilkovitch-Miagkova et al., 2003) and mediates tumor cell transformation by jaagsiekte sheep retroviruses in lung cancers (Rai et al., 2001). Clearly, activation of ECM-degrading enzymes such as HYAL-2 appears to be closely associated with tumor progression.

CD44 (AN HA RECEPTOR) IN TUMOR PROGRESSION

CD44 denotes a family of cell-surface glycoproteins which are expressed in a variety of cells and tissues including tumor cells and carcinoma tissues (Gunther et al., 1991; Iida and Bourguignon, 1995; Iida and Bourguignon, 1997; Zhu and Bourguignon, 1998; Auvinen et al., 2005; Bourguignon, 2001; Bourguignon et al., 1997; 1998; 1999; 2000; 2001a,b; 2002; 2003; 2004; 2006; 2007; Turley et al., 2002). Nucleotide sequence analyses reveal that many CD44 isoforms (derived by alternative splicing mechanisms) are variants of the standard form, CD44s (Screaton et al., 1992; 1993). The presence of high levels of CD44 variant (CD44v) isoforms

is emerging as an important metastatic tumor marker in a number of cancers (Gunther et al., 1991; Iida and Bourguignon, 1995; Iida and Bourguignon, 1997; Zhu and Bourguignon, 1998; Auvinen et al., 2005; Bourguignon et al., 1997; 1998a,b; 1999; 2000; 2001a,b,c; 2002; 2003; Turley et al., 2002). Recent studies have shown that CD44 is also expressed in tumor stem cells which have the unique ability to initiate tumor cell-specific properties (Al-Hajj et al., 2003). In fact, CD44 is proposed to be one of the important surface markers for cancer stem cells (Al-Hajj et al., 2003). All CD44 isoforms contain an HA-binding site in their extracellular domain and thereby serve as a major cell surface receptor for HA (Underhill, 1992). Both CD44v isoforms and HA are overexpressed at sites of tumor attachment (Toole et al., 2002). It has been determined that HA binding to CD44v isoforms is involved in the stimulation of both receptor kinases (e.g. ErbB2, EGFR, and TGFβ receptors) (Bourguignon et al., 1997; 2001b; 2002; 2006; 2007; Wang and Bourguignon, 2006a,b) and non-receptor kinases (e.g. c-Src and ROK) (Bourguignon et al., 1999; 2001b; 2003) required for a variety of tumor cell-specific functions leading to tumor progression. Some of the cellular and molecular mechanisms controlling the ability of HA to activate CD44v isoform-associated kinases in tumor cells to growth, migrate, and invade other tissues will be described below.

HA-Mediated CD44 Interaction with Receptor Kinases in Tumor Cell Activation

HA-Mediated CD44 Interaction with ErbB2 ($p185^{HER2}$) Kinase

The HER2 oncogene (also called ErbB2 or neu) encodes a 185 kDa membrane protein ($p185^{HER2}$) which contains a single transmembrane spanning region, and a tyrosine kinase-associated cytoplasmic domain (Bargmann et al., 1986). This protein, initially discovered as an activated oncogenic variant (neu), is overexpressed in many epithelial tumors including mammary and ovarian carcinomas (Press et al., 1990; Reese and Slamon, 1997; Meden and Kuhn, 1997). Overexpression or amplification of the HER2 oncogene (ErbB2) in both breast and ovarian cancers is generally associated with a poor prognosis (Raspollini et al., 2006; Malamou-Mitsi et al., 2007; Sasaki et al., 2007). In recent years, much excitement has surrounded the remarkable therapeutic effects of HER-2/neu-blocking antibodies, such as Herceptin™ (Bookman et al., 2003; Longva et al., 2005). Nevertheless, the molecular mechanisms by which HER-2/neu enhances the growth and survival of cancer cells, and induces tumor progression are not completely understood.

Hyaluronan (HA) is known to constitutively regulate ErbB2 tyrosine kinase activity and to influence ErbB2 interaction with CD44 signaling in tumor cells (Bourguignon et al., 1997; 2001b; 2007). Previously, we determined that CD44 and ErbB2 are physically linked to each other via

interchain disulfide bonds in human ovarian tumor cells (Bourguignon et al., 1997). Most importantly, HA binding to a CD44-ErbB2 complex activates the ErbB2 tyrosine kinase activity and promotes tumor cell growth (Bourguignon et al., 1997). HA-mediated CD44-ErbB2 signaling complexes in ovarian tumor cells are also associated with molecular scaffolds and adaptors such as Vav2 (a Rac-specific GEF) and Grb2 (Bourguignon et al., 2001b). HA treatment induces the recruitment of both Vav2 and Grb2 into CD44v3-ErbB2 ($p185^{HER2}$) containing multi-molecular complexes leading to the co-activation of Rac1 and Ras signaling and ovarian tumor cell growth and migration (Bourguignon et al., 2001b). Most recently, we have found that HA/CD44–ErbB2 interaction [mediated by N-WASP (Neural Wiskott-Aldrich Syndrome Protein–an actin-cytoskeleton activator)] promotes β-catenin signaling and actin-cytoskeleton functions leading to certain tumor-specific behaviors (e.g. transcriptional activation and tumor cell migration) and ovarian cancer progression (Bourguignon et al., 2007). Clearly, there are important direct signaling interactions among HA, CD44, and ErbB2 during tumor cell activation (summarized in Fig. 6.1).

HA-Mediated CD44 Interaction with EGFR

Epidermal growth factor receptor (EGFR) contains a single transmembrane spanning region, and a tyrosine kinase-associated cytoplasmic domain (Cohen et al., 1982). Previous studies have found that guanidine nucleotide (GDP/GTP) exchange on Ras is significantly stimulated by tyrosine phosphorylation of EGFR (Cohen et al., 1982). Thus, it appears that EGFR activation mediates Ras-mediated stimulation of a downstream kinase cascade which includes the Raf-1/MEK/MAPK pathway leading to tumor cell growth (Cohen et al., 1982). EGFR is overexpressed in many tumors including head and neck squamous cell carcinomas (HNSCC) (Bonner et al., 2002; Choong and Cohen, 2006; Hoyek-Gebeily et al., 2007). Overexpression or amplification of EGFR in head and neck cancer is generally associated with a poor prognosis (Bonner et al., 2002; Choong and Cohen, 2006; Hoyek-Gebeily et al., 2007). Recently, much excitement has surrounded the remarkable therapeutic effects of FDA-approved EGFR-blocking antibodies, such as cetuximab (Gebbia et al., 2007). Nevertheless, the molecular mechanisms by which EGFR enhances the growth and survival of HNSCC, and induces resistance to chemotherapy are not completely understood.

Hyaluronan (HA) is known to constitutively regulate EGFR tyrosine kinase activity and to influence EGFR interaction with CD44 signaling in HNSCC (Bourguignon et al., 2006; Wang and Bourguignon, 2006a,b). Previously, we determined that CD44 and EGFR are physically linked to each other in human HNSCC (Bourguignon et al., 2006). Most importantly,

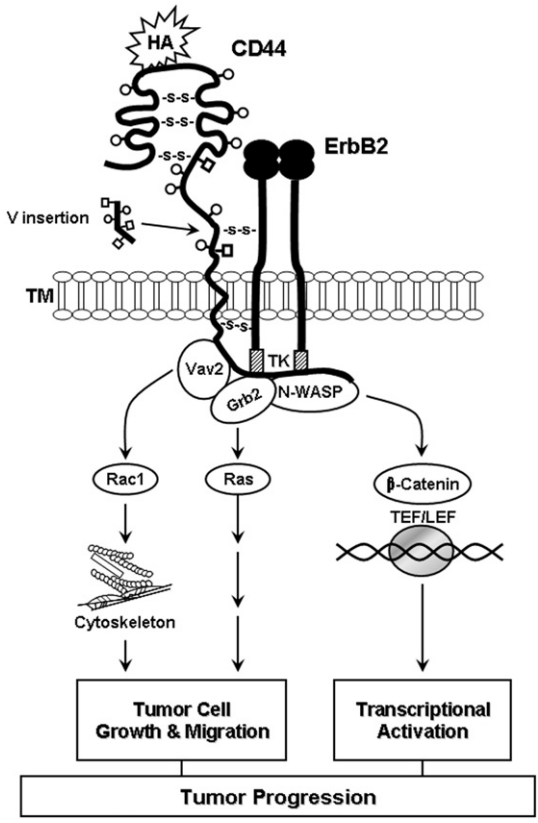

FIGURE 6.1 HA-mediated CD44 interaction with ErbB2 signaling in tumor cells. The binding of HA to CD44 promotes ErbB2 kinase activation leading to Vav2-mediated Rac1 signaling and Grb2-associated Ras activation. The co-activation of Rac1 and Ras induces tumor cell growth and migration. HA binding to CD44 also stimulates N-WASP-mediated activation and β-catenin signaling leading to transcriptional upregulation. All these events (e.g. transcriptional activation, tumor cell growth, and migration) are required for tumor progression.

HA binding to a CD44–EGFR complex activates the EGFR tyrosine kinase activity and promotes HNSCC functions (Bourguignon et al., 2006; Wang and Bourguignon, 2006a,b). HA-mediated CD44–EGFR signaling complexes in HNSCC are also associated with molecular scaffolds and adaptors such as LARG (Leukemia-Associated RhoGEF, a RhoA-specific GEF) (Bourguignon et al., 2006). HA treatment induces the recruitment of LARG into CD44–EGFR containing multimolecular complexes leading to the co-activation of RhoA and Ras signaling and HNSCC cell growth and migration (Bourguignon et al., 2006). Most recently, we have found that HA/CD44–EGFR interaction also promotes Ca^{2+} signaling (Bourguignon et al., 2006; Wang and Bourguignon, 2006a) and cytoskeleton functions (Bourguignon et al., 2006) as well topoisomerase II activation (Wang et al., 2007) leading, HNSCC migration, growth, and multidrug resistance (Wang and Bourguignon, 2006a, b; Wang et al., 2007). Clearly, there are important direct signaling interactions among HA, CD44, and EGFR during HNSCC activation (summarized in Fig. 6.2).

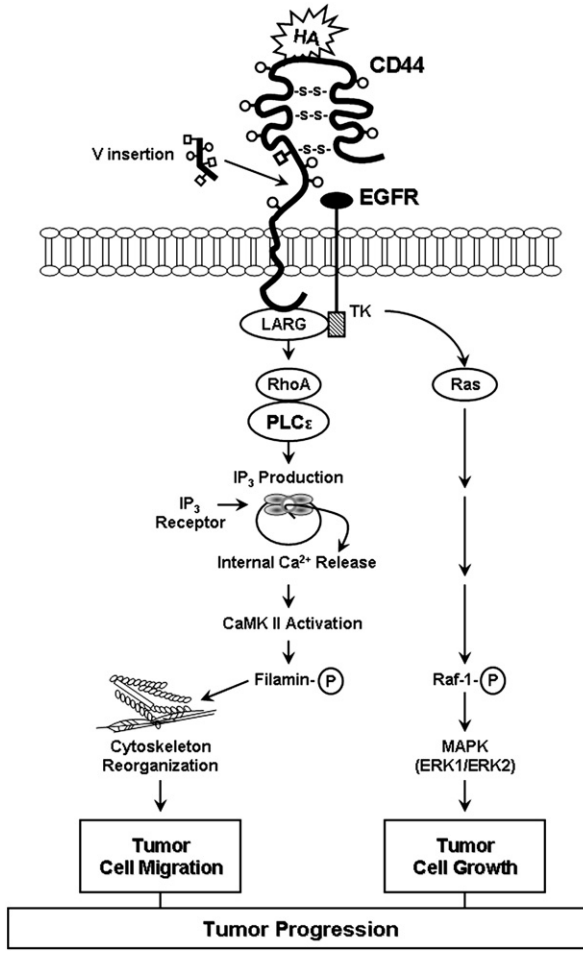

FIGURE 6.2 HA-mediated CD44 interaction with EGFR signaling in tumor cells. HA–CD44 binding is tightly coupled with LARG and EGFR in a complex which can induce LARG-mediated RhoA activation and EGFR signaling-regulated Ras activation. HA/CD44 and LARG-activated RhoA then stimulates PLCε-mediated IP3 production and IP3 receptor-triggered intracellular Ca2+ mobilization resulting in CaMKII activation. CaMKII then phosphorylates the cytoskeletal protein, filamin leading to cytoskeleton reorganization and tumor cell migration. In the meantime, Ras activation by HA-mediated CD44–LARG–EGFR complex formation also promotes Raf-1 phosphorylation, MAPK (in particular, ERK1 and ERK2) activation, and tumor cell growth. Together, we believe that CD44–LARG interaction with EGFR plays an important role in promoting HA-dependent RhoA and Ras co-activation leading to the concomitant stimulation of HNSCC growth and migration required for tumor progression.

HA-Mediated CD44 Interaction with TGFβ Receptor Signaling

Cytokines, such as the transforming growth factor β (TGFβ) superfamily are multifunctional peptides that are known to regulate a diverse set of cellular processes by binding to their specific surface receptors (Blobe et al., 2000). Three mammalian TGFβ isoforms (TGFβ1, TGFβ2, and TGFβ3), coded by different genes, have been identified (Messague et al., 1992). TGFβ interacts with three surface receptors known as type I (TGFβRI), type II (TGFβRII), and type III (TGFβIII) receptors (Blobe et al., 2000). TGFβ1 mediates its activity by high affinity binding to the type II (TGFβII) receptor, which has been identified as a 70–80 kDa transmembrane protein with a cytoplasmic serine/threonine kinase domain (Blobe et al., 2000). For cellular signaling, the TGFβRII requires both its kinase activity and association with members of a series of related 55 kDa TGFβRIs [designated as activin receptor-like kinase-ALK (1 to 6 different subtypes)]. Of these, only ALK5 has been shown to represent a functional TGFβRI (Blobe et al., 2000). Subsequently, the TGFβ signal is propagated from the plasma membranes (via TGFβRII/TGFβI kinases) by phosphorylation of the Smad proteins which belong to a class of intracellular mediators known to regulate transcriptional responses and gene expression in the nucleus (Shi, 2001). The type III (TGFβIII) receptor also binds TGFβ and may function in capturing TGFβ for presentation to the signaling receptors (Lopez-Casilla et al., 1991; Wang et al., 1991). In cancers, the TGFβ receptors on tumor cells are often mutated or functionally defective (Carcamo et al., 1995). For example, defective ligand binding to the cell surface caused by the absence of TGFβRII, or expression of a truncated form or splice variant of TGFβRII, may account for the resistance to activated TGFβ in cancer cells (Kadin et al., 1994; Park et al., 1994). Some studies also indicate that decreased expression of TGFβRII may contribute to breast cancer progression, and is related to a more aggressive phenotype in both *in-situ* and invasive carcinomas (Oft et al., 1998; Yin et al., 1999; Yoneda et al., 2001).

In order to gain a better understanding of TGFβ receptor signaling in breast cancer cells, we have examined the interaction between CD44 and the transforming growth factor β (TGFβ) receptors (a family of serine/threonine kinase membrane receptors) in human metastatic breast tumor cells (MDA-MB-231 cell line). Immunological data indicate that both CD44 and TGFβ receptors are expressed in MDA-MB-231 cells; and that CD44 is physically linked to the TGFβ receptor I (TGFβRI) [and to a lesser extent to the TGFβ receptor II (TGFβRII)] as a complex *in vivo*. Scatchard plot analyses and *in vitro* binding experiments show that the cytoplasmic domain of CD44 binds to TGFβRI at a single site with high affinity [an apparent dissociation constant (K_d) of ~1.78 nM]. These findings indicate that TGFβRI contains a CD44 binding site (Bourguignon et al., 2002).

Furthermore, we have found that the binding of HA to CD44 in MDA-MB-231 cells stimulates TGFβRI serine/threonine kinase activity which, in turn, increases Smad2/Smad3 phosphorylation and parathyroid hormone-related protein (PTHrP) production (well-known downstream effector functions of TGFβ signaling). Most importantly, TGFβRI kinase activated by HA phosphorylates CD44 which enhances its binding interaction with the cytoskeletal protein, ankyrin, leading to HA-mediated breast tumor cell migration. Overexpression of TGFβRI by transfection of MDA-MB-231 cells with TGFβRIcDNA stimulates formation of the CD44-TGFβRI complex, the association of ankyrin with membranes, and HA-dependent/CD44-specific breast tumor migration. (Bourguignon et al., 2002). Taken together, these findings strongly suggest that CD44 interaction with the TGFβRI kinase promotes concomitant activation of both HA-dependent and TGFβ-specific signaling pathways required for ankyrin–membrane interaction, tumor cell migration, and important oncogenic events (e.g. Smad2/Smad3 phosphorylation and PTHrP production) during metastatic breast tumor progression (summarized in Fig. 6.3).

HA-Mediated CD44 Interaction with Non-Receptor Kinases in Tumor Cell Activation

HA-Mediated CD44 Interaction with c-Src Signaling

The Src family kinases are classified as oncogenic proteins due to their ability to activate cell proliferation (Barone and Courtneidge, 1995; Broome and Hunter, 1996), spreading (Kaplan et al., 1995; Rodier et al., 1995), and migration (Hansen et al., 1996; Hall et al., 1996; Rahimi et al., 1998) in many cell types including epithelial tumor cells (Tanaka et al., 1996; Summy and Gallick, 2003). The amino terminus of Src contains a myristoylation (or palmiotylation) site which is important for membrane association (Thomas and Brugge, 1997; Schlessinger, 2000). Src also contains several functional domains including SH3 and SH2 (Src homology) domains, the catalytic protein tyrosine kinase (PTK) core, and a conserved regulatory tyrosine phosphorylation site (Thomas and Brugge, 1997; Schlessinger, 2000). Certain amino acid residues in the c-Src molecule play an important role in modulating its kinase activity. Mutations of specific key amino acids result in either upregulation or downregulation of c-Src kinase activity. For example, replacement of tyrosine 527 with phenylalanine (e.g. Y527F, the dominant-active form of c-Src kinase) strongly activates c-Src kinase transforming capability and enzyme activities (Kmiecik and Shalloway, 1987). Mutation of Lysine 295 to Arginine (e.g. K295R, the dominant-negative form of c-Src kinase) renders c-Src kinase defective and reduces c-Src kinase-mediated biological activities (Kmiecik and Shalloway, 1987; Bagrodia et al., 1991).

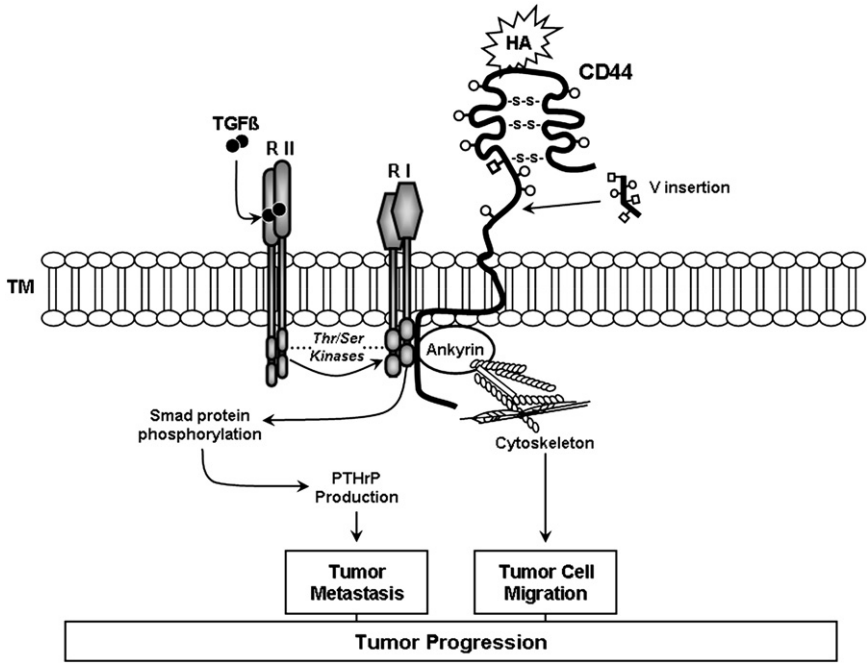

FIGURE 6.3 HA-mediated TGFβ receptor signaling in tumor cells. CD44 is tightly complexed with TGFβRI. This CD44-associated TGFβRI kinase can be activated by HA and/or TGFβ leading to phosphorylation of Smad proteins (Smad2 and Smad 3) and PTHrP production which is known to cause metastasis. Moreover, HA and/or TGFβ-activated CD44-TGF-βRI kinase is also capable of phosphorylating CD44. Most importantly, CD44 phosphorylation enhances its binding to the cytoskeletal protein, ankyrin, which, in turn, interacts with the cytoskeleton and induces tumor cell migration. Both oncogenic signaling events (e.g. Smad2/Smad3 phosphorylation and PTHrP production) and ankyrin-cytoskeleton function contribute to tumor progression.

In addition, it has been observed that the interaction between Src kinase and membrane-linked molecules regulates receptor signaling and various cellular functions (Thomas and Brugge, 1997; Schlessinger, 2000). In fact, CD44-mediated cellular signaling has been suggested to involve Src kinase family members (Taher et al., 1996; Ilangumaran et al., 1998). For example, Lck, one of the Src kinase family members is found to be closely complexed with CD44 during T-cell activation (Taher et al., 1996). CD44 also selectively associates with active Src family tyrosine kinases (e.g. Lck and Fyn) in glycosphingolipid-rich plasma membrane domains (lipid rafts) of human peripheral blood lymphocytes (Ilangumaran et al., 1998). Moreover, the cytoplasmic domain of CD44s has been shown to be involved in the recruitment of the Src family (e.g., Src, Yes, and Fyn) in prostate tumor cells during anchorage-independent colony growth (Zhu and Bourguignon,

1998). Collectively, all these observations support the notion that c-Src kinases participate in CD44-mediated cellular signaling.

Overexpression or increased activity of c-Src is frequently detected in human ovarian cancers, implicating the involvement of c-Src in the etiology of ovarian carcinomas (Summy and Gallick, 2003; Tanaka et al., 2004). CD44 is known to be tightly coupled with c-Src kinase in ovarian tumor cells (SK-OV-3.ipl cells) (Bourguignon et al., 2001c). Our previous work determined that the cytoplasmic domain of CD44 binds to c-Src kinase at a single site with high affinity (Bourguignon et al., 2001a). Most importantly, HA interaction with CD44 stimulates c-Src kinase activity which, in turn, increases tyrosine phosphorylation of the cytoskeletal protein, cortactin. Subsequently, tyrosine phosphorylation of cortactin alters its interaction with actin-cytoskeleton leading to tumor cell migration (Bourguignon et al., 2001c). Therefore, HA/CD44-mediated cellular signaling clearly involves Src kinase family members during tumor cell activation (summarized in Fig. 6.4).

HA-Mediated CD44 Interaction with RhoA-ROK Signaling and Cytoskeleton Function

Members of the Rho subclass of the Ras superfamily [small molecular weight GTPases, (e.g. RhoA, Rac1, and Cdc42)] are known to transduce a variety of signals regulating many different cellular processes (Hall, 1998). Overexpression of certain RhoGTPases in human tumors often correlates with a poor prognosis (Fritz et al., 1999; Li and Lim, 2003). In particular, coordinated RhoGTPase signaling is considered to be a possible mechanism underlying cell proliferation and motility, an obvious prerequisite for metastasis (Fritz et al., 1999; Li and Lim, 2003).

Several enzymes have been identified as possible downstream targets for RhoGTPases (e.g., RhoA) in regulating cytoskeleton-mediated cell motility (Kimura et al., 1996; Amano et al., 1997; Fukata et al., 1999). One such enzyme is Rho-Kinase (ROK, also called Rho-binding kinase) which is a serine-threonine kinase (Kimura et al., 1996; Amano et al., 1997; Fukata et al., 1999). ROK interacts with RhoA in a GTP-dependent manner and phosphorylates a number of cytoskeletal proteins such as adducin (Fukata et al., 1999) and myosin phosphatase (Kimura et al., 1996). Structurally, ROK is composed of catalytic (CAT), coiled-coil, Rho-binding (RB), and pleckstrin-homology (PH) domains (Kimura et al., 1996; Amano et al., 1997; Fukata et al., 1999). Overexpression of the Rho-binding domain (a dominant-negative form) of ROK by transfecting breast tumor cells with RB cDNA induces reversal of tumor cell-specific phenotypes (Bourguignon et al., 1999; 2003). A previous study showed that ROK is responsible for the phosphorylation of CD44-associated cytoskeletal proteins during actin filament and plasma membrane interaction. When ROK is overexpressed

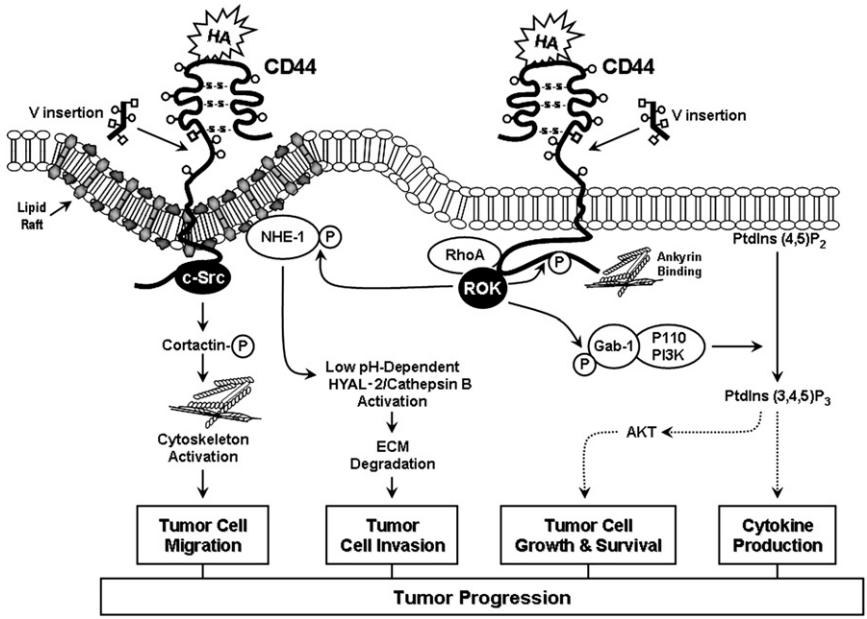

FIGURE 6.4 HA-mediated c-Src signaling and ROK activation in tumor cells. The binding of HA to CD44 stimulates Src signaling in lipid rafts leading to cortactin phosphorylation and cytoskeleton-mediated tumor cell migration. HA binding to CD44 also promotes RhoA-mediated ROK activation. Activated ROK then phosphorylates NHE1 (in lipid rafts). Most importantly, the phosphorylation of NHE1 by ROK promotes Na^+-H^+ exchange activity, intra-endosomal/lysosmal pH changes, and extracellular acidification leading to a concomitant activation of at least two low pH-dependent enzymes-HYAL-2 (located at lipid rafts) and cathepsin B (secreted in the medium) required for extracellular matrix (ECM) degradation, HA modification and tumor cell invasion. HA/CD44-activated ROK is also capable of phosphorylating the cytoplasmic domain of CD44. CD44 phosphorylation by ROK enhances its binding interaction with ankyrin. Finally, HA/CD44-activated ROK phosphorylates the linker molecule, Gab-1. Most importantly, phosphorylation of Gab-1 by ROK promotes the membrane localization of Gab-1 and PI3 kinase to CD44 and activates certain isoforms of PI3 kinase to convert PtdIns $(4,5)P_2$ to PtdIns $(3,4,5)P_3$ leading to AKT activation, cytokine production, and tumor cell behaviors (e.g. tumor cell growth, survival, and invasion) required for tumor progression.

or constitutively activated, changes in actin-cytoskeleton organization occur which are similar to those observed during normal Rho-activated conditions (Bourguignon et al., 1999; 2003). ROK is overexpressed in breast tumor cells and is capable of phosphorylating the cytoplasmic domain of CD44 (Bourguignon et al., 1999). Moreover, phosphorylation of the cytoplasmic domain of CD44 by ROK enhances its binding interaction with ankyrin (Bourguignon et al., 1999). Overexpression of the Rho-binding domain (a dominant-negative form) of ROK by transfecting breast tumor cells with RB cDNA induces reversal of tumor cell-specific phenotypes

(Bourguignon et al., 1999). These findings support the notion that ROK plays a pivotal role in CD44–ankyrin interaction and RhoA-mediated oncogenic signaling required for membrane-cytoskeleton function and metastatic tumor cell migration.

HA/CD44-activated RhoA also promotes ROK phosphorylation of the linker molecule, Grb2-associated binder-1 (Gab-1) (Bourguignon et al., 2003). Most importantly, phosphorylation of Gab-1 by ROK promotes the membrane localization of Gab-1 and phosphatidylinositol 3-kinase (PI3 kinase) to CD44 and activates certain isoforms of PI3 kinase to convert PtdIns (4,5)P_2 to PtdIns (3,4,5)P_3 leading to AKT activation, cytokine production, and tumor cell behaviors (e.g. tumor cell growth, survival, and invasion) required for breast tumor progression (Bourguignon et al., 2003). These findings suggest that there is a strong connection between RhoA-ROK activation and Gab-1-associated PI3 kinase activation during the stimulation of breast tumor cells by HA–CD44 interaction.

Previous studies indicate that Na^+-H^+ exchanger-1 (NHE1) is one of the principal intracellular pH (pHi) regulatory molecules in breast tumor cells (Boyer and Tannock, 1992). NHE1 appears to be involved in the aberrant regulation of both extracellular pH (pH_e) and intracellular pH (pH_i) in human breast tumor cells under nutrient-depleted conditions (Reshkin et al., 2000). Certain observations on the localization and regulation of certain NHE isoforms suggest that there are connections between NHE activity and cholesterol-enriched lipid rafts (Poli et al., 1991). Interestingly, NHE1 also serves as one of the cellular substrates for RhoA-activated Rho-kinase (ROK) (Tominaga et al., 1998). NHE1 phosphorylation by RhoA-activated ROK induces actin stress fiber assembly (Tominaga et al., 1998). Recent evidence indicates that the binding of HA to CD44 activates RhoA-ROK activity which, in turn, promotes NHE1 phosphorylation and Na^+-H^+ exchange activity leading to intracellular acidification and creates an acidic extracellular matrix environment. These events result in hyaluronidase (HYAL-2)-mediated HA catabolism, HA modification, cysteine proteinase (cathepsin B)-mediated matrix degradation, and cancer progression (Bourguignon et al., 2004) (summarized in Fig. 6.4).

In conclusion, we believe that the binding of HA to CD44 is capable of stimulating the activation of receptor kinases (e.g., ErbB2, EGFR, and TGFβ receptor) and non-receptor kinases (e.g., c-Src kinases and ROK) in a variety of tumor cells. These events result in a coordinated ''cross-talk'' among multiple signaling pathways (see Figs 6.1–6.4) leading to oncogenesis and subsequent cancer progression.

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CHAPTER

7

Adhesion and Penetration: Two Sides of CD44 Signal Transduction Cascades in the Context of Cancer Cell Metastasis

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INTRODUCTION: HA, CD44, THE TUMOR MICROENVIRONMENT, AND METASTASIS

Hyaluronan (HA) is a major glycosaminoglycan (GAG) of the extracellular matrix, constituted by a variable number of repeating glucuronic acid and *N*-acetylglucosamine disaccharide subunits. Synthesized on the inner face of the plasma membrane by three distinct isoforms of

hyaluronan synthase (HAS), this GAG is then extruded into the surrounding extracellular space (Toole, 2004). Increased HA deposition in the stroma has been observed in many cancers and is believed to result from increased HA synthesis by both malignant cancer cells and tumor stromal cells. Elevations in stromal HA levels have been correlated with poor prognosis and reduced survival. For example, elevated HA expression in the tumor stroma in breast cancer has been shown to correlate with poorly differentiated tumors, auxiliary lymph node status, and short overall survival (Auvinen et al., 2000; Karihtala et al., 2007). It is proposed that the tumor-progressing effect of HA is mediated through a combination of its biophysical, hydrodynamic properties on the matrix and its capacity to activate a number of different cell-surface receptors that are expressed on tumor and stromal cells. This chapter will focus on how one of the principal HA receptors, CD44, is associated with conferring the malignant progression of tumor cells, by contributing to the modulation of cancer cell adhesion and cancer cell invasion within the tumor microenvironment.

CD44 is a cell-surface glycoprotein receptor with a well-defined HA binding domain within its amino-terminal ectodomain. As a consequence of the insertion of differential exon products or alternatively, through extensive addition of carbohydrate moieties, the extracellular domain of CD44 has also been shown to bind other ligands besides HA, including osteopontin and fibroblast growth factor. The intracellular carboxy-terminal tail of the receptor has been shown to interact with a number of adaptor proteins including ankyrin, ezrin, GAB1, Src kinases, and Rho-family GTPases (Ponta et al., 2003; Thorne et al., 2004). Consequently, through the association with these intracellular proteins, CD44 is coupled to a range of distinct signaling pathways. As a result of the diversity of signaling pathways induced downstream of CD44, HA has been shown to modulate cell adhesion, cell motility, cell proliferation, cell survival, and cancer cell invasion (see Toole, 2004). In this regard, these experimental observations in cell-based studies provide evidence to implicate HA-induced CD44 signaling in conferring many of the properties essential for tumor cells to successfully negotiate each of the defined stages of the metastatic cascade.

In addition to the cancer cell, CD44 is also expressed on numerous other cell types within the tumor microenvironment, including endothelial cells and specialist cells such as osteoclasts. Therefore, given that cancer cells synthesize and secrete HA and OPN into the extracellular space, these signaling competent molecules may bind to CD44 and induce responses in other cell types present within the tumor stroma. For example, HA is a chemotactic factor for fibroblasts and breast cancer cells (Tzircotis et al., 2005; Tzircotis et al., 2006), thus cancer cell-derived HA may enhance fibroblast recruitment at the tumor site. Other studies show that activation of CD44 is critical in driving osteoclast motility within the bone (Chellaiah

et al., 2003a,b). This suggests a potential role for breast cancer cell-derived HA or OPN in stimulating osteoclasts to resorb bone, a common clinical feature of advanced stages of this disease. Therefore, activation of CD44 receptors in multiple cell types present within the tumor microenvironment may have added significance to cancer metastasis, besides that of simply modulating cancer cell function. Indeed, the potential importance of host-cell CD44 expression to cancer metastasis is inferred from studies conducted exploiting the CD44 knock-out mouse in which CD44 deficiency was shown to lead to an inability of osteosarcoma cells to develop spontaneous metastasis (Weber et al., 2002).

Interest in the significance of CD44 with regard to tumor progression has also been fueled by the characterization of this receptor as a cell surface marker of cancer stem cells (Al-Hajj et al., 2003; Dontu et al, 2005). In respect of metastasis, the capacity of stem cells to disseminate and colonize distant organs during the early stage of disease has been suggested to underpin a later re-emergence of metastatic disease during patient relapse. Breast cancer cells that disseminate to the bone marrow cavity during the early stage of disease have been shown to express the characteristic breast cancer stem cell signature, i.e., high CD44+/low CD24– expression (Balic et al., 2006). Further clinical studies have reported that the prevalence of CD44+/CD24– cells also favors distant metastasis in breast cancer patients (Abraham et al., 2005). Therefore, experimental and clinical studies both suggest a key role for HA and CD44 in promoting metastasis of cancer. While HA and CD44 signaling has been reported to increase epithelial cell proliferation, the predominant pathological function of CD44 is associated with the role of this receptor in orchestrating and co-ordinating cell–cell and cell–matrix interactions within the tumor microenvironment. Consequently, this has implications in facilitating the passage of malignant cells through the matrix at the primary tumor site, in addition to promoting the initial arrest, penetration, and outgrowth of metastatic cancer cells at secondary tissues.

PENETRATION: CD44 PROMOTED INVASION AND INTRAVASATION

The capacity of tumor cells to invade their surrounding space is regarded as one of the ascribed ''hallmarks of cancer'' (Hanahan and Weinberg, 2000) and is a key factor in the acquisition of malignancy. CD44 signal transduction has been associated in regulating cell migration, cell invasion, and cell adhesion; properties that facilitate the cell–cell and cell–matrix interactions that underlie tumor-promoted angiogenesis, localized cellular invasion, the entry (intravasation) and exit (extravasation) of cancer cells from the bloodstream, and ultimately, penetration and

colonization of a secondary organ or tissue. Loss of E-cadherin expression has been reported in the early stages of localized epithelial tumor invasion. Decreased E-cadherin expression not only facilitates adherens junction disassembly, but is reported to lead to aberrant activation of the β-catenin transcription factor resulting in increased transcription of genes driving cell proliferation, survival, and cell invasion. Studies conducted in colorectal epithelium revealed that mutation of the tumor suppressor protein APC, a negative regulator of β-catenin activation, resulted in enhanced expression of CD44 in these epithelial cells, suggesting that this cell surface receptor is a downstream transcriptional target of β-catenin (Wielenga et al., 1999). Therefore, deregulation of β-catenin transcription resulting from either the inactivation of tumor suppressor genes or alternatively, as suggested in colorectal cancer, from the disruption of adherens junctions may contribute to the emergence of increased CD44 expression in epithelial cells during the early stages of tumor development (Kim et al., 1994). More recently, CD44 signaling cascades have also been shown to induce activation of the β-catenin signaling pathway suggesting that disruption of β-catenin signaling may serve as an important initiating and potentiating event in facilitating the localized tumor invasion promoted by CD44 (Bourguignon et al., 2007).

HA-induced activation of CD44 and/or RHAMM has been shown to induce the invasion of multiple cancer cell lines. In addition to facilitating a cytoskeletal-dependent promotion of cell migration, our increasing understanding of how CD44 signaling regulates the activity of an expanding list of extracellular proteases is fundamental to the ability of cancer cells to degrade the physical barriers provided by the extracellular matrix and basement membrane. Enzymes of the matrix metalloproteinase (MMP), serine protease, and cysteine protease families have each been reported to underpin cancer cell invasion and metastasis (Deryugina and Quigley, 2006; Mohamed and Sloane, 2006; Gocheva and Joyce, 2007; Lopez-Ortin and Matrisian, 2007). It is increasingly acknowledged that there is significant interplay between these different protease families in promoting tumor invasion. For example, cysteine proteases have been shown to regulate the activity of serine proteases through the promotion of enzymatic cleavage and removal of pro-peptide sequences. In addition, the differential patterns of enzymatic expression, their complex mode of activation, the breadth of substrate-specificity and indeed the redundancy amongst the enzymes have all been contributing factors in the sub-optimal targeting of these species in the clinic. Consequently, targeting of individual proteases may not necessarily provide a favorable therapeutic intervention. Instead, understanding how the expression and/or activity of these species is co-ordinately regulated may identify alternative means of controlling the spectrum of proteolytic activity acquired by malignant cancer cells and may show enhanced clinical benefit in treating locally

advanced or metastatic cancer. As discussed below, CD44 has been shown to regulate proteolytic activity in cancer cells as a consequence of the signal transduction properties of the receptor and its role in serving as a physical scaffold for proteases on the cell surface (Fig. 7.1).

MMP-9 or Gelatinase B has high avidity to degrade type IV collagen, an extracellular matrix protein that is enriched in the basement membrane. MMP-9 has been shown to associate with the large ectodomain of CD44 in murine mammary carcinoma cells (Yu and Stamenkovic, 1999).

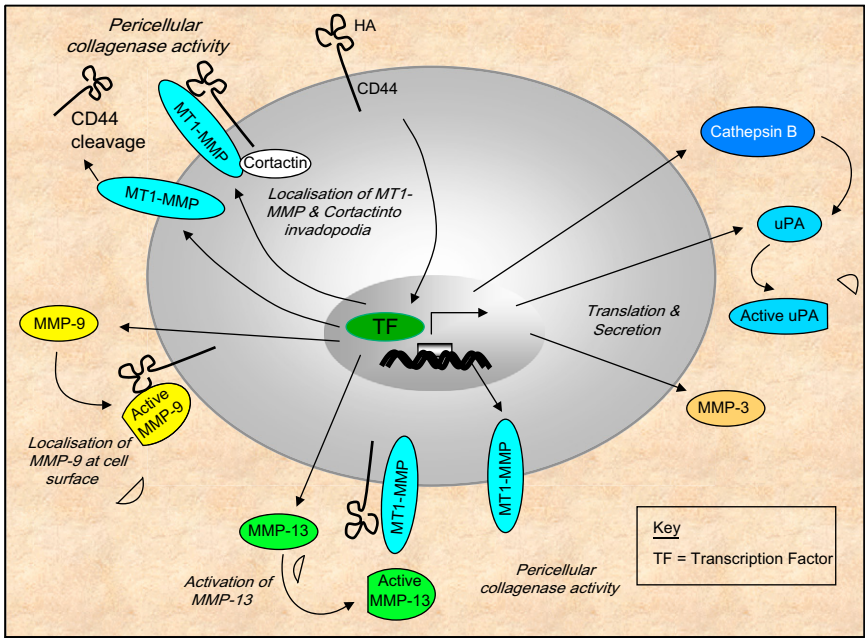

FIGURE 7.1 Schematic depiction of the role played by CD44 in regulating and co-ordinating proteolytic-dependent invasion of cancer cells through extracellular matrix. CD44 signaling regulates the transcription of multiple proteolytic species, whose activation in the extracellular space is promoted through autocatalysis or following protease-mediated cleavage of the inactive precursor, e.g., cathepsin B-mediated activation of urokinase plasminogen activator (uPA). In addition, CD44 serves to recruit the membrane-tethered matrix metalloproteinase MT1-MMP and increase the expression of the cytoskeletal protein cortactin to the invasive front of cells to promote the formation of invadopodia, membrane protrusions that underpin cell invasion. The association of CD44 with MT1-MMP and the secreted MMP-9 concentrates the extracellular enzymatic activity at the invasive front of the cell, enabling cells to exhibit the pericellular collagenolytic activity that is essential for degrading collagen-enriched matrices. In addition, the capacity of MT1-MMP to associate and cleave CD44 results in the shedding of the ectodomain of CD44 from the cell surface, decreasing the adhesion of the cell to substrates in the extracellular matrix. The resultant loss of adhesion at the invasive edge of the cell facilitates further propagation of cell motility.

Consequently, this interaction localizes MMP-9 proteolytic activity to the invasive surface of the cells, promoting physical degradation of the matrix in the immediate vicinity of the cell. In concentrating MMP-9 activity at the invasive front of the cell, the emergence or increased expression of CD44 in epithelial cells may thus enhance their capacity to disrupt the integral structure of the basement membrane and facilitate the access of these tumor cells to the underlying connective tissue.

More recent studies have identified a different mode through which HA-induced CD44 signaling may regulate cellular proteolytic activity; at the level of transcription. Addition of HA-oligosaccharides to several different cell cultures has demonstrated the capacity to regulate a number of MMP species. For example, transcription of two potent collagenases, MMP-3 and MMP-13 was induced by stimulation of articular and temporomandibular joint chondrocytes with HA-oligosaccharides (Ohno et al., 2005; Ohno et al., 2006) while addition of HA increased the transcription of the genes encoding MMP-9 and MMP-13 in murine 3LL tumor cells and primary fibroblasts (Fieber et al., 2004). Each of these responses was partially disrupted through inhibition of CD44 signaling, suggesting that signaling through CD44 and/or additional HA receptors including RHAMM may be functionally coupled to the transcriptional regulation of protease gene expression in these cells. Similar responses have been observed in tumor cells. Studies conducted by our own laboratory have demonstrated that HA-induced CD44 signaling regulates the transcription of not only soluble MMPs, but in addition, induces the transcription of the membrane-tethered MMPs. We have shown that the induction of CD44 expression in the non-invasive MCF-7 breast cancer cell line underpins HA-induced transcription of the gene encoding the membrane-tethered MMP, MT1-MMP, a potent collagenase that has been described as an essential component of pericellular invasion of collagen-enriched matrices (Itoh et al., 2006; Szabova et al., 2007). Interestingly, this membrane-tethered collagenase has also been shown to complex with CD44 in the plasma membrane of cells, promoting the cleavage of the CD44 ectodomain (Kajita et al., 2001; Mori et al., 2002; Ueda et al., 2003). The cleavage of the ectodomain of the receptor is proposed to attenuate the CD44 promoted adhesion of the cell with substrates that are enriched within the extracellular matrix. Accordingly, this reduces the avidity or strength of the interaction between the cell cytoskeleton and the extracellular matrix, thus increasing the potential of cell motility.

Furthermore, CD44-regulated protease activity is not solely restricted to the MMP family but may also drive transcription of serine protease and cysteine cathepsin genes in invasive breast cancer cells. For example CD44 has been shown to induce expression and activity of the serine protease urokinase plasminogen activator (uPA) in chondrosarcoma cells (Kobayashi et al., 2002). Using a tetracycline-regulated CD44 expression

system in the weakly invasive MCF-7F breast cancer cell line, in addition to several other metastatic breast cancer cell lines, we have shown that HA-CD44 signaling induces the transcription of cysteine proteases including cathepsins B and K (Hill et al., 2008) and increases expression of uPA. Of particular interest, cysteine cathepsins are known to induce cleavage-promoted activation of uPA in the extracellular milieu, suggesting that CD44 signaling may regulate the transcription and/or secretion of several intermediate species within this pathway, thus defining a mechanism by which this cell adhesion receptor can regulate a co-ordinated activation of this proteolytic cascade. Similarly, in the context of regulating MMP activity, MT1-MMP has been shown to induce proteolytic cleavage of MMP-13 and MMP-2, processing the inactive zymogen to the mature activated species (Atkinson et al., 1995; Knauper et al., 2002). Accordingly, CD44 signaling may function as a key upstream regulator through which a number of proteolytic cascades may be induced and co-ordinately regulated in invasive cancer cells.

In addition to degrading the basement membrane that separates epithelial cells from the underlying connective tissue, proteolytic enzymes are essential in enabling cancer cells to transmigrate across the vessel wall of capillaries within the extracellular matrix. In localizing MMP-9 activity to the invasive front (Yu and Stamenkovic, 1999), one can rationalize an argument for CD44 contributing to the invasion of cancer cells across the type IV collagen-rich basement membrane of blood vessels. However, somewhat contrary to expectation, the inhibition of MMP-9 activity has been reported to potentiate rather than inhibit the intravasation of fibrosarcoma cells in an *in vivo* model. Instead, activation of uPA activity was shown to differentiate those fibrosarcoma cells with high intravasation potential from low intravasation potential (Madsen et al., 2006). Furthermore, pharmacological inhibition of uPA activity attenuated the rate of fibrosarcoma cell intravasation. Therefore, although the linkage of CD44 to the regulation of uPA activity remains to be validated in a range of cancer cell-based models, the emerging experimental data from *in vitro* experiments suggest that CD44 signaling may increase the spectrum of proteolytic activity that enhances the efficiency with which malignant tumor cells gain access to circulatory systems. This is also supported by recent observations from *in vivo* experiments that have reported a marked enrichment of CD44 in endocrine-resistant breast cancer cells that have actively disseminated to the lymphatic vessels or formed metastases in the regional lymph nodes (Harrell et al., 2006).

However, there are several caveats to the proposition that CD44 actively contributes to the intravasation and extravasation of cancer cells from circulatory systems. Firstly, the paradigm that uPA is a universal determinant of a cancer cell's ability to successfully complete the process of intravasation remains to be established in different experimental models.

Similarly, it remains to be established whether the lack of involvement of MMP-9 activity in the case of fibrosarcoma cells is universal or whether this gelatinase may contribute to the intravasation of other cancer cell lines. In addition, the role of uPA, MMP-9, and other proteases in promoting the degradation of the basement membrane at the endothelial boundary at the secondary site of metastasis, in order to permit the escape of the cancer cell from the circulation, remains to be established. Finally, the involvement of CD44 in contributing to and/or regulating the rate of intravasation and extravasation of cancer cells remains to be directly investigated in relevant experimental systems.

Functionally, CD44 expression and activation are clearly linked with regulating cancer cell invasion of experimental matrices. Neutralizing antibodies that block the CD44 receptor attenuate the invasion of numerous cancer cell lines including representative models of gastric colorectal, ovarian, breast, and prostate cancer. We have observed that RNAi-mediated depletion of CD44 expression also reduces the efficiency with which metastatic breast cancer cells invade through a collagen I-enriched matrix, reflecting the emerging functional importance of this cell-surface receptor in regulating collagenase expression, activation, and distribution (Hill et al., 2008). In the context of metastasis, collagen I is a significant constituent of the extracellular matrix in numerous tissues, including the specialized matrices of bone and skin, and soft tissues including the lungs and liver, all tissues to which breast cancer preferentially disseminates to. Further research is likely to expand the number of proteolytic enzymes whose expression is regulated by CD44 signaling and which contribute to the invasion promoted by HA-induced signaling through this receptor. In extending the characterization of such activity, a more detailed appreciation of the functional significance of CD44 to each of the proteolytic-dependent processes of the metastatic cascade will also emerge.

CD44 signaling may also contribute to tumor invasion through additional mechanisms. Several key effector signaling proteins such as ankyrin, the ezrin-radixin-moiesin (ERM) family of proteins, and Neural–Wiskott–Aldrich syndrome protein (N-WASP) are reported to couple to the intracellular domain of CD44 (Ponta et al., 2003; Thorne et al., 2004; Bourguignon et al., 2007), facilitating the ability of this receptor to modulate the actin-cytoskeleton (discussed in more detail in a further chapter of this volume). The formation of elongated actin-cytoskeletal projections at the leading edge of a motile cell has been shown to promote the migration and invasion of cells through tissue matrix. Recent studies demonstrate a strong interplay between the cytoskeletal protein cortactin and the matrix-metalloproteinase MTI-MMP within the invadopodia (membranous projections) of invasive breast cancer cells (Artym et al., 2006). These invadipodia are key sites of proteolytic enzyme activity,

where the enzymes are concentrated to degrade the matrix components at the leading edge of the cell. Therefore, CD44 may have a dual function in invadopodia, promoting the phosphorylation of cortactin to promote actin-cytoskeletal projections (Bourguignon et al., 2001; McFarlane et al., 2008) while acting as a scaffold protein to localize MT1-MMP activity to these sites on the cell membrane (Mori et al., 2002). In addition, since CD44 signaling regulates the transcription of the genes encoding cortactin and MT1-MMP (Hill et al., 2006; 2008), the resulting increase in cortactin and MT1-MMP expression identifies a potential mechanism that potentiates the timeframe and magnitude of the invasive response that is initiated by the activation of CD44.

CELL–CELL ADHESION: ROLE OF CD44 IN PROMOTING EXTRAVASATION

The ability of CD44 to regulate the expression, activation, and distribution of proteolytic enzymes on the cell surface of cancer cells indicates a role for this receptor in facilitating localized tumor invasion, and ultimately, a potential role in promoting the entry (intravasation) of invasive cancer cells into the bloodstream. However, work from a number of laboratories suggests that CD44 may further contribute to metastasis at later stages of the cascade. For example, the regulation of proteolytic activity is equally important in enabling the penetration of invasive cancer cells into and through the extracellular matrix at the secondary site of tumor growth. In addition, CD44 has been proposed to contribute in part to the establishment of pericellular HA coats around chondrocytes, endothelial cells, and invasive cancer cells (Knudson et al., 1996; Nandi et al., 2000; Draffin et al., 2004). The maintenance of a HA coat around metastatic cancer cells has been proposed to enable them to withstand the vascular shear stresses encountered during passage through capillaries, promote their survival during their transportation in the circulation, enable cancer cells to escape immune surveillance, and finally, as discussed in detail below, contribute to the promotion of cell–cell adhesion at vascular endothelial cell boundaries (Simpson et al., 2002). In addition, pericellular HA has been shown to promote adhesion-independent growth of cancer cell lines, suggesting an important role in enabling cancer cells to new environment and promote outgrowth of a new tumor (Itano et al., 2004; Zoltan-Jones et al., 2003).

The role of CD44 in contributing to cell–cell adhesion has been confirmed by studies from several laboratories examining both its physiological and pathophysiological significance. Studies conducted by Siegelman and colleagues were the first to confirm that CD44 was functionally important in initiating the rolling and adhesion of T-lymphocytes

on endothelial cells *in vitro*. The physiological significance of their findings was also demonstrated from observations that the inhibition of CD44 abrogated the infiltration of lymphocytes into sites of peritoneal infection *in vivo* (DeGrendele et al., 1996; 1997). Subsequently, CD44 was shown to promote integrin receptor activation, in order to mediate the arrest and firm adhesion of these cells on endothelium (Siegelman et al., 2000; Nandi et al., 2004). Like lymphocytes, cancer cells are proposed to exploit a similar "docking and locking" mechanism in order to exit the bloodstream at capillary beds and infiltrate into underlying tissues (Fig. 7.2). Consequently, we and others have demonstrated the role of CD44 in promoting the adhesion and transmigration of myeloma, prostate cancer and breast cancer cells to bone marrow endothelial cell monolayers (Okada et al., 1999; Draffin et al., 2004). Specifically, we have confirmed that CD44 expression on breast and prostate cancer cell lines correlates with their ability to adhere to bone marrow endothelial cells. Blockade of CD44 function using neutralizing antibodies or inhibition of CD44 expression using siRNA attenuated the adhesion of the PC3 prostate cancer cell line or the MDA-MB-231 and MDA-MB-157 breast cancer cell lines. Furthermore, overexpression of CD44 in the DU145 prostate cancer cell line or the T47D breast cancer cell line (each shown to exhibit negligible endogenous CD44 expression), increased the capacity for these cells to adhere to bone marrow endothelial cells. Therefore, the capacity with which metastatic cancer cell lines adhere to this endothelial boundary can be dictated by manipulating the expression of CD44 in these cancer cells.

Pericellular HA has also been shown to contribute to the promotion of cell–cell adhesion. Enzyme (hyaluronidase)-mediated inhibition of a pericellular HA matrix around PC3 cells was shown by McCarthy and colleagues to attenuate the ability of these cells to adhere to bone marrow endothelial cell monolayers (Simpson et al., 2001; 2002). Furthermore, inhibition of HAS-2 and HAS-3 expression in PC3 cells also attenuated their adhesion suggesting that the HA promoting the formation of the pericellular HA coat and driving adhesion was sourced from the cancer cell. This was confirmed by further experiments in which hyaluronidase treatment of PC3 cells but not the bone marrow endothelial cells failed to affect the adhesion of prostate cancer cells to this monolayer (Simpson et al., 2001; Draffin et al., 2004). In addition, we demonstrated that CD44 expression on the surface of endothelial cells facilitated the adhesion of the HA-presenting cancer cell (Draffin et al., 2004). In contrast, at sites of inflammation, CD44-positive T-cells adhere to a HA layer deposited on the surface of the endothelial boundary. The action of inflammatory cytokines and chemokines have been shown to stimulate HAS expression and activity in the endothelial cells, ultimately generating this HA coating (Estess et al., 1999). Thus, the experimental data indicate that the

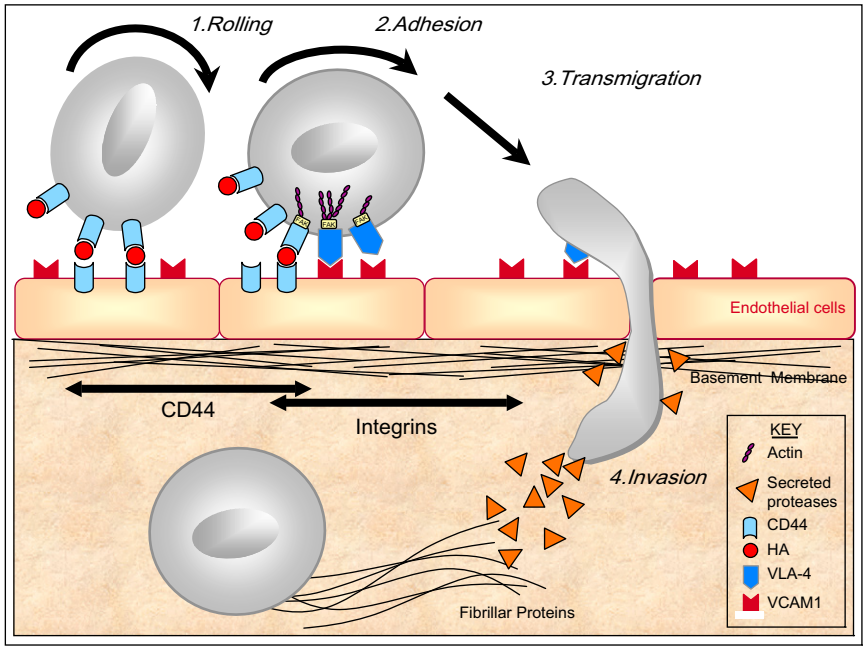

FIGURE 7.2 Schematic depiction of CD44-promoted adhesion to distal endothelium and penetration of the underlying matrix. CD44 initiates the rolling and arrest of metastatic breast cancer cells upon the distal endothelium. CD44 expressed on the cancer cell contributes to the retention of a pericellular HA coat that is presented to and interacts with CD44 receptors expressed on the surface of the receptive endothelium. The engagement of CD44 receptors initiates the activation of α4β1-integrin (VLA-4) receptors on the cancer cell that then engage with their counter-receptor on the endothelium (VCAM). The activation of CD44 and VLA-4 results in the activation of signaling cascades that promote cytoskeletal reorganization promoting the assembly of focal adhesions to underpin the arrest of the cell on the endothelium. The transmigration of the arrested cancer cell across the endothelium may be enhanced by CD44 signaling events regulating the contraction of the endothelium and secondly, through the action of CD44-regulated gelatinase activity (MMP-2/MMP-9) facilitating the degradation of the basement membrane. CD44 signaling may also contribute to the subsequent invasion of the cancer cell into the underlying tissue while HA/CD44 signaling may assist in promoting the survival and outgrowth of the cancer cell within the new tissue environment.

orientation of HA presentation in mediating cancer cell arrest is markedly different from that engaged in promoting the tethering of lymphocytes at sites of inflamed endothelial boundaries. This also suggests that cancer cells that acquire elevated HAS activity and express CD44 receptors, perhaps in direct response to increased autocrine/paracrine cytokine and chemokine signaling, may define a sub-population of cancer cells that have increased potential to adhere to vascular endothelial boundaries and thus have increased metastatic potential.

The activation of integrin receptors is fundamental in promoting the firm adhesion of both lymphocytes and cancer cells upon the endothelial cell. CD44 was originally shown to promote the activation of the α4β1-integrin heterodimer in T-lymphocytes, driving a latrunculin-sensitive cell–cell adhesion response (Nandi et al., 2004). Studies conducted in breast cancer cell lines have also identified that cross-linking mediated activation of CD44 facilitates an α4β1-integrin-dependent adhesion (Wang et al., 2005). We have also characterized a complex interaction between HA-induced CD44 signaling and integrin receptor expression and activation in metastatic breast cancer cells. We have shown that stimulation with HA induces a rapid change in β1-integrin subunit expression and using conformation-specific antibodies confirmed that HA increases the pool of activated β1-integrin receptors in these adhesive breast cancer cells (McFarlane et al., 2008). Furthermore, we have demonstrated that the adhesion of MDA-MB-231 breast cancer cells and the PC3 prostate cancer cell line to bone marrow endothelial cells is inhibited by concurrent administration of neutralizing antibody to the α4β1-integrin heterodimer but not by an antibody blocking the activation of α2β1-integrin receptors. In addition, we have shown that a HA/CD44-induced activation of a cortactin-paxillin signaling pathway that underpins cell–cell adhesion, is attenuated by co-administration of a neutralizing antibody to the α4β1-integrin heterodimer. Consequently, the results from several laboratories suggest a physical interaction of CD44 with the α4β1-integrin heterodimer, to regulate the cytoskeletal reorganization that underpins cell–cell adhesion. The precise nature of the intermolecular association of these two cell-surface receptors remains unknown but may result from a direct physical interaction of the ectodomains of the receptors or alternatively, by the action of scaffolding/adaptor proteins interacting with the cytoplasmic tails of these receptors.

Further studies also indicate that CD44 may contribute to cell–cell adhesion through interactions with P-selectins. This ability of CD44 to interact with selectins is associated with the extensive incorporation of O-linked sialofucosylated glycans within the variant exons of CD44. In a series of recent studies primarily conducted on colorectal cancer cell lines, the expression of variant CD44 was shown to selectively slow the rolling and promote adhesion of these cancer cells on P-selectin with diminished effects reported in facilitating rolling and/or adhesion to either E- or L-selectin (Hanley et al., 2006; Napier et al., 2007).

As such, the expression of standard and variant isoforms of CD44 on cancer cells may contribute to the promotion of cell–cell adhesion through their ability to engage in (i) a HA-dependent reciprocal interaction with CD44 expressed on endothelial cells; (ii) through regulating inside-out activation of integrin receptors; and finally (iii) through serving as a ligand for selectins expressed on the surface of endothelial cells.

THE ROLE OF CD44 IN PROMOTING TISSUE-SPECIFIC METASTASIS

Tumors show a high degree of selectivity with regard to the organs in which secondary tumors develop. The development of metastases within the liver is a prevalent clinical feature of advanced and aggressive colorectal cancers. CD44 expression, particularly that of large molecular weight variant isoforms of CD44, has been shown to correlate with increased metastasis of colorectal cancer cells to the liver (Yue et al., 2003; Ohji et al., 2007; Kuhn et al., 2007). However, other diseases such as breast cancer metastasize to a diffuse number of tissues including the lungs, liver, brain, skin, and bone. Therefore, does CD44 expression on cancer cells promote tissue-specific homing of cancers? Tetracycline-regulated induction of CD44 expression in MCF-7 breast cancer cells has recently been shown to potentiate their dissemination from the mammary fat pad to the liver, without incidence of histologically detectable metastasis in the lungs or other soft tissues (Ouhtit et al., 2007). In contrast, consistent with CD44 promoting an efficient adhesion of breast cancer cells to bone marrow endothelium cells in *in vitro* models, clinical reports have confirmed an enrichment of CD44 expression (studied in the context of the breast cancer stem-cell markers) in breast cancer cells resident within the bone marrow of patients diagnosed with early stage breast cancer (Balic et al., 2006). Enrichment of CD44 expression has also been detected in endocrine-resistant breast cancer cells that have disseminated to the lymphatic vessels or regional lymph nodes in athymic mice (Harrell et al., 2006), experimental findings that are again supported by a prior correlation of CD44 expression with nodal metastasis in breast cancer patients (Berner and Nesland, 2001). The apparent disparity of the results observed from these range of studies with breast cancer cells may reflect the fact that the resultant site of metastasis observed can be heavily influenced by the cell line exploited, the stresses that they are exposed to, and by the route of tumor cell administration to the animal. The use of *in vivo*-selected, tissue-homing derivatives of breast cancer cells in experimental models of metastasis allied with an extensive pathological examination of metastatic lesions from patients will be useful in elucidating the role of CD44 in promoting the dissemination of breast cancer to particular target organs. The current evidence from clinical and laboratory-based studies suggests that CD44 expression correlates with the escape of breast cancer cells to the lymph nodes, liver, and bone marrow cavity. Although some studies suggest a differential role for standard and variant isoforms, expression of CD44 in multiple tumor types typically correlates with an increased metastatic capability as opposed to directing tissue-specific metastasis.

CONCLUSIONS

Expression of CD44 on the surface of cancer cells facilitates their adhesion to other cells and the avidity with which they may adhere to substituents of the extracellular matrix. In addition to regulating adhesion, CD44 facilitates the penetration of cancer cells through tissue by acting as a scaffold to concentrate proteases on the invasive surface of cells and by regulating protease expression in cancer cells. Further understanding of the downstream signaling proteins and mediators of CD44-promoted adhesion and penetration will provide novel therapeutic opportunities to attenuate the metastasis-promoting action of HA and CD44 within the tumor microenvironment. Alternatively, an increased biophysical knowledge of CD44 and its interacting proteins may identify unique strategies to uncouple CD44 from proteases and other cell-surface adhesion receptors, providing more selective strategies to target the adhesion and penetration of cancer cells.

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CHAPTER

8

Involvement of CD44, a Molecule with a Thousand Faces, in Cancer Dissemination

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PROLOGUE

Cancer progression and metastasis are complex processes associated with multiple and highly organized sequential steps that are both well coordinated and organ selective. The dissemination of malignant cells is dependent on growth and local invasion at the primary site. First, the cells must lose local adhesiveness, e.g., in epithelial cells by breaking homophilic interactions mediated by cadherins. The cells, which are detached from the local growth (now designated as metastatic cells) penetrate into the lymphatic system and the blood circulation. The metastatic cells migrate in the lymph vessels and blood vasculature until they become trapped at remote sites through adhesion to endothelial cells. Then they transverse the endothelium (in a process known as trans-endothelial migration) and establish new colonies of malignant cells at the extravascular site (Yeatman and Nicolson, 1993). Well orchestrated interplay of adhesion molecules and chemoattractants is indispensable for the cell's locomotion in the blood-lymph circulation, transmigration through the endothelium and colonization within the target organ (Stetler-Stevenson et al., 1993). Organ-derived chemokines, present in soluble form as well as confined to the cell membrane or extracellular matrix (ECM), are involved in the selective navigation of metastatic cells into specific organs (Yeatman and Nicolson, 1993). Adhesion molecules expressed on metastatic cells and their specific countermolecules (ligands) expressed on endothelial cells and on cells of the extravasculature are also involved in the selective lodgment of the disseminating cancer cells in the target tissues. In this context we have found (Zahalka et al., 1995) that spleen invasion by lymphoma cells is mediated by β2-integrin, whereas CD44, expressed on the same lymphoma cells, navigates these cells to the lymph nodes. Possibly, integrin ligand exclusively expressed in the spleen and CD44 ligand exclusively expressed in the lymph node differentially arrest the lymphoma cells in the respective organs. The docking of the metastatic cell in the target tissue is another essential step in the malignant process, as their adherence to substrate blocks delivery of apoptotic signals and thereby rescues the cancer cell from apoptotic death (Meredith and Schwartz, 1997; Tian et al., 2000).

The three steps model of Dr. Springer (Springer, 1994) describes the mechanism of cell locomotion in the vasculature and the cell trans-endothelial migration. In the first step, cells flowing in the blood vessel form lose attachment with the endothelial cells, resulting in cell rolling interactions mediated by adhesion de-adhesion processes (i.e. the cells lose and gain adhesiveness). In the second step, chemoattractants derived from the extravascular tissue or from the endothelium stimulate G-protein-like receptors on the rolling cells. The stimulated G-protein receptors activate cell surface integrins. In the third step, the activated integrins generate firm

attachments with the endothelial cells resulting in extravasation and cell accumulation in the tissue. Acquisition of rolling capacity by a cell of multicellular organism is highly dependent on the ability of the cell to gain and lose adhesiveness. In order to move, the affinity of cell surface molecules to the substratum countermolecules must be intermediate. Too strong affinity may arrest the cell on the endothelium, whereas too weak affinity may allow the blood shear stress to release the cell from the endothelium. The process of cell motility is essential for embryonic development, and reoccurs in the adult organism at sites of wound healing or tissue remodeling and also during metastasis, where malignant cells probably recapitulate processes of embryonic behavior.

The interplay between chemokines, cytokines, growth factors, cell-bound or tissue-bound enzymes, adhesion and homing molecules, as well as apoptosis-promoting and survival-inducing signals, determines the tumor fate for life or death. Therefore, comprehensive understanding of the sophisticated biological networks is a prerequisite for developing novel therapeutic strategies. Realizing that the cell migration machinery is an important factor for tumor spread and knowing that adhesion and homing molecules are critical elements for this function, we have focused our research on these molecules. The malignant lymphoma LB cell line was used as an experimental model in our study, since we were familiar with its structural and functional phenotype, that has been extensively explored in our laboratory (e.g., see Ish-Shalom et al., 1995; Pillemer et al., 1992; Sharon et al., 1993). Of the two dominant families of adhesion/homing molecules that could be involved in the support of LB cell migration, i.e. integrins or selectins, we focused our attention on the former, simply because the latter is not expressed on these cells (Zahalka et al., 1993). Using the protocol described by Zahalka et al. (1993), we found that injection of the β2-integrin-specific anti-CD18 monoclonal antibodies (mAb) into BALB/c mice almost completely blocked the spleen colonization by LB lymphoma cells, as indicated by measuring the tumor cell proliferation in the organ, using the H^3-thymidine incorporation assay. Surprisingly, the invasion of proliferating LB cells into the lymph nodes was not affected by targeting the β2-integrin with the same antibody. Furthermore, spleen-derived LB cells (i.e., LB cells that were recovered from the spleen) colonized upon their subcutaneous (s.c.) injection both in the spleen and the lymph nodes, whereas lymph node-derived LB cells colonized in the lymph nodes only (Zahalka et al., 1993). These findings suggest that LB cells trapped in the lymph nodes lose their spleen homing receptor, whereas LB cells trapped in the spleen retain both their spleen and lymph node homing receptors. We subsequently asked, if not β2 integrin, which adhesion/homing receptor guides LB cells into the lymph node? At that time a report by Mackay et al. (1990) attracted our attention. They showed that CD44

expression on activated lymphocytes correlated with their localization in the peripheral lymph nodes. Ultimately, we decided to explore the involvement of CD44 in lymph node colonization by LB cells.

CD44, A POLYGAMIC MOLECULE, INTERACTING WITH MULTIPLE LIGANDS

CD44 Structure

CD44 is a single chain glycoprotein encoded by a single copy of a gene located on the short arm of chromosome 11 in humans and on chromosome 2 in mice, spanning ~50 kb of genomic DNA. The CD44 glycoprotein is an acidic molecule whose charge is largely determined by sialic acid. The $t_{1/2}$ of CD44 turnover is estimated to 8 h. Mouse CD44 contains 20 exons. In the mouse, the first 5 exons (1 to 5) are constant. The next 10 exons (6 to 15, also numbered v1 to v10) are variant, i.e., they are differentially incorporated into the CD44 molecule by alternative splicing. Exons 16, 17, and 18 are again constant whereas exons 19 and 20 are variant. Exons 16 and 17 encode the C′ terminus part of the extracellular domain, exon 18 encodes the transmembrane spanning domain, and exon 19 or 20 differentially encodes either a short (three amino acids) cytoplasmic tail or the more abundant long one (72 amino acids). The human CD44 does not contain exon v1 (the first variant exon of human CD44 is numbered v2). The differential utilization of the 10 variant exons generates multiple CD44 variants (CD44v) with different combinations of variant exon products (reviewed in Naor et al., 2002; Naor et al., 1997; Ponta et al., 2003). Theoretically, over 800 membrane-bound CD44 isoforms can be generated, although apparently not all combinations are expressed (Naor et al., 2002; Naor et al., 1997). To date, tens of different CD44 isoforms have been discovered; the most common one is standard CD44 (CD44s), in which exon 5 is spliced directly to exon 16, skipping the entire variant exon sequence.

The amino terminus of the CD44 molecule contains six cysteine residues, which are possibly utilized to form a globular domain or three globular subdomains. A stretch of 92 amino acids (from position 12 to position 103) at the N-terminus of the human CD44 receptor displays ~35% homology with a sequence from a family of proteins that bind hyaluronic acid (HA). This group of proteins, known as hyaladherins or the link protein superfamily, includes cartilage link protein, aggrecan, versican, tumor necrosis factor-inducible protein-6 (TSG-6) and lymphatic vessel endothelial HA receptor-1 (LYVE-1), but not the receptor for hyaluronate-mediated motility (RHAMM) or its variant: intracellular HA binding protein (IHABP), both of which are structurally unrelated to the

hyaladherins (Naor et al., 2002). A structural model of the CD44 link-homologous region was constructed based on the TSG-6 link module determined by NMR (Bajorath, 2000). Residues Arg-21, Tyr-22, Arg-58, and Tyr-59 of human CD44 form a cluster critical for HA binding.

The structural variability of the CD44 molecule is further enriched by posttranslational modifications. Massive N-linked and O-linked glycosylations, as well as glycosaminoglycan (GAG) attachments double the molecular mass of the CD44s core protein from 37 kDa to 80–95 kDa. The molecular mass of the different CD44 isoforms ranges from ~80 kDa to ~250 kDa. Both alternative splicing and posttranslational modifications contribute to this diversity. Most of the potential N-linked glycosylation sites (Asn-residues) are found in the extreme N-terminal of the extracellular domain, which includes the link module, and in the alternatively spliced variable region. The potential O-linked glycosylation sites (Ser/Thr residues) and GAG attachments (Ser-Gly motifs) are distributed in the more carboxyl terminal regions of the extracellular domain, including the membrane proximal region, and the variable region (Naor et al., 2002). The human CD44 cytoplasmic tail contains 6 potential serine phosphorylation sites. Schematic maps depicting the CD44 molecule are illustrated in our review articles (Naor et al., 2002; Naor et al., 1997) and in a more recent review by Ponta et al. (2003).

CD44 Ligands and the Mechanism of Ligand Binding

The principal ligand of the CD44 receptor is hyaluronic acid (HA, hyaluronate, hyaluronan), a linear polymer of repeating disaccharide units [D-glucuronic acid (1-β-3) *N*-acetyl-D-glucosamine (1-β-4)]$_n$. CD44 can, however, interact with several additional molecules such as galectin-8 (Eshkar Sebban et al., 2007), collagen, fibronectin, fibrinogen, laminin, chondroitin sulfate, mucosal vascular addressin, serglycin/gp600, osteopontin (OPN) and the major histocompatibility complex class II invariant chain (Ii), as well as L-selectin and E-selectin (reviewed in Naor et al., 2002, and Naor et al., 1997; and see also Hanley et al., 2005; Napier et al., 2007). In many cases CD44 does not bind to its ligand unless activated by external stimuli. As both CD44 and its ligand are ubiquitous molecules, this mechanism should avoid unnecessary engagement of the receptor. In fact, three states of CD44 activation have been identified in cell lines and normal cell populations (Lesley et al., 1995): active CD44, which constitutively binds HA; inducible CD44, which does not bind HA or binds it only weakly, unless activated by inducing factors (e.g., mAbs, cytokines, or phorbol ester); and inactive CD44, which does not bind HA, even in the presence of inducing factors. In many cases the N-glycosylation pattern of the CD44 receptor dictates the status of its HA binding capacity, where active CD44 is the

least glycosylated, inactive CD44 the most glycosylated, and inducible CD44 holds the intermediate position (Lesley et al., 1995). Complete or partial removal of *N*-glycans from the CD44 molecule prevents HA from binding to the receptor of some types of cells, whereas in others partial removal enables HA binding. Trimming of the exterior sugar unit, alpha 2,3-linked sialic acid, confers HA binding upon some CD44 molecules that normally do not engage this ligand. Acquisition or enhancement of HA binding can be achieved through desialylation of cell surface CD44 or by treating cells expressing this molecule with TGF-β1 (Cichy and Pure, 2000) or phorbol 12-myristate 13-acetate (PMA) (Rochman et al., 2000). Desialylation reduces the negative charge of CD44 thereby seemingly enabling the interaction with the negatively charged HA. Exposure of HA binding sites, either directly or due to a conformational change and/or increase in the net positive charge of the receptor, could account for the acquisition of HA binding by the CD44 modified molecule. O-glycosylation may negatively influence HA binding in several, but by no means all, cell lines. Additionally, GAG modifications may negatively or positively influence HA binding (Naor et al., 2002; Naor et al., 1997). For example, decoration of CD44 by heparan sulfate (HS) or chondroitin sulfate (CS) is required for binding of several growth factors, fibronectin or collagen (Ehnis et al., 1996; Jalkanen and Jalkanen, 1992), and for acquisition of migration capacity on collagen and fibrinogen substrates by melanoma and endothelial cells, respectively (Faassen et al., 1992; Henke et al., 1996; Knutson et al., 1996). In some cell lines HA binds exclusively to CD44s, whereas in others only CD44 variants bind HA (reviewed in Naor et al., 1997). Expression of variant epitopes may enforce aggregation of the CD44 receptors, leading to acquisition of HA binding (Sleeman et al., 1996), or it might simply expose the distal HA binding site for interaction with the ligand. Note that HA aggregation also enhances its binding to the CD44 receptor (Zhuo et al., 2006).

THE INTERACTION BETWEEN THE CD44 OF LB CELLS AND HYALURONATE – *IN VITRO* STUDIES

Flow cytometry analysis revealed that LB cells co-express CD18 and CD44 (Zahalka et al., 1995). Before analyzing how the CD44 molecule supports the invasion of LB cells into mouse lymph nodes, we will discuss first our *in vitro* findings, which elucidate the mechanism of interaction between the CD44 of LB cells and hyaluronate. Having this information in mind, it might be easier to evaluate the data derived from animal studies. LB cells directly obtained from culture or from local

tumor growth (following s.c. injection), as well as from spleen or axillary lymph nodes (again, after their s.c. injection into BALB/c mice), expressed pan-CD44 (detected by flow cytometry, using anti-CD44 mAb recognizing a constant epitope shared by all CD44 isoforms) and v6-containing CD44 isoform (detected with anti-CD44v6 mAb recognizing epitope included in the v6 variant exon product). These cells expressed to a lesser extent v4-containing epitope detected with anti-CD44v4 mAb (Sionov and Naor, 1997). Analyzing the CD44 transcript repertoire with RT-PCR provides additional information related to the CD44 expression at the nucleotide level: lymphoma dissemination, especially to the lymph node, is associated with enhanced expression of cell surface CD44 variants not expressed on parental LB cells (Wallach et al., 2000). However, all these types of lymphoma cells neither bound soluble fluoresceinamine-labeled HA (Fl-HA), as indicated by flow cytometry, nor attached to HA immobilized on plastic. Pretreatment of the culture or animal-derived cells with hyaluronidase did not restore the HA binding capacity, implying that the lack of binding cannot be attributed to external-bound HA, derived from culture or animal, that blocks the CD44 receptor. In independent experiments we showed that pretreatment of HA9 cells (a subline of LB cells that constitutively bind HA) with hyaluronidase does not affect the subsequent binding of Fl-HA to HA9 cells (see below and Sionov and Naor, 1997). Acquisition of HA binding to all types of LB cells was detected by both methods after their incubation with PMA (Rochman et al., 2000; Sionov and Naor, 1997; 1998). Increasing the dose of PMA gradually enhanced the upregulation of CD44 on LB cells, finally reaching a threshold of cell surface CD44 expression, which allows HA binding (Sionov and Naor, 1998). A constitutive HA-binder HA9 cell line was derived from low HA-binder LB2.3 subline (S. Elbaz from our laboratory) by repeatedly selecting for cells binding to immobilized HA. HA9 cells expressed CD44 and CD44 variants more intensively than the parental LB cells, as indicated by both flow cytometry (Sionov and Naor, 1997) and RT-PCR (Wallach et al., 2000). Flow cytometry revealed that this difference was maintained even after PMA activation, which enhanced the CD44 expression on both cell types. Linear relationships were registered for CD44 upregulation and acquisition of HA binding by HA9 cells and PMA-activated LB cells, the latter after reaching an expression threshold level (Sionov and Naor, 1998). Interestingly, presence of IM7.8.1 anti-CD44 mAb (but not anti-CD44v6 or anti-CD44v4 mAbs) reduced the binding of Fl-HA to HA9 cells, yet, excess of soluble HA, while blocking the binding of Fl-HA to HA9 cells, did not interfere with the binding of the anti-CD44 mAb to the same cells (Sionov and Naor, 1997). This finding suggests that HA and the anti-pan CD44 mAb interact with distinct sites on the CD44 molecule, but the antibody allosterically reduced the binding of the CD44 molecule for HA. Immobilized HA

treated with hyaluronidase lost its ability to bind PMA-activated LB cells (Zahalka et al., 1995) or HA9 cells (Sionov and Naor, 1997).

To acquire HA binding potential, LB cells must be activated either *in vitro* (e.g. by PMA) or *in vivo* by a still unknown factor. We suggest that the *in vivo*-activated LB cells are a rare cell population, represented by HA9 cells isolated in our study by serial repeating selections of LB cells attached to immobilized HA. These cells are characterized by their ability to form CD44-dependent aggregates (Wallach et al., 2000). Hence, the activation of LB cells by PMA and HA9 cells, respectively, represent two phases in HA binding process: the induction phase mediated by phorbol ester stimulation and the effector phase of constitutively HA-binder cells. This discrepancy enables to distinguish between intracellular signals involved in the induction phase and those involved in the effector phase of HA binding. The *de novo* synthesis of cell surface CD44 of LB cells activated with PMA as well as the acquisition of HA binding potential by these cells were inhibited by cycloheximide. The basic expression of cell surface CD44 on non-activated LB and HA9 cells as well as the spontaneous HA binding capacity of HA9 cells were not affected by addition of cycloheximide, suggesting a relative long half life of CD44. In contrast, after PMA activation, the newly synthesized cell surface CD44 and the accompanied acquisition of HA binding were inhibited by this reagent (Sionov and Naor, 1998). PMA is a well-known protein kinase C (PKC) activator; e.g. such a stimulated PKC is involved in activation of CD44, leading to its ability to mediate chemotaxis (Tzircotis et al., 2006). However, staurosporine, sphingosine, polymyxin B, and quercetin that inhibit PKC activity or genestein that inhibits tyrosine protein kinase, did not affect PMA-induced HA binding by LB cells (Sionov and Naor, 1998), indicating that these signaling pathways are not involved in the acquisition of HA binding capacity. In contrast, the PMA-induced HA binding by LB cells (and the corresponding enhanced expression of CD44), but not the constitutive binding of HA9 cells, was strongly suppressed by calmodulin antagonists (chlorpromazine, trifluoperazine, W-7), the calcium channel blocker verapamil, and the calcium ionophore ionomycin (possibly owing to overdose paralyzing effect) (Sionov and Naor, 1998). Hence, in LB cells, PMA-induced calcium mobilization leads to cell surface CD44 activation and acquisition of HA binding potential. We propose that these effects are mediated by calmodulin, that following calcium binding, upregulates Ca^{2+}/calmodulin-dependent kinase II (CaMKII). Mishra et al. (2005) demonstrated that calmodulin and CaMKII are involved in upregulation of monocyte CD44 after stimulation with TNFα, but not lipopolysaccharide. Lipopolysaccharide, however, induced CD44 transcription through JNK and Egr-1. The mechanisms by which calmodulin affect CD44 expression and HA binding capacity, are still unknown. There is one report showing that CaMKII phosphorylates CD44 at Ser325 (Lewis et al., 2001).

This modification was essential for CD44-dependent migration on HA. It remains to be determined whether calmodulin is involved in CD44 deglycosylation (or desialylation), or alternatively, in induction of differential splicing, both functions are associated with acquisition of HA binding (see below).

The PMA-induced HA binding acquisition described above is an example of inside-out activation process which generates oncogenic phenotype, as interaction of cell surface CD44 with HA supports cell migration and resistance to apoptosis (Naor et al., 2002). Dr. Bourguignon and colleagues described a reversed outside-in model, which also leads to oncogenic phenotype. Using a head and neck squamous cell carcinoma (HNSCC) cell line, it was demonstrated that interaction of HA with cell surface CD44 leads to calcium mobilization, CaMKII activation, and filamin (cytoskeletal protein) phosphorylation, resulting in reorganization of the cytoskeleton and tumor cell migration (Bourguignon et al., 2006). In these cells calcium mobilization, induced by HA–CD44 interaction, generates signals that enhance also cell survival and growth as well as the subsequent resistance to the anti-cancer cytotoxic drug cisplatin (Wang and Bourguignon, 2006). Hence, calcium signaling elements such as calmodulin and CaMKII as well as upstream factors such as phospholipase C and inositol 1,4,5-triphosphate are potential therapeutic targets in malignant diseases, using, for example, the relevant small interfering RNA (siRNA) approach. The question whether interaction between the cytoplasmic domain of CD44 and cytoskeletal proteins is important for HA binding is controversial and possibly cell type-related (Naor et al., 2002). However, integrated cytoskeleton is not required for PMA-induced acquisition of HA binding by LB cells or the constitutive HA binding by HA9 cells. PMA-activated LB cells and HA9 cells lost their ability to bind HA following treatment with microfilament-disrupting dose of cytochalasin D (which prevents actin polymerization) or with microtubule-disrupting dose of colchicine (which blocks microtubule formation) (Rochman et al., 2000; Sionov and Naor, 1998). Both functions are essential for cytoskeleton activity. Furthermore, we found that the acquisition of HA binding is not related to changes in the macroaggregation state of cell surface CD44 or to changes in membrane microviscosity (Rochman et al., 2000; Sionov and Naor, 1998). It should be emphasized that the question how PMA confers HA binding upon LB cells is only partially resolved.

ELUCIDATION OF HA BINDING MECHANISM IN LB CELLS

PMA activation enhanced the expression of standard CD44 and the expression and number of CD44 variants on LB cells as shown by western

blot, flow cytometry, and RT-PCR (Wallach et al., 2000; Rochman et al., 2000; Sionov and Naor, 1997). Although we cannot role out the significance of quantitative changes in CD44 expression (i.e. upregulation of this receptor), which allow HA binding to LB cells, our data rather point to the significance of quantitative alterations in the context of this effect. Parental LB cells, that do not bind HA, acquired such binding potential after treatment with neuraminidase (Rochman et al., 2000). This enzyme cleaves sialic acid from cell surface CD44 glycoform, thereby increasing the net positive charge of this molecule. This in turn enables its interaction with the negatively charged HA (Naor et al., 1997). Neuraminidase treatment did not enhance HA binding to PMA-activated LB cells or HA9 cells, either because such cells are deficient in CD44 sialic acid and/or they display enhanced expression of CD44 variants. Both phenotypes may be involved, either separately or in combination, in enhancing the positive net charge of this molecule. Indeed, we presented evidence for the existence of these two parameters in PMA-activated LB cells and HA9 cells (Rochman et al., 2000; Sionov and Naor, 1997), which may explain their ability to bind HA. Glycosaminoglycans (GAGs; e.g. HA, heparin, heparan sulfate, chondroitin sulfate, or keratan sulfate) can be co-precipitated with their glycoproteins by the cationic detergent cetylpyridinium chloride (CPC). CD44 western blot analysis of the precipitated proteins revealed that HA, but not the other GAGs, binds exclusively to CD44 variants of PMA-activated LB cell extracts, but not to parental LB cell extracts. These data show that it is possible to confer HA binding upon LB cells by either removing sialic acid with neuraminidase, or by enhancing CD44 variant expression by phorbol ester (Rochman et al., 2000). Our results further suggest that CD44 variants can bind HA even if their sialic acid (if exists) was not removed (Rochman et al., 2000), since some of the alternative splicing products (but not standard CD44) may carry sufficient net positive charge to allow their interaction with HA. Low concentrations of neuraminidase and PMA, which by themselves were insufficient to confer HA binding upon LB cells, displayed a significant synergistic binding effect when combined. Similarly, tunicamycin, which inhibits N-glycosylation of cell surface glycoproteins, does not confer HA binding upon LB cells unless combined with sub-optimal doses of PMA. Interestingly, CD44 expression on LB cells under these experimental conditions (tunicamycin plus low doses of PMA) was similar to the CD44 expression on parental LB cells, yet HA binding could be detected on the double treated cells only (Rochman et al., 2000). This finding clearly shows that enhanced expression of CD44 is not obligatory for HA binding to LB cells, but qualitative changes are rather essential. The simultaneous effect of increasing the CD44 positive charge by desialylation with neuraminidase (or by deglycosylation with tunicamycin) and augmenting the CD44 variant repertoire by treatment with PMA may explain this synergistic effect. It should be stressed that the

desialylation or deglycosylation procedures can affect, in addition to CD44, other cell surface proteins, whose modified version may indirectly influence HA binding to CD44 of LB cells. However, it has been observed by other investigators that desialylation of CD44-coated beads (Katoh et al., 1995) as well as desialylation or deglycosylation of soluble CD44 (Skelton et al., 1998) generates HA binding sites in the absence of other proteins, implying that the modified CD44 can independently interact with hyaluronan.

We have seen that standard CD44 of LB cells can interact with HA only after desialylation with neuraminidase, whereas CD44 variants of PMA-activated LB cells interact with HA, but not with other GAGs, even without chopping their sialic acid (Rochman et al., 2000). The last observation was supported by transfection experiments. LB cells were transfected with CD44v4-v10 (CD44v) cDNA or with standard CD44 (CD44s) to overexpress these isoforms. The CD44v-transfected LB cells bound HA and chondroitin sulfate, whereas the CD44s did not. Anti-CD44 mAb and excess of soluble HA blocked the binding of Fl-HA to CD44v-transfected LB cells, implying that CD44 receptor is involved in this interaction. The CPC precipitation assay revealed that CD44 variants (but not standard CD44) of cell extracts derived from CD44v-transfected LB cells can bind HA and other GAGs, but standard CD44 of cell extracts derived from CD44s-transfected LB cells cannot (Wallach-Dayan et al., 2001). Yet, CD44 variants from PMA-activated LB cells bind HA only, as indicated by the CPC assay (Rochman et al., 2000). This is not surprising since the CD44 variant repertoire of CD44v-transfected LB cells differs from the variant repertoire of PMA-activated LB cells.

Measuring LB cell binding under dynamic conditions, rather than static conditions, reflects more precisely the physiological *in vivo* state when the cells are bound to the endothelium of the blood vessels under the flow pressure of the blood (known as a wall shear stress; the physiological shear stress is around 2 dyn/cm^2). The LB transfectants were settled on substrates coated with different GAGs, assembled in flow chamber apparatus, and then subjected to increasing shear flow. The number of cells accumulating and rolling under the shear flow was counted. Three to four times more CD44v-transfected LB cell accumulated and rolled on HA substrate (but not on other GAGs) under shear stress of 2 dyn/cm^2 than did CD44s-transfected LB cells. The accumulation and rolling of the CD44v-transfected LB cells was blocked with anti-CD44 mAb or excess of soluble HA. In conclusion, LB cells transfected with a CD44 variant resist shear stress and remained attached to the HA substrate at much higher rates than parental LB cells or LB cells transfected with standard CD44 (Wallach-Dayan et al., 2001). Extrapolating these *in vitro* data into the animal milieu may predict that the *in vivo* migration of CD44v-transfected LB cells should be much more

efficient than that of parental LB cells or LB cells transfected with standard CD44. The HA binding site of CD44v4-v10 was destroyed by point mutation, replacing arginine with alanine at position 43. This construct was transfected into LB cells. LB cells expressing the CD44 mutant, although normally presenting their CD44 isoforms, lost their ability to bind soluble Fl-HA from the solution, and to display rolling interactions on HA substrate under shear stress (Wallach-Dayan et al., 2001). Since only CD44 variants bind HA, we must assume that the variable region, generated by alternative splicing, influences the configuration of the constant domain, hence, creating an HA binding site.

THERAPY OF THE MALIGNANT LYMPHOMA: BLOCKING THE INTERACTION BETWEEN CD44 AND ITS LIGAND BY TARGETING THE CD44 VARIANT WITH RELEVANT ANTIBODY

If the *in vitro* migratory advantage of CD44v-transfected LB cells is also reflected *in vivo*, we may find that these cells disseminate faster and more efficiently than CD44s-transfected LB cells or parental LB cells. To allow detection of the disseminated lymphoma, all cell lines were labeled by transfected green fluorescence protein (GFP). Indeed, the skin local tumor growth and the lymph node invasion of CD44v-transfected LB cells were much faster than that of the corresponding CD44s-LB cells and parental LB cells. Furthermore, the mutated CD44v-LB cells, that lost their ability to bind HA, lost also their ability to develop local tumors and to invade the lymph nodes following s.c. injection (Wallach-Dayan et al., 2001). Hence, we may suggest a parallel between the *in vitro* and the *in vivo* findings, showing that the interaction between cell surface CD44v and HA is essential for both cell accumulation/rolling *in vitro* and tumor cell progression *in vivo*. However, LB cells invade the spleen and the lymph nodes substantially faster than HA9 cells, which display constitutive HA binding potential (Sionov and Naor, 1997). This finding suggests that the high affinity of CD44 isoforms expressed on HA9 cells to tissue HA slows their migration to the lymphoid organs. Hence, the following hierarchy is suggested in the context of dissemination capacity (faster $>$ slower) of the lymphoma cells: CD44v-transfected LB cells $>$ CD44s-transfected LB cells $=$ LB cells $>$ HA9 cells. To explain this hierarchy we should assume that intermediate affinity (represented by CD44v-transfected LB cells) between cell surface CD44 and tissue HA is required to allow a rapid dissemination. Too high affinity (represented by HA9 cells) slows the migration capacity and too weak affinity allows release of the cells from the tissue substrate, e.g. by the shear stress flow. The remaining cell lines adjust their positions between these two extremes.

As indicated earlier, s.c. inoculated LB cells invade the spleen and the peripheral lymph nodes. Intravenous injection of anti-CD18 mAb blocked spleen, but not lymph node invasion (Zahalka et al., 1995). It was predicted, therefore, that LB cells exploit a different molecule for lymph node infiltration. At that time it was shown (Mackay et al., 1990) that CD44 expression on activated lymphocytes correlated with their localization in the peripheral lymph nodes. We hypothesized, therefore, that the lymph node invasion by LB lymphoma cells is CD44-depended and subsequently challenged this hypothesis by analyzing the ability of anti-CD44 mAb to block the tumor infiltration into this organ. Subcutaneously injected LB cells enter the spleen via the blood and the lymph nodes - via the afferent lymphatics. LB cells cannot enter the lymph node via the high endothelial venule (HEV) even when injected intravenously (Zahalka et al., 1995). This is hardly surprising, because LB cells do not express MEL-14, a molecule essential for HEV binding. Therefore, to efficiently avoid LB cell invasion into the lymph node the relevant antibody should be s.c. injected adjacent to this organ, whereas to avoid spleen invasion the relevant antibody should be injected intravenously (i.v.). Indeed i.v. injection of anti-CD18 mAb (directed against the β2 integrin) suppressed the invasion of LB cell into the spleen, but not into the lymph nodes, whereas s.c. injection of anti-CD44 mAb or its Fab' fragments suppressed the invasion of this lymphoma into the axillary and brachial lymph nodes, but not into the spleen (LB cells co-express CD18 and CD44), as indicated by measuring the proliferation of the organ-infiltrating cells using ^{3}H-thymidine incorporation assay (Zahalka and Naor, 1994; Zahalka et al., 1995). This anti-CD44 mAb, designated I.M.7.81, recognizes a constant epitope, shared by standard CD44 and all CD44 variants. The antibody was injected (150 μg mAb per injection, per mouse) two hours after the tumor inoculation and then every other day until the termination of the experiment (day 12). We further found that at the end of the antibody treatment, the number of the lymphoma cells invading the lymph nodes decreased by at least two orders of magnitude, from hundreds of thousands to several hundreds dpm (Zahalka et al., 1995). Using fluorescent-labeled lymphoma cells (transfected with GFP) and tracing the labeled cells in the local tumor growth as well as in the peripheral lymph node, we found that the I.M.7.81 anti-CD44 antibody suppressed both the local growth and remote lymph node invasions by CD44v-transfected LB cells even when injected 6 days after the tumor inoculation (Wallach-Dayan et al., 2001). Our *in vitro* studies demonstrated that the rolling attachments of CD44-transfected LB cells were restricted to HA substrate. Moreover, CD44v-transfected LB cells mutated at the HA binding site could not invade the local site and remote peripheral lymph nodes. Hence, digestion of tissue HA by the enzyme hyaluronidase may also disrupt the tumor progression. Using the protocol of antibody treatment, s.c. injection of hyaluronidase either two

hours (Zahalka et al., 1995) or 6 days (Wallach-Dayan et al., 2001) after tumor inoculation suppressed the local lymphoma growth and lymph node invasion, in most but not all experiments (Sionov and Naor, 1997). These apparent contradictable findings can be reconciled when considering the double-edged sword role of hyaluronidase (Kovar et al., 2006; Lokeshwar et al., 2005). Delicate balance determines whether hyaluronidase enhances the lymph node metastases by destroying the matrix resistance to cell migration or retards them by tuning and targeting the enzymatic activity to the HA-depended tumor migration phase. When the two vectors display an equal power the enzyme effect cannot be detected. Further apparent contradiction was raised by the hyaluronidase experiment. We reported here earlier that lymph node-infiltrating LB cells do not bind HA, yet in some experiments we showed that hyaluronidase injection reduces the lymph node invasion by the lymphoma. If lymph node-infiltrating LB cells do not recognize HA, how could they be sensitive to enzyme treatment? This paradox can be reconciled by assuming that the metastatic LB cells acquire HA binding capacity before their invasion into the lymph node, e.g. to support their migration, in the afferent lymphatics, but they lose it upon penetration into the lymphoid organ. Alternatively, we may hypothesize that only a very small fraction of lymph node-infiltrating LB cells (which is not detected by flow cytometry) binds HA. This cell fraction, which includes perhaps cancer stem cells (Lobo et al., 2007), is responsible for the HA-depended lymph node colonization and therefore displays hyaluronidase sensitivity.

The enhanced accumulation of CD44v-transfected LB cells over CD44s-transfected LB cells or parental LB cells in the lymph nodes may be attributed to more efficient cell migration to these organs or to more extensive cell proliferation inside them. We found that, after their lymph node invasion, all the above mentioned cell lines (including CD44v- and CD44s-transfected LB cells) display similar proportions of cells in S-phase, i.e. they show almost the same proliferation rate inside the lymphoid organ. This finding implies that the enhanced accumulation of CD44v-transfected LB cells in the lymph nodes must be attributable to the efficient migration into the lymphoid organ rather than accelerated cell division inside it (Wallach-Dayan et al., 2001).

GENE VACCINATION WITH CD44 VARIANT CONSTRUCT, RATHER THAN WITH STANDARD CD44 CONSTRUCT, GENERATES RESISTANCE TO TUMOR GROWTH

The lesson that we have learned from the LB cell lymphoma model in particular and from reviewing the literature in general (Naor et al., 2002; Naor et al., 1997; Ponta et al., 2003) focused our attention not only on the

substantial involvement of CD44 in tumor progression, but also to the role of the specific CD44 isoforms in the support of tumor spread and metastasis. We have seen that CD44 variant(s) of LB cells, rather than standard CD44, is engaged in binding the cells to HA and in supporting their resistance to detachment from HA substrate when subjected to physiological shear stress flow. Moreover, the same CD44 variant(s) is involved in the *in vivo* interaction of LB cells with HA, which is essential for the local tumor growth and its accumulation in remote lymph nodes. If this is the case, the therapeutic strategy should target the relevant CD44 variant on the tumor cells, rather than targeting constant epitopes that are shared by both CD44 variants and standard CD44. As CD44 variants associated with a certain tumor are less ubiquitous than the other CD44 isoforms, including standard CD44, the exclusive targeting of the tumor-expressing variant(s) should rescue all innocent cells that express the other CD44 isoforms, including a substantial number of cells that express standard CD44 only. In other words, limiting the therapeutic targeting to the CD44 variant(s) of the tumor cells should markedly reduce the potential of a side effect damage, which would ultimately be generated if standard CD44 is targeted. To cope with such a challenge we must first identify the CD44 variant(s) of the tumor, then show that this variant(s) is essential for the tumor survival and finally tailor a ''therapeutic suit'' that would selectively silence or kill the relevant tumor. The ''therapeutic suit'' could be either an antibody against the relevant tumor antigen (e.g., CD44v), or even better, a vaccine or a cDNA vaccine of such an antigen. Vaccination, in general, and DNA vaccinations, in particular, have some advantages over passive immunization by antibody. They provide prolonged antigen exposure that continuously stimulates the immune system and offer a unique opportunity to enhance the immunogenicity of the antigen in question by modifying the potential immunizing cDNA sequence. Using the above indicated principles, we generated a cDNA vaccine against CD44v and CD44s of DA3 mammary tumor of BALB/c mice. Vaccination with CD44v cDNA, but not with CD44s cDNA, generated resistance to the tumor growth (Wallach-Dayan et al., submitted).

EPILOGUE

CD44 isoforms support the local growth and the metastatic spread of LB cell lymphoma, DA3 mammary adenocarcinoma (this review article), and many other hematological and solid tumors described in our previous review articles (Naor et al., 2002; Naor et al., 1997). CD44 variants, but not necessarily standard CD44, are associated with the malignant phenotype (i.e., their migratory potency and the metastasis outcome) of the tumors described in this review as well as with many other tumors (Bourguignon

et al., 2001; Carvalho et al., 2006; Hong et al., 2006; Wang et al., 2007; Naor et al., 2002; Naor et al., 1997). However this observation is not the rule. There are examples (Bartolazzi et al., 1994; Sy et al., 1991) that show that standard CD44, rather than its variants, enhances tumor progression. Even more, in some cases (e.g., prostate cancer and cervical neuroendocrine carcinoma) CD44 functions as a metastasis suppressor molecule (Gao et al., 1997; Kuo et al., 2006), which locks the tumor cells in the primary site, thus preventing the malignant spread (reviewed in Naor et al., 2002). Under such circumstances only CD44 downregulation allows release of the tumor from the primary site and the subsequent metastatic phase. However, later studies reported that CD44 rather supports prostate cancer progression (Desai et al., 2007; Patrawala et al., 2007). The internal and external environment of the tumor possibly influences the structure–function relationship between the tumor CD44 and its local substrate. A certain combination of conditions dictates very high affinity of CD44 to its tissue ligand (an example prostate cancer), resulting in tumor arrest in the local growth and a minimal metastatic phase. On the other hand, a different combination of conditions activates, in different tumors, either standard CD44 or CD44 variants, which their intermediate affinity to the substrate rather generates a migratory metastasis-supportive phenotype. Hence, the CD44 structure–function relationship must be separately analyzed in each tumor, and accordingly the therapeutic suit should be tailored. Note that the heterogeneity in CD44-dependency can be sometimes detected even in tumors derived from the same histological origin (e.g., prostate cancer).

The alternative splicing machinery when intensively activated, e.g., by inflammatory cascade (Nedvetzki et al., 2003) or perhaps malignant environment (Huang et al., 2007), may generate, especially at the splicing junction of cell surface CD44, modified sequences, which further enrich the CD44 repertoire and create new, sometimes disease-related structural specificities. For example, we have found that the transcript CD44v3-v10 of synovial fluid cells from joints of rheumatoid arthritis (RA) patients contains an extra tri-nucleotide CAG, which allows translation of an extra alanine between exon v4 and exon v5, without interfering with the reading frame. The extra CAG, was transcribed from the end of the intron, bridging exon v4 to exon v5, exactly at the splicing junction (Nedvetzki et al., 2003). This CD44 version of RA patients, that was designated CD44vRA, while being detected on synovial fluid inflammatory cells, was rarely found on peripheral blood leukocytes (PBLs) of the same patient and never found on synovial fluid cells of osteoarthritis patients or keratinocytes of normal donors, who do express the wild type variant CD44v3-v10 (Golan et al., 2007). The cell surface CD44vRA variant accumulates fibroblast growth factor from the environment and presents it at right orientation to cells with cognate receptor (e.g., fibroblasts, endothelial cells) thereby

accelerating the RA inflammatory cascade, leading to bone and cartilage destruction (Nedvetzki et al., 2003). Furthermore, we suggest that soluble CD44vRA, that was enzymatically cleaved from the RA synovial fluid cell membrane, can interact with galectin-8 and reduce its ability to deliver apoptotic signals to the inflammatory cells, thereby rescuing their pathological activity (Eshkar Sebban et al., 2007). We recently generated anti-CD44vRA mAbs that induce apoptosis in synovial fluid cells of RA patients, but neither in PBLs of the same patients nor in synovial fluid cells of osteoarthritis patients. Furthermore, the anti-CD44vRA mAb induced resistance to joint inflammation in mice with collagen-induced arthritis (Golan et al., 2007). If the malignant process also creates specific modifications in the CD44 splice variants (Vela et al., 2006) as does the inflammatory cascade, mAbs recognizing this alteration may silent the malignant activity of the relevant tumor without causing damage to normal cells engaged in essential physiological functions. This prediction should be challenged, first in animal models, and if found true, in clinical settings.

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CHAPTER

9

RHAMM/HMMR: An Itinerant and Multifunctional Hyaluronan Binding Protein That Modifies CD44 Signaling and Mitotic Spindle Formation

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INTRODUCTION

RHAMM was originally isolated as a secreted fibroblast protein in a model of wounding *in culture* (Fig. 9.1) (Turley, 1982). In this study, embryonic heart tissue was explanted and the supernatant medium was

collected for further analysis between 18 and 24 hours after explantation, when fibroblasts were rapidly migrating and producing high levels of HA. Sampling at this time excluded analysis of dividing cells and interphase cells that had formed actin stress fibers/focal contacts, since these events do not occur until approximately 36 hours after explantation (Fig. 9.1). Hyaluronan binding proteins were isolated using hyaluronan sepharose chromatography, analyzed on SDS-PAGE, and purified proteins were extracted and used for preparation of polyclonal and monoclonal antibodies (Turley et al., 1985; 1987). Expression cloning using these reagents resulted in the isolation of RHAMM (Hardwick et al., 1992). RHAMM is a coiled coil protein that first appeared in vertebrates and is not found in lower organisms or in insects (Fig. 9.2).

Given its roles in such important cellular processes as motility and cell division *in culture* (Turley et al., 2002; Slevin et al., 2007), it is surprising that germline genetic deletion of RHAMM does not result in a detectable phenotype during embryogenesis or adult homeostasis (Tolg et al., 2003; 2006). However, loss of RHAMM does affect the tissue response to injuries

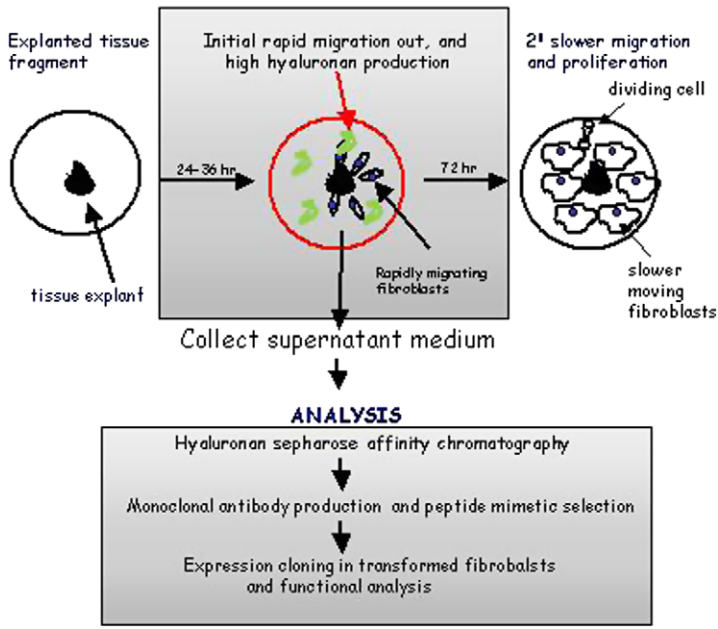

FIGURE 9.1 Schemata used to isolate secreted RHAMM from rapidly migrating fibroblasts *in culture*. Embryonic chick hearts were explanted and fibroblasts allowed to rapidly migrate out of the explants for 24–36 hours. Cell division and a secondary stage of slower cell migration does not occur until 72 hours. The supernatant medium was decanted and hyaluronan binding proteins were captured using hyaluronan-Sepharose affinity columns. Monoclonal antibodies were prepared against the captured proteins and these were used for expression cloning (Turley, 1982).

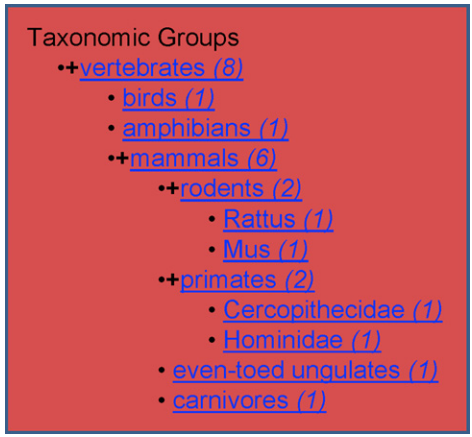

FIGURE 9.2 Taxonomic groups in which RHAMM has emerged. RHAMM first appears in vertebrates and has been identified in mammals, birds, and amphibians. No structural orthologues have yet been identified in lower organisms.

including excisional cutaneous wound repair and bleomyocin-induced lung damage (Tolg et al., 2006; Slevin et al., 2007). Excisional cutaneous wound, repair occurs via several well-regulated stages that include initial inflammation, proliferation, and remodeling (Dorsett-Martin, 2004; Gibran et al., 2007; Oberyszyn, 2007). The inflammatory and proliferative (fibrogenesis) stages are subtly altered by loss of RHAMM (Tolg et al., 2006). For example, neutrophil accumulation remains high throughout the inflammatory and post-inflammatory stages of wound repair in RHAMM$^{-/-}$ mice whereas these cells have undergone apoptosis within several days following injury in wild-type animals. Fibrogenesis of RHAMM$^{-/-}$ wounds aberrantly decreased due to reduced fibroblast infiltration compared to fibrogenesis/fibroblast infiltration in wild-type animals. Mesenchymal differentiation during fibrogenesis is also modified and/or inappropriately regulated. For example, granulation tissue of RHAMM$^{-/-}$ wounds is filled with adipocytes and Rhamm$^{-/-}$ fibroblasts explanted from wounds undergo adipogenesis *in culture* to a much greater extent than wild-type wound fibroblasts. Myofibroblast activity is reduced in Rhamm$^{-/-}$ wounds, as reflected by the relative lack of differentiation markers such as smooth muscle actin. Furthermore, muscle differentiation within the wound is impaired (Tolg et al., 2006). The many similarities between responses to tissue injury and cancer suggest that RHAMM may be important in the progression of malignant tumors.

RHAMM AND CANCER

Although RHAMM mRNA is rarely detected in most homeostatic adult tissues, it is commonly hyperexpressed in human cancers. Cell surface RHAMM has been detected as a tumor antigen in several malignancies,

including leukemia, breast, melanoma, prostate, and ovarian cancers (Greiner et al., 2006; Schmitt et al., 2008). Intracellular RHAMM expression is also elevated above normal tissue levels in brain tumors (e.g., astrocytomas, gliomas, meningiomas) (Panagopoulos et al., 2008), gastric cancer (Li et al., 2000a), colorectal cancer (Zlobec et al., 2008), multiple myeloma (Maxwell et al., 2004), oral squamous cell carcinoma (Yamano et al., 2008), endometrial carcinomas (Rein et al., 2003), and bladder cancer (Kong et al., 2003). RHAMM is often most highly expressed in carcinomas at later stages of progression and the levels of RHAMM have been correlated to tumor stage and in some cases to prognosis. For example, RHAMM hyperexpression is an independent prognostic indicator of poor clinical outcome in breast cancer (Wang et al., 1998; Pujana et al., 2007), colorectal cancer (Zlobec et al., 2008), multiple myeloma (Maxwell et al., 2004), and oral squamous cell carcinoma (Yamano et al., 2008).

The prognostic value of RHAMM is linked to specific subtypes of organ specific malignancies, which have distinct anatomic and molecular pathologies that are correlated to different outcomes. For example, colorectal cancers can be grouped into DNA mismatch repair-proficient, MLH1 negative and presumed Lynch syndrome. Although RHAMM is expressed at all Dukes stages of ungrouped colorectal cancers (Yamada et al., 1999), hyperexpression of RHAMM mRNA is an independent prognostic factor for poor outcome and ranks higher than T stage, vascular invasion, tumor budding, and tumor grade in its association with increased risk of peripheral metastasis and with worse outcome in patients with metastatic disease (Zlobec et al., 2008a, b). RHAMM is a prognostic factor in DNA-mismatch repair-proficient (MMR-proficient) and presumed Lynch syndrome forms of colorectal cancer but not in MLH1 negative colorectal tumors (Duval et al., 2001; Lugli et al., 2006; Zlobec et al., 2008c). Furthermore, combining RHAMM hyperexpression with another prognostic factor, loss of p21, identifies a subgroup of MMR-proficient tumors with a high incidence of microsatellite instability that has a particularly poor prognosis (Zlobec et al., 2008c). These results predict that the association of elevated RHAMM expression with neoplastic disease may be selective in terms of tumor subtype. It may therefore be possible to use RHAMM levels as one marker to further classify specific tumors or to target RHAMM for therapy in such tumor subtypes.

In addition to increased levels of expression, distinct RHAMM isoforms that arise through multiple mechanisms are associated with tumors and some of these isoforms appear to behave as multifunctional oncogenes. Two major alternatively spliced mRNA species have been identified, which differ in the presence of exon 4, (which encodes a 15 amino acid sequence in the amino terminal region of RHAMM). For example, $\text{Rhamm}^{-\text{exon4}}$ is expressed by aggressive multiple myeloma subsets (Maxwell et al., 2004).

Other tumor types (including breast carcinoma, astrocytoma, multiple myeloma, melanoma, gastric carcinoma, and astrocytomas), which have been reported to hyperexpress RHAMM mRNA also express truncated RHAMM protein forms (Li et al., 2000a, b; Ahrens et al., 2001; Zhou et al., 2002; Hamilton et al., 2007). Although the basis for the formation of these smaller proteins (which range from 40 to 70 kD compared to 86 kD full length Rhamm protein) is not known, it is possible that they arise as a result of partial proteolytic processing and/or the use of multiple start codons, which are present within the coding sequence of RHAMM. Human astrocytomas express a 70 kD tumor specific RHAMM isoform in addition to the full length protein (86 kDa) which is expressed in low amounts by normal astrocytes (Zhou et al., 2002). Gastric carcinomas, breast carcinomas, and melanomas also express multiple RHAMM isoforms (52–70 kDa) in addition to the full-length form (Li et al., 2000b; Ahrens et al., 2001; Hamilton et al., 2007). Highly invasive human breast cancer cell lines such as MDA-MB-231 similarly express a 70 and 52 kD Rhamm protein compared to less aggressive lines such as MCF7, which primarily express the full length RHAMM protein (Hamilton et al., 2007). It is also possible that some of these additional lower molecular weight isoforms are created from low abundance splice variants of RHAMM mRNA, however no such variants have been identified to date.

Several studies suggest that mutations in RHAMM may affect oncogenic properties. RHAMM contains mononucleotide repeat coding sequences that are mutated in human MMR type colorectal cancers with high microsatellite instability (Duval et al., 2001). These mutations result in conversion of intronic 3′ non-coding to a coding sequence which creates additional protein sequence resembling the HA binding region of RHAMM. Malignant peripheral nerve sheath tumors arising from neurofibromatosis type 1 (risk is 10%) often exhibit an allelic (N = 1) deletion of a RHAMM gene (Levy et al., 2004; Mantripragada et al., 2008). Germ line polymorphisms upstream of the coding sequence in the RHAMM gene are also associated with a predisposition to breast cancer suggesting that mutated RHAMM may function as a novel breast cancer susceptibility gene (Pujana et al., 2007).

Finally, one of the more intriguing characteristics of RHAMM that is likely associated with oncogenesis is the change in both the subcellular distribution and functions of the protein (Maxwell et al., 2008). In examples such as gastric and highly malignant breast cancers, RHAMM is exported to the cell surface where it functions as a hyaluronan-responsive motogenic protein (Li et al., 2000a; Hamilton et al., 2007). Extracellular RHAMM is detected on a number of tumor cell lines cultured *in vitro*. In this case, cell surface RHAMM is more pronounced in subconfluent, rather than confluent cultures (Turley, 1982; Hardwick et al., 1992) and the addition of hyaluronan acts to further increase the amount of cell surface RHAMM

(Gao et al., 2008). As has been observed with many other non-conventionally exported proteins such as autocrine motility factor and galectins 1 and 3 (Radisky et al., 2003; Nickel, 2005; Prudovsky et al., 2008), extracellular RHAMM functions, at least in part, to enhance motility as well as invasion of tumor cells (Hamilton et al., 2007). However, in certain tumors such as mesenchymal desmoid tumors, alterations in RHAMM may function to promote both tumor initiation and as invasion (Tolg et al., 2003). RHAMM is highly expressed in human mesenchymal desmoid tumors (fibromatoses). In an animal model of desmoid tumor susceptibility, both the number (which equates to initiation) and size (which equates to growth and invasiveness) of tumors are decreased when RHAMM is germ line deleted as a result of homologous recombination (Tolg et al., 2003).

Although the molecular mechanisms for the unconventional export and other subcellular changes in the distribution of RHAMM during tumorigenesis are not yet known, similar changes occur during normal wound healing and tissue repair (Zaman et al., 2005; Tolg et al., 2006; Slevin et al., 2007), which are processes that require cellular infiltration and remodeling of the microenvironment. Thus, some of the functions of extracellular RHAMM in tumors may indeed mirror those observed in wound healing, which like tumor progression requires both cellular infiltration and tissue remodeling.

EXTRACELLULAR AND INTRACELLULAR RHAMM ONCOGENIC FUNCTIONS *IN CULTURE*

RHAMM was originally identified as a "secreted" hyaluronan binding protein that was isolated from the supernatant medium of rapidly locomoting, sparsely cultured chick embryonic fibroblast (Turley, 1982) (Fig. 9.1). The binding of purified, secreted RHAMM protein to fibroblast monolayers is both saturable and of high affinity (Turley et al., 1985). These and other data led to the prediction that secreted RHAMM acts as a co-receptor that associates with binding sites or other receptors located at the cell surface (Hardwick et al., 1992). More recently, cell surface RHAMM has been shown to function as a motility receptor in both dermal fibroblasts responding to injury and in aggressively invasive breast cancer cell lines that have undergone EMT by forming complexes with CD44 (Tolg et al., 2006; Hamilton et al., 2007). RHAMM coated beads were used to demonstrate that RHAMM interactions with the cell surface enhances the cell surface display of CD44 (Fig. 9.3). Additional studies using RHAMM$^{-/-}$ fibroblasts have shown that RHAMM-stimulated motility is sensitive to antibodies against CD44 and that RHAMM stimulates Erk1,2 activity through its interaction with CD44 (Tolg et al., 2006). These and other data (summarized below) indicate that cell

surface RHAMM functions as a co-receptor for CD44 in wound fibroblasts and in breast cancer cells that have undergone an EMT (e.g., MDA-MB-231 cells) (Tolg et al., 2006; Hamilton et al., 2007). Furthermore, this interaction is necessary for sustained activation of motogenic signaling pathways through ERK1,2. Cell surface RHAMM also associates with RON (gene name, MST-1R), a tyrosine receptor kinase of the c-Met family, whose ligand is macrophage stimulating protein (MSP) (Manzanares et al., 2007). Thus extracellular RHAMM functions as a co-receptor with one or more adhesion/growth factor receptors on the cell surface, with the ability to alter the intensity and/or duration of key signal transduction pathways.

Extracellular export of RHAMM may be particularly relevant to tumor therapy. For example, patients with certain malignant tumors were shown to have increased levels of RHAMM in their circulation predicting that this protein might be used as a tumor specific marker (Greiner et al., 2006). Indeed, dendritic vaccination using RHAMM peptides has shown efficacy in phase I clinical trials of patients with acute myeloid leukemia (AML) and multiple myeloma (Schmitt et al., 2008). Furthermore, vaccination of mice using dendritic cells transfected with RHAMM mRNA induces an anti-tumor effect associated with increased activation of the immune system in a model of mouse glioma (Amano et al., 2007). Thus, tumors may be susceptible to these and other RHAMM-targeted therapies as a result of the increased levels of cell surface protein expressed on these tumors.

While initial reports on the function of RHAMM focused on its extracellular functions, the cloning and further characterization of RHAMM primary structure revealed the protein lacks a signal peptide for classical secretion through the Golgi/ER or a membrane spanning sequence typical of traditional transmembrane receptors (e.g., CD44) (Maxwell et al., 2008). Several subsequent studies therefore focused on identifying the functions of intracellular RHAMM protein forms, since its sequence was more in line with that of a cytoplasmic or nuclear protein. These studies demonstrated that intracellular RHAMM is associated with interphase microtubules, it can be localized to the nucleus (Hofmann et al., 1998a; Liska et al., 2004; Shakib et al., 2005), and it also co-distributes with centrosomes/mitotic spindles (Maxwell et al., 2003; Evanko et al., 2004). This complex subcellular distribution of RHAMM in tumor cells has created challenges for developing models to study the importance of cell surface verses intracellular RHAMM.

The addition of soluble, recombinant extracellular RHAMM to Ras-transformed fibroblasts was originally observed to inhibit TGF-β induced progression through the cell cycle by inhibiting cyclin B1 and cdc2 expression thus restricting passage through G2/M (Mohapatra et al., 1996). Additionally, it has been shown using an unbiased expression array screen in synchronized, proliferating cells that RHAMM mRNA levels peak at or near the G2/M boundary of the cell cycle (Cho et al., 2001;

Whitfield et al., 2002; Groen et al., 2004; Liska et al., 2004; Yang et al., 2005) and RHAMM is one of the numerous mitotic spindle proteins that are phosphorylated during spindle formation (Nousiainen et al., 2006). Furthermore, intracellular RHAMM is required for progression of certain cell types through G2/M (Maxwell et al., 2003). Collectively these studies imply that control of G2/M by RHAMM may be coordinated by both extracellular and intracellular forms of this protein. However, the precise relationship between the mitotic functions of these two pools of RHAMM has not yet been established.

Cells derived from RHAMM$^{-/-}$ animals provide an important tool to address this problem. Studies using recombinant RHAMM-coated beads to challenge RHAMM$^{-/-}$ fibroblasts have shown that these beads are sufficient to stimulate the motility of these cells over 4 hours indicating that cell surface RHAMM alone is sufficient to promote short spurts of motility (Tolg et al., 2006). On the other hand, recombinant RHAMM coated beads do not rescue the aberrant mitosis of RHAMM$^{-/-}$ fibroblasts (Tolg et al., 2006). Collectively, the evidence indicates that intracellular RHAMM protein levels (e.g. too much or too little) are necessary and sufficient to control mitotic spindle formation and stability. More recently, the spindle formation function of RHAMM has been shown to be part of the BRCA/BARD1 E3 ubiquitin ligase pathway (Pujana et al., 2007; Joukov et al., 2006). This pathway has the potential for promoting genomic instability in tumors in which BRCA1 is compromised by mutation. The mitotic spindle functions of RHAMM may also, at least in part, explain the importance of RHAMM as a novel breast tumor susceptibility gene.

These motogenic and mitogenic effects of RHAMM have been demonstrated *in culture*, and although they have the potential to contribute to cancer initiation and progression, is there any evidence that RHAMM performs similar motogenic and mitogenic functions *in vivo*? As noted above, RHAMM polymorphisms and hyperexpression have been linked to cancer susceptibility and poor clinical outcome in a number of human cancers. However, analyses of RHAMM gene dysregulation using animal models have not been extensively reported. To date, *in vivo* analyses of the mitogenic and motogenic effects of RHAMM have been reported in a mouse model of collagen-induced arthritis (Nedvetski et al., 2004), during lung repair of bleomycin induce injury (Zaman et al., 2003), during repair of full thickness, excisional skin wounds in RHAMM$^{-/-}$ mice (Tolg et al., 2006), and in desmoids/upper intestinal tract tumor formation in a transgenic mouse model of these tumors (Tolg et al., 2003).

Functions of RHAMM in Animal Models of Repair and Disease

Collagen-induced arthritis in both wild-type and CD44$^{-/-}$ mice is dependent upon the production of hyaluronan since destruction of this

polysaccharide by hyaluronidase injected into the toe joint blocks its development (Naor et al., 2007). In wild-type mice, blocking CD44 function also stops the appearance of arthritis in joints but genetic loss of CD44 intensifies rather that abrogates arthritis development. In this model, extracellular RHAMM compensates for CD44/hyaluronan binding function since injection of anti-RHAMM antibodies or recombinant RHAMM reduces the inflammatory response in the absence of CD44 (Nedvetski et al., 2004). However, compensation is accompanied by an intensified disease course due to the upregulation of pro-inflammatory cytokines that are highly expressed in the absence of CD44. The molecular mechanisms by which RHAMM affects inflammation in this model have not yet been identified but the process is associated with increased accumulation of hyaluronan in the toe joints of $CD44^{-/-}$ animals, which acts to promote signaling through cell surface RHAMM. Although RHAMM partners with CD44 in some systems (see below), this is not possible in the $CD44^{-/-}$ mice, suggesting that cell surface RHAMM may partner with an as yet unidentified integral receptor on spleenocytes (Nedvetski et al., 2004).

Macrophage infiltration into injured lung tissues also requires cell surface expression of RHAMM (Zaman et al., 2005). In this model of lung injury, TGFβ1 production is stimulated by bleomycin and this cytokine promotes expression of cell surface RHAMM and motogenic influx of macrophages to the site of injury. Although macrophages express both cell surface RHAMM and CD44, blocking the motogenic function of cell surface RHAMM with anti-RHAMM antibodies alone reduced macrophage influx and the concomitant fibrotic response resulting from bleomycin. Collectively, these two studies using injury models demonstrate a role for cell surface RHAMM in controlling influx of inflammatory cells into wounded tissues thereby affecting the course of tissue repair.

Germline genetic deletion of RHAMM results in the wound mesenchymal defects noted above (Tolg et al., 2006). In $RHAMM^{-/-}$ mice, reduced fibroplasia is related to a motogenic defect associated with reduced responsiveness *in culture* to motility-promoting stimuli such as PDGFBB and hyaluronan oligosaccharides (Tolg et al., 2006). Surprisingly, neither increased apoptosis nor reduced proliferation is detected in the wound site although markers for motility are aberrantly expressed. A motogenic defect of $RHAMM^{-/-}$ fibroblasts is retained *in culture* and results from reduced display of cell surface CD44 and aberrant activation kinetics of ERK1,2. This study also demonstrated that CD44 and RHAMM co-associate to regulate the duration of ERK1,2 motogenic signaling during tissue repair. Furthermore, the motogenic defect of $RHAMM^{-/-}$ wound fibroblasts can be rescued either by the stable expression of constitutively active MEK1 or by addition of extracellular recombinant RHAMM, which was prevented from intracellular uptake by linkage to Sepharose beads (Fig. 9.3).

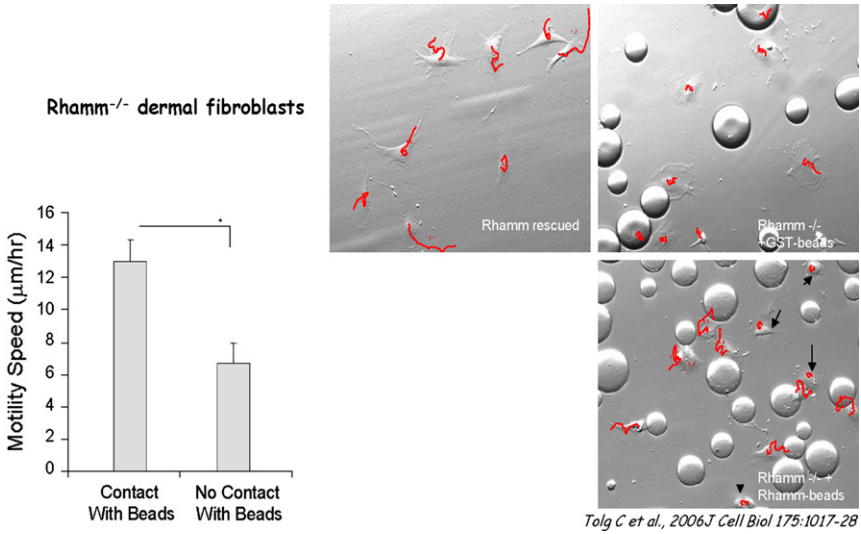

FIGURE 9.3 Cell surface but not intracellular RHAMM is required for acute migration in response to hyaluronan or growth factors. RHAMM$^{-/-}$ dermal fibroblasts were exposed to recombinant GST-RHAMM protein linked to Sepharose beads or to GST alone. GST-RHAMM-beads that touch RHAMM$^{-/-}$ fibroblasts promote migration while GST-beads or untouched beads (detected by real time analysis) do not. Since cells cannot internalize beads coated with recombinant RHAMM, showing that cell surface RHAMM is sufficient to promote migration, at least over the 4 h assay period. From Tolg et al. (2006).

Two studies have also investigated the roles of RHAMM in facilitating the growth of tumors *in vivo*. In a xenograft injection model using MDA-435 tumor cells, expression of the hyaluronan binding region of RHAMM (Liu et al., 2004) results in decreased growth rate compared to control tumor cells lacking this peptide. The slower growth produced by the expression of the hyaluronan binding domain of RHAMM results from the ability of this peptide to activate caspase 3 and 8 as well as poly-(ADP-ribose) polymerase, thereby promoting apoptosis of the transfected tumor cells. The effect of RHAMM expression in the initiation and growth of desmoid tumors in a syngeneic mouse environment has also been evaluated (Tolg et al., 2003). For these studies, RHAMM$^{-/-}$ mice were crossed to Apc/Apc1638N transgenic mice [which are predisposed to desmoids (fibromatoses) and upper intestinal tract tumors]. RHAMM deficiency significantly reduces both the number (equated to initiation) and size (equated to invasion) of desmoids but not upper intestinal tract tumors. These effects of RHAMM on tumorigenesis are apparently related to a desmoid tumor cell proliferation deficiency that is observed at low but not high cell density *in culture*. Currently therefore, these *in vivo* data support a role for RHAMM in both cell motility and

proliferation and further suggest a potential role in apoptosis. Is there any clinical evidence that RHAMM performs similar functions in human cancers?

Functions of RHAMM in Human Tumors

The selectively high expression of RHAMM in tumor cell subsets (Wang et al., 1998; Pujana et al., 2007) and at the invading front of primary breast cancers (Assmann et al., 1998) is consistent with the ability of RHAMM to promote motogenic/invasive activities of tumors that have been identified *in culture*. Proteomics and integrative genomics of human cancers also point to a role for RHAMM in mitosis. For example, DNA microarray analyses have revealed that RHAMM expression is cell cycle G2/M regulated in hepatic carcinoma (Yang et al., 2005) and RHAMM expression is correlated with centrosomal structural abnormalities in multiple myeloma (Maxwell et al., 2005). These studies are consistent with evidence indicating that intracellular RHAMM has a mitotic spindle/centrosomal function *in culture*. But how do the multiple RHAMM protein forms control these disparate cellular functions?

RHAMM PROTEIN FORMS CONTROL MULTIPLE SIGNALING NETWORKS

Neither structural nor phylogenetic analyses of RHAMM (Fig. 9.2) have provided clues as to the molecular mechanisms by which the protein products of this gene influence cell motility and cell division. The secondary structure of RHAMM is largely a hydrophilic coiled coil (Turley et al., 2002; Groen et al., 2004), with the exception of an N-terminal 162 amino acid globular region (encoded by exons 1–4) and additional interspersed linker regions connecting the helical domains of the coiled coils (Fig. 9.4). These analyses suggest intracellular RHAMM performs mainly structural functions yet the majority of experimental data point to the importance of this protein in activation of signal transduction pathways. RHAMM primary sequence contains neither a signal peptide nor a membrane spanning sequence that would predict its ability to be exported from the cell or to activate signaling cascades once it is exported. Yet again, evidence *in vitro* shows a clear role for extracellular RHAMM in motogenic signaling (Hofmann et al., 1998b; Maxwell et al., 2008). Furthermore, although it contains putative nuclear localization and export sequences (and is found in the cell nucleus), it does not contain sequences that predict its localization to the mitotic spindle, mitochondria, interphase microtubules, or actin filaments. These results suggest that interaction of RHAMM with these subcellular

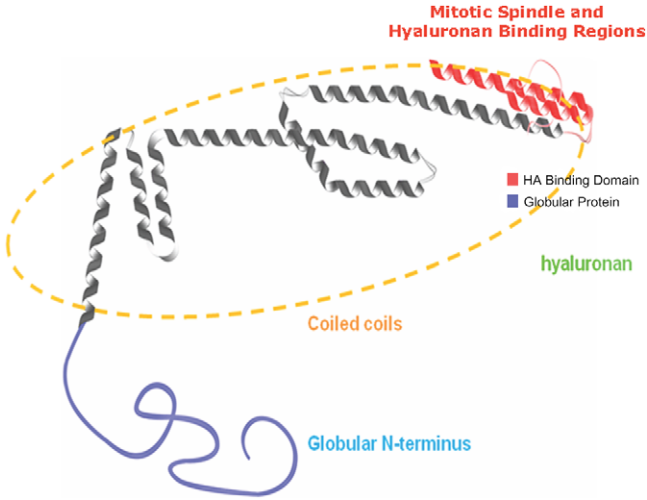

FIGURE 9.4 The predicted secondary structure of RHAMM. RHAMM is largely an acidic coiled coil protein. Approximately ¾ of its sequence occurs as a coiled coil similar to TACC, myosin, and other coiled coil proteins. The N-terminal 163 amino acids form a globular peptide. The mitotic spindle binding (a leucine zipper) and hyaluronan binding (highly basic region) are localized to the carboxyl terminus of RHAMM.

components is either indirect or involves unique structural domains that mediate binding.

Phylogenetic analysis of RHAMM sequence has also not clarified the molecular mechanisms by which RHAMM could affect such basic cell functions as motility and mitosis. RHAMM orthologs first appeared in vertebrates but there is no significant homology to proteins in lower organisms such as *Drosophila*, worms, or yeast that might provide hints as to the functions of this gene (Fig. 9.2). As a result, a conundrum has arisen surrounding not only the motogenic and mitogenic functions of RHAMM protein forms but also when and where these functions come into play. On the one hand experimental (but not proteomic) analysis of RHAMM shows it performs essential motogenic and cellular functions as a cell surface co-receptor and mitogenic functions as a mitotic spindle binding protein *in culture* and during tissue repair *in vivo*. The conundrum then is this: How can a cell surface co-receptor also function as a mitotic spindle protein?

Growing evidence has shown that this conundrum is not restricted to RHAMM. In recent years a number of proteins that are classified as cytoplasmic (due to lack of Golgi/ER export sequence and membrane spanning sequence) are secreted by unconventional means and associate with integral receptors to influence cell signaling (Fig. 9.5) (Radisky et al., 2003; Nickel, 2005; Maxwell et al., 2008; Prudovsky et al., 2008). The

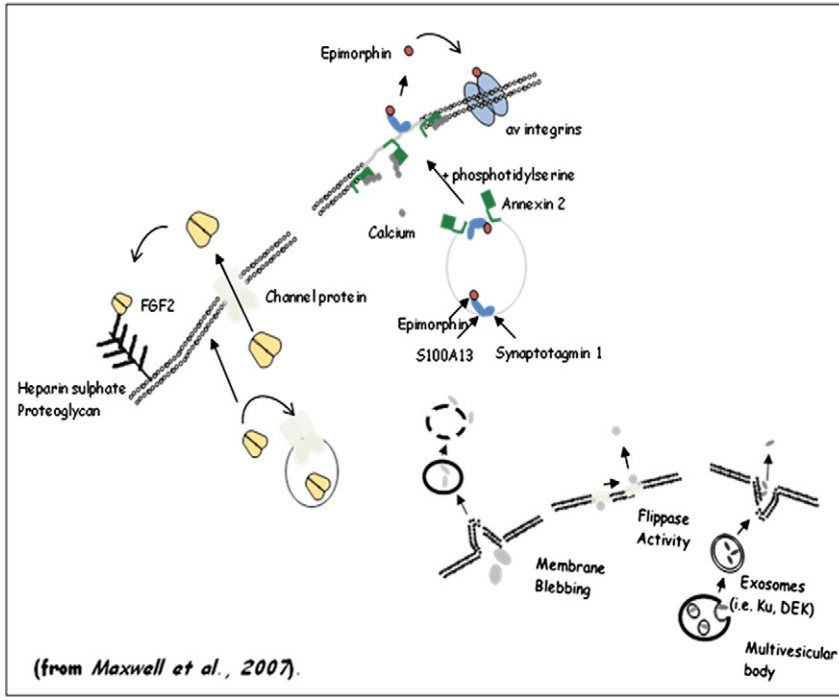

FIGURE 9.5 Diagram of mechanisms for unconventional export of cytoplasmic proteins. Several mechanisms have been identified that permit release of cytoplasmic proteins in the absence of cell death. These include export through channels, via an export protein complex,

signaling functions of these multifunctional or "moonlighting" proteins at the cell surface so far appear to be unrelated to their intracellular functions. RHAMM is one example of this class of proteins and others include epimorphin/syntaxin 2; galectins 1, 3, autocrine motility receptor, and bFGF1,2. Export mechanisms are still being characterized but to date appear to involve one of five types: passage through membrane channels, flipping through the plasma membrane via protein export complexes, membrane blebbing, flippase activity, and exosomes (Fig. 9.5) (Maxwell et al., 2008).

Although the mechanisms for unconventional export of RHAMM have not yet been defined, it is secreted in response to specific stimuli as is the case with other unconventionally exported proteins. For example, TGFβ1 and hyaluronan both promote export of RHAMM to the extracellular compartment. The extent to which the extracellular functions of RHAMM are coordinated remain to be determined; however evidence suggests these apparently discrete pools of RHAMM are somehow linked. Further

characterization of these RHAMM functions may provide unique paradigms for future structure/function analyses. At the very least, the puzzle of why the mitotic and motogenic functions are essential for wound repair (and appear to be utilized by tumors) but not for embryogenesis or adult homeostasis suggest that our current ability to relate function to structure is far from complete. These findings also imply that cell division and motility are differently regulated during embryogenesis/homeostasis compared to what occurs during wound repair/neoplasia. If so, our appreciation of the regulatory complexity of these fundamental cell functions is also still rudimentary. Although little insight into molecular mechanisms of these multiple documented RHAMM functions has been attained by proteomics, what has experimentation told us?

The motogenic signaling ability of RHAMM requires the cell surface display of this protein (Slevin et al., 2007; Turley et al., 2002; Tolg et al., 2006), which results from unconventional export in response to such motogenic factors as TGFβ1 (Samuel et al., 1993) and hyaluronan (Gao et al., 2008). In fact, cell surface RHAMM alone is sufficient to promote migration over short time periods (e.g. 4 hours) since exogenous RHAMM added to RHAMM$^{-/-}$ fibroblasts rescues their ability to migrate in response to serum and hyaluronan (Tolg et al., 2006). *In culture*, unconventional export of RHAMM also appears to be time (after plating) and cell density dependent (Turley, 1982; Hardwick et al., 1992). Stimulated export is a characteristic of unconventionally secreted proteins that differs from classical export through the Golgi-ER, which is constitutive in nature.

Exported RHAMM promotes activation of signaling cascades such as protein tyrosine kinases (e.g., c-abl, src, MST-1R, FAK, Erk1,2, PI3 kinase and Ca^{++} fluxes in response to PDGF, hyaluronan, serum, bFGF, and MSP (Turley et al., 2002; Greiner et al., 2006; Evanko et al., 2007; Naor et al., 2007; Slevin et al., 2007). RHAMM, which lacks membrane-spanning sequence and is a likely peripheral surface protein, appears to associate with integral receptors in order to promote activation of signaling cascades. As described above, RHAMM physically associates with integral receptors (e.g., CD44 and MST-1R) (Tolg et al., 2006; Hamilton et al., 2007; Manzanares et al., 2007). Although a co-association between cell surface RHAMM and CD44 has been described in aggressive human breast cancer cell lines and in dermal wound fibroblasts, it clearly partners with other as of yet unidentified cell surface receptors in the absence of CD44. The RHAMM/CD44 pairing promotes cell surface retention of CD44 and sustained activation of ERK1,2 possibly through formation of RHAMM/CD44 dimers. However, this partnering may not always transduce signaling in response to hyaluronan. For example, hyaluronan-promoted invasion of glioma cells into brain slices requires RHAMM but not CD44 even though individual tumor cells express both proteins (Akiyama et al.,

2001). As another example, hyaluronan oligosaccharides promote protein tyrosine phosphorylation of focal adhesion kinase (FAK), paxillin, and ERK1,2 in endothelial cells (Lokeshwar and Selzer, 2000; Slevin et al., 2007; Gao et al., 2008). These signaling events require RHAMM although do not necessarily involve CD44. Furthermore, although both CD44 and RHAMM are required for endothelial tube formation, CD44 is uniquely required for endothelial cell adhesion while only RHAMM is required for migration of endothelial cells in 2D culture (Savani et al., 2001). Importantly, RHAMM but not CD44 is required for bFGF-induced neo-vascularization in mice (Slevin et al., 2007).

These results indicate that CD44 and cell surface RHAMM share some functions, which likely require their physical association, but they also regulate distinct processes possibly in part by partnering with distinct membrane and/or signaling components. For example, the results and evidence discussed earlier that a cell surface form of RHAMM compensates for the genetic loss of CD44 in a hyaluronan-dependent collagen-induced form of arthritis (Nedvetski et al., 2004) predict that RHAMM binds to additional hyaluronan receptors and/or to other integral membrane proteins. Indeed, the physical association of cell surface RHAMM with MST-1R in airway epithelia provides an example of a RHAMM partner that mediates growth factor (MSP1) regulated signaling (Manzanares et al., 2007).

To date, mitotic spindle/centrosome functions of intracellular RHAMM forms have been reported but the presence of RHAMM in multiple intracellular compartments (e.g. mitotic spindle, interphase microtubules, actin stress fibers, cell-substratum adhesions, nucleus, Fig. 9.6) and their association with disparate proteins such as BCL2 (Xu et al., 2003), calmodulin (Lynn et al., 2001), and glucose regulated proteins p78 and 75 (Kuwabara et al., 2006) predict additional intracellular functions. The mitotic spindle functions of RHAMM were first revealed when microinjected anti-RHAMM antibodies were observed to result in aberrant mitotic spindle formation (Maxwell et al., 2003). Consequently, several studies showed that RHAMM complexes with other mitotic spindle binding proteins including TPX2 and NuMa (Groen et al., 2004; Joukov et al., 2006; Pujana et al., 2007). Addition of recombinant RHAMM or use of anti-RHAMM antibodies to block endogenous RHAMM protein function results in aberrant mitotic spindle formation in extracts of Xenopus or HeLa cells (Joukov et al., 2006). Quantifying the amount of RHAMM in these cell extracts reveals that excess RHAMM protein results in formation of multiple and structurally aberrant spindles. The E3 ubiquitin ligase activity of BRCA1/BARD1 complexes attenuates the mitotic spindle functions of RHAMM by regulating the level of this protein at specific points of the cell cycle (Joukove et al., 2006). The proposed mechanisms by which cell surface RHAMM/CD44 activates motogenic signaling cascades and intracellular RHAMM/TPX2/BRCA1/

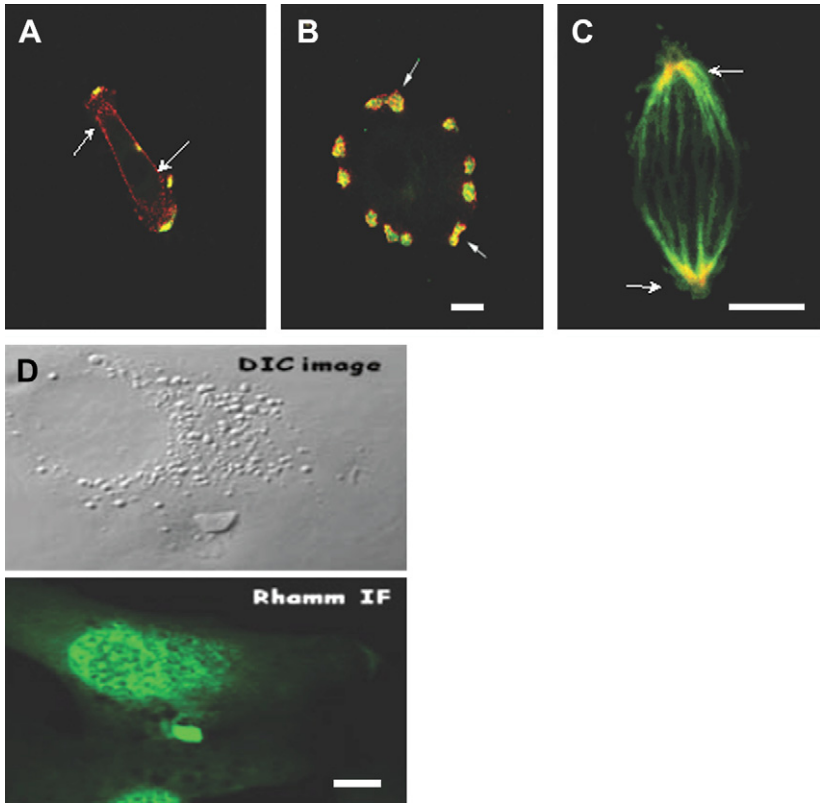

FIGURE 9.6 RHAMM proteins are localized to multiple subcellular compartments. Confocal analyses of fibroblasts show that compartmentalization of RHAMM proteins change over time after subculture. Thus RHAMM protein is (**A**) initially found at the cell membrane as cells flatten onto the growth surface [red is RHAMM (arrow), green is cortactin] then (**B**) becomes distributed in podosomes (red is RHAMM, green is cortactin, arrows show co-localization of RHAMM and cortactin). Bar = 20μm (**C**) RHAMM decorates the apex of mitotic spindles (arrow, Bar = 5μm) of dividing cells (red is RHAMM and green is phospho-Erk1,2) and is often present in the (**D**) nuclei of interphase fibroblasts. Bar = 25μm. (See Page 4 in Color Section at the back of the book).

BARD1 complexes regulate mitotic spindle integrity have recently been reviewed (Maxwell et al., 2008).

Subsequent studies using yeast two hybrid screens identified 28 additional protein partners for intracellular RHAMM of which several (BRCA1,2, CSPG6) were confirmed to associate with RHAMM in mammalian cells using immunoprecipitation assays (Pujana et al., 2007). From these and other published data a RHAMM "interactome" was constructed mainly of intracellular proteins that are components of various signaling pathways that impact upon cytoskeleton or centrosome integrity.

These analyses have cemented a dogma that the cell surface signaling and mitotic spindle protein of RHAMM are distinct. But are the known intracellular and extracellular functions of RHAMM somehow linked? Certain studies (discussed above) suggest that some functions of extracellular and intracellular forms of RHAMM might be linked in mitosis. To reiterate, extracellular and intracellular RHAMM forms both affect progression through G2/M, and RHAMM levels increase during transit through the cell cycle. One way to develop testable models to address this question of relatedness is to use a systems biology approach to construct potential functional interactomes, which predict networks of interacting or functionally linked proteins. For example, this approach was used to construct a RHAMM/centrosome interactome implicated in spindle stability (Pujana et al., 2007). We constructed a similar interactome *in silico* using the Ingenuity Pathway Analysis Software-Complete Pathways Database functional network program (http://www.ingenuity.com). With this program, we interrogated the functional interactions amongst reported RHAMM partner proteins, including both the intracellular and cell surface protein partners reviewed above. Four functional networks were identified but only one of these incorporated most of the well characterized RHAMM binding partners. This network (Fig. 9.7) includes RHAMM/HMMR, CD44, ERK1,2, BRCA1, 2, BARD1, RON, PDGFBB, PDGFR, MAD1L1, MAD2L1, HGS, XP06, and nuclear pore proteins. The functions of this network include cell cycle, cellular assembly, and organization, consistent with the dynamic nature of both cell motility and mitotic spindle formation.

The two signaling pathways incorporated with the highest level of significance within this functional network account for the role of BRCA1 in DNA damage response ($p < 0.000180$) and PDGF signaling ($p < 0.001$). However, these interactomes do not include the previously published BRCA1 pathway in mitotic spindle formation/stability, although many of the components of this pathway are included (Fig. 9.7). The role for RHAMM in PDGF and other growth factor signaling is well documented (Turley et al., 2002; Slevin et al., 2007), as is an involvement of ERK1,2 in RHAMM controlled signaling (Turley et al., 2002; Slevin et al., 2007). The direct interaction between RHAMM and CD44 is represented in Fig. 9.7 and CD44 is predicted to link RHAMM to PDGFR. The top physiological system associated with this signaling network is connective tissue function ($p < 0.000005$) and the top disease category is cancer ($p < 0.000005$), in particular tumor morphology. Collectively, these results support numerous experimental data which suggest that both cell surface and intracellular RHAMM forms contribute to a functionally interconnected network that controls processes essential to cell motility and mitosis important for both connective tissue function and cancer progression.

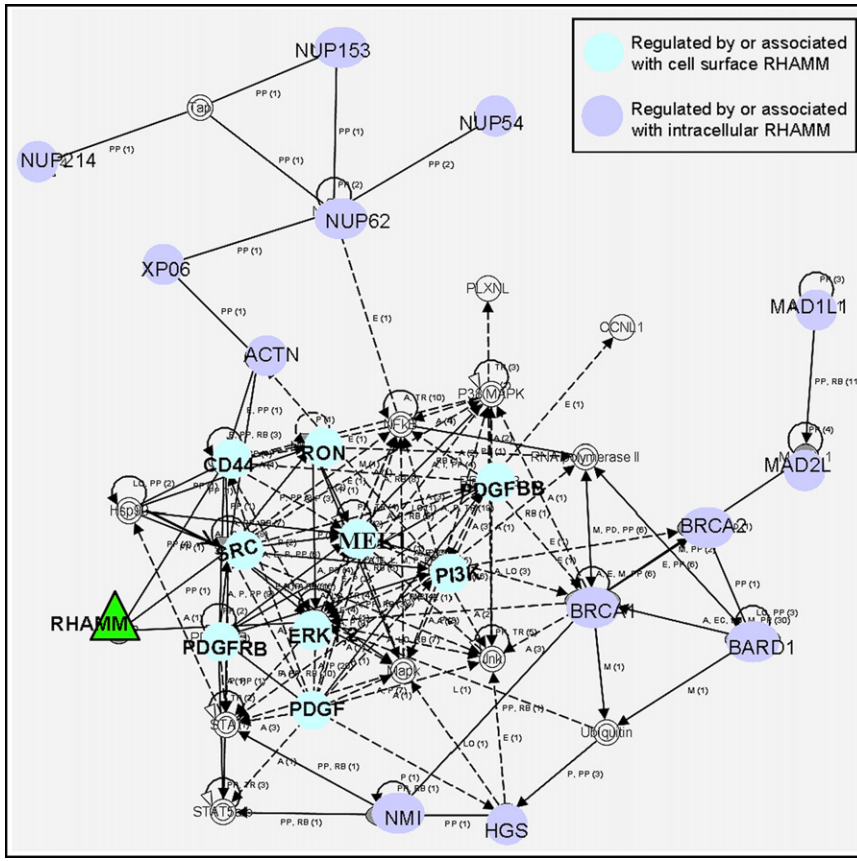

FIGURE 9.7 Functional signaling network regulated by cell surface and intracellular RHAMM. Using published RHAMM protein intracellular and extracellular partners, functional networks were constructed using Ingenuity pathway analysis. Several networks were derived from this analysis, but the network were shown here reveals that most of the proposed oncogenic effects of RHAMM can be functionally linked. For example, this network shows that CD44, RON, PDGF, MEK1/ERK1,2 are functionally linked to BRCA1,2, BARD 1 nuclear pore proteins and centrosomal proteins. The cell surface activities are shown in red and the intracellular activities are shown black. These results predict that at least some of the extracellular and intracellular functions of RHAMM may be coordinated. RHAMM (HMMR, receptor for hyaluronan mediated motility); RON (MSTR-1, macrophage stimulating 1 receptor); ACTN (alpha actinin); MEK1 (mitogen-activated protein kinase kinase); ERK1,2 (mitogen-activated protein kinase); PDGFRB (platelet derived growth factor receptor beta); PDGFBB (platelet derived growth factor beta dimer); SRC (Rous sarcoma oncogene); PI3K (phosphoinositide-3-kinase); CD44 [CD44 molecule (Indian blood group)]; BRCA1 (breast cancer 1); BRCA2 (breast cancer 2); BARD1 (BRCA1 associated RING domain 1); NMI [N-myc (and STAT) interactor]; HGS (hepatocyte growth factor-regulated tyrosine kinase substrate); MAD2L (MAD2 mitotic arrest deficient-like 1); MAD1L1 (MAD1 mitotic arrest deficient-like 1); XPO6 (Exportin 6); NUP62 (nucleoporin 62); NUP54 (nucleoporin 54); NUP153 (nucleoporin 153); and NUP214 (nucleoporin 214).

CONCLUSIONS

RHAMM is a gene that belongs to a multifunctional group of cytoplasmic proteins, which are unconventionally exported to the cell surface in response to specific stimuli. This process is highly active during response to injury and tumorigenesis and in certain situations/phenotypic backgrounds, RHAMM, or structural variants of RHAMM, can act as an oncogene. RHAMM is poorly expressed in normal tissues but highly expressed and required for response-to-injury processes, in particular affecting inflammation and the subsequent mesenchymal response for repair. It is also found in numerous human tumors and hyperexpression of RHAMM is associated with poor clinical outcome of many of these cancers. Cell surface RHAMM performs co-receptor functions by affecting signaling through hyaluronan and growth factor receptors while intracellular RHAMM is a mitotic spindle protein that interacts with BRCA1/BARD1 complexes to control mitotic spindle stability. Further analyses of the molecular mechanisms by which RHAMM regulates these processes will undoubtedly identify new ''inside–outside'' paradigms that control motility and mitosis and may lead to the identification of novel key signaling/structural nodes that can be targeted in the treatment of chronic inflammation or cancer.

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CHAPTER

10

Altered Hyaluronan Biosynthesis in Cancer Progression

Naoki Itano and Koji Kimata

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INTRODUCTION

Hyaluronan (HA) is a sugar-chain macromolecule in which *N*-acetylglucosamine and glucuronic acid are linked together by alternating β-1,3 and β-1,4 linkages (Fig. 10.1) (Laurent and Fraser, 1992). Despite its apparently simple structure, HA exhibits multiple properties depending on its molecular size and its binding molecules (Fraser and Laurent, 1989). For instance, high molecular weight HA forms part of the extracellular matrix (ECM) by linking HA-binding molecules into macromolecular aggregates and regulating a variety of cell behaviors, such as cell adhesion, motility, growth, and differentiation. HA oligosaccharides also

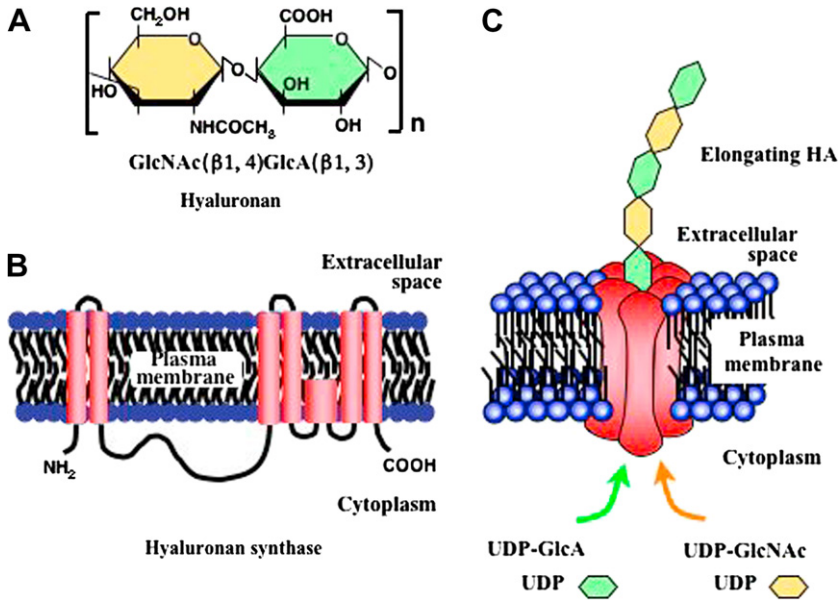

FIGURE 10.1 Scheme illustrating the HA structure (**A**), a predicted structure of mammalian HAS (**B**), and a proposed secretion process of HA (**C**).

regulate such cell behaviors in different ways by acting on intracellular signaling pathways through interaction with cell surface receptors (Toole et al., 1989). Accumulating evidence has demonstrated that the production of HA is excessive in cancer malignancies (Knudson, 1996; Toole, 2004); increased serum levels and deposition in tumor tissue are often associated with malignant progression in many cancers, including breast cancer and colorectal cancer (Ponting et al., 1992; Ropponen et al., 1998).

Although the close association of HA production in the progression of cancer cells is now being established, the entire picture of diverse and complex HA functions in cancer progression remains to be elucidated. Fortunately, animal models with genetically manipulated HA Synthase (HAS) expression provide powerful tools for understanding the *in vivo* function of HA, particularly in connection with cancer cell behavior. Thus the central aim of the present review is to highlight the role of HA in cancer progression from the viewpoint of abnormal HA biosynthesis.

HA BIOSYNTHESIS

The discovery of three members of the HAS gene family (HAS-1, HAS-2, and HAS-3) has enabled great strides in understanding the unique process of HA biosynthesis and mode of chain elongation (Weigel et al., 1997; Itano and Kimata, 2002). Structurally, all HAS proteins are composed of multiple

membrane-spanning regions and large cytoplasmic loops (Fig. 10.1). Unlike typical glycosyltransferases, the cytoplasmic loop in HAS molecules possesses two active sites which participate in the transfer of UDP-GlcNAc and UDP-GlcA substrates. Characterization of the three HAS isoforms has revealed differences in enzymatic properties, particularly in their ability to form HA matrices and determine product size (Itano et al., 1999a). The expression profiles of HAS genes are temporally and spatially regulated during embryogenesis and pathogenesis (Sugiyama et al., 1998; Kennedy et al., 2000; Recklies et al., 2001; Pienimaki et al., 2001), and divergence in the transcriptional regulation of HAS genes during these processes can be explained to some extent by upstream signaling pathways that are triggered by various growth factors, cytokines, cellular stress, and so on. The dynamic turnover of HA is therefore tightly regulated by altering the expression profiles of HAS isoforms to have different enzymatic properties (Weigel et al., 1997; Itano and Kimata, 2002).

ALTERED HA SYNTHESIS IN CANCER

The malignant transformation of cells frequently impairs regulation of HA synthesis and induces excessive HA production (Hamerman et al., 1965; Hopwood and Dorfman, 1977; Leonard et al., 1978). During this process, multiple transcriptional regulation of HAS genes allows cells to optimize the extracellular environment for tumor growth and malignant progression, and a transcriptional switch in HAS isoforms has been demonstrated in cells undergoing oncogenic transformation (Itano et al., 2004). Of the three HAS isoforms, only HAS-2 gene expression was increased in *v-Ha-ras* transformed cells, which showed lowered malignancy. Conversely, both HAS-1 and HAS-2 expression were elevated in highly malignant cells transformed with *v-src*. This implies that HAS isoforms may be involved in different stages of malignant tumor progression. From this point of view, it is increasingly necessary to confirm the relationship between HAS expression and prognosis by statistical analysis using clinical samples. Thus far, these clinicopathological studies have indicated that elevated HAS-1 expression and/or intronic gene splicing correlate with poor prognosis in human colon cancer, ovarian cancer, and multiple myelomas (Yamada et al., 2004; Yabushita et al., 2004; Adamia et al., 2005).

Emerging evidence is providing new insight into the functional aspect of this polysaccharide, particularly in respect to the involvement of HA in tumor malignancy; forced expression of HAS-2 and HAS-3 genes results in excess HA production and enhanced tumorigenic ability of fibrosarcoma and melanoma cells (Kosaki et al., 1999; Liu et al., 2001; Li and Heldin, 2001). Moreover, induced expression of HAS-1 restores the metastatic potential of mouse mammary carcinoma mutants, previously having low

levels of HA synthesis and metastatic ability (Itano et al., 1999b). Inversely, suppression of HAS-2 or HAS-3 decreases HA production and reduces the tumorigenic potential of various cell lines (Simpson et al., 2002a, b; Nishida et al., 2005; Udabage et al., 2005). Although the above clearly demonstrates the important role of HA in tumorigenesis, the tumor promoting ability of excess HA is still somewhat controversial since HAS-2 overexpression also suppresses the tumorigenesis of glioma cells (Enegd et al., 2002). Furthermore, the *in vitro* growth of human prostate cancer cells decreased dramatically when transfected with an HAS-2-expression plasmid, but co-expression of HAS-2 and hyaluronidase HYAL-1 restores the growth of these cancer cells (Bharadwaj et al., 2007). Here, since HA accumulation is the result of a balance between the activities of HAS and hyaluronidases, the presence of hyaluronidase may have promoted HA turnover in the cancer cells and overcome the tumor suppression by excess amounts of HA. Alternatively, the biphasic effects of HA on tumorigenesis can be explained by considering its dose-dependent properties (Itano et al., 2004). To assess this idea, we generated stable transfectants expressing various levels of HAS genes and examined their tumorigenicity in nude mice. Although significant growth promotion was observed within a narrow range of HAS-2 expression, this growth was inhibited at high expression levels. The dose-dependency of HA may help us consider statements regarding the physiological significance of changes in HA concentration with tumor grade or stage, since HA accumulation in clinical samples varies and occasionally shows little statistical changes with tumor grade.

The involvement of HAS in tumor progression has also been evaluated using genetically manipulated animal models. In one study, a transgenic (Tg) mouse model allowing overexpression of murine HAS-2 in mammary glands was generated in order to simulate hyperproduction of HA found in human breast cancer (Koyama et al., 2007). In this model, the expression of exogenous HAS-2 was conditionally controlled by the expression of Cre-recombinase driven by a mammary epithelial cell-specific MMTV promoter. By intercrossing the Tg mice with a mouse mammary tumor model expressing rat c-*neu* protooncogenes in mammary epithelial cells, mammary tumors with aggressive growth rates were developed. Histologically, these tumors were classified as poorly differentiated adenocarcinomas with numerous intratumoral stroma (Fig. 10.2).

TUMOR–STROMAL INTERACTION

Most aggressive tumors are composed not only of cancer cells, but also of many host stromal cells (Kalluri and Ziesberg, 2006), and the importance of interactions between cancer cells and their surrounding stroma in facilitating tumor progression has been demonstrated both by clinical and

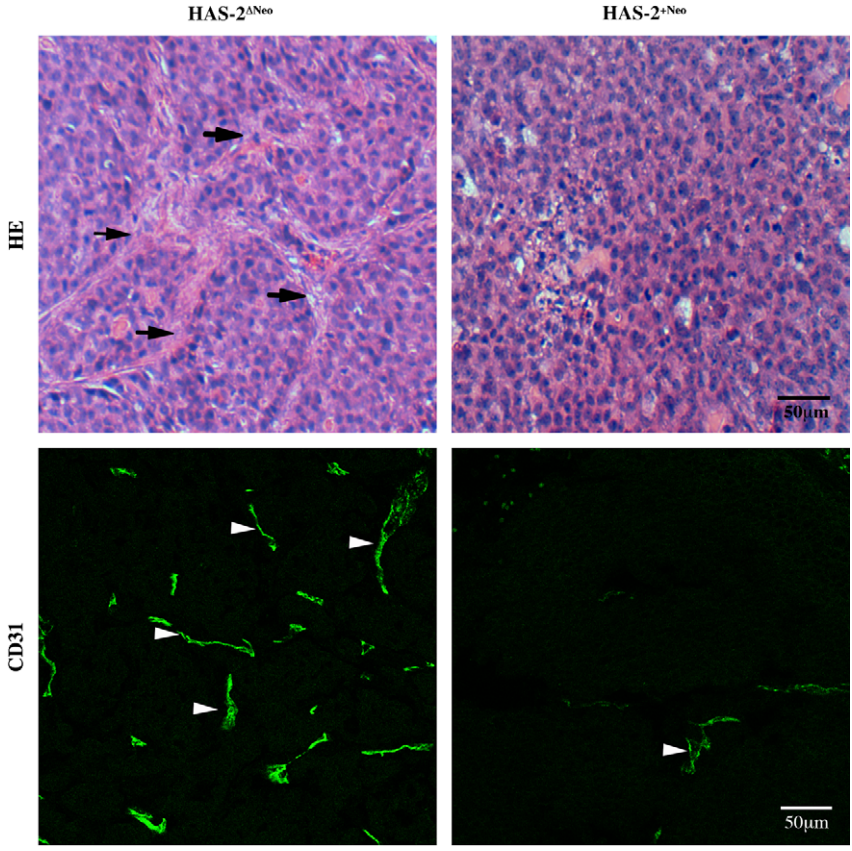

FIGURE 10.2 HA overproduction promotes the formation of intratumoral stroma. Tumor sections from HAS-2-overexpressing transgenic (HAS-$2^{\Delta Neo}$) and control (HAS-2^{+Neo}) mice were stained with hematoxylin and eosin (upper panels). The most prominent histological feature of the HAS-$2^{\Delta Neo}$ tumors was increased formation of intratumoral stroma (arrows). In contrast, control tumors had the characteristics of ductal carcinoma with much less stroma. Tissue sections from HAS-$2^{\Delta Neo}$ and HAS-2^{+Neo} tumors were stained with an antibody against murine CD31 (lower panels). Tumor microvessels (arrowheads) of smaller

experimental evidence (Bhowmick and Moses, 2005). Carcinoma cells actively recruit several distinct stromal cells, such as inflammatory cells, vascular cells, and fibroblasts within the tumor, by secreting chemoattractant factors (Desmouliere et al., 2004; Mantovani et al., 2006). Furthermore, crosstalk between carcinoma cells and adjacent stromal cells influences the composition and arrangement of the tumor microenvironment to support tumor progression by allowing angiogenesis and facilitating the invasion and metastasis of tumor cells (Bhowmick et al., 2004).

Each cell type can potentially communicate with other cells, or among themselves, through the release of auto-/paracrine factors and formation of a complex ECM network (Orimo et al., 2005; Shekhar et al., 2003). A strong correlation has been drawn between cancer progression and the degree of HA accumulation within it, especially in the invading edges of carcinomas (Setala et al., 1999; Auvinen et al., 2000). As such, HA-rich tumor microenvironments, which may be favorable for cancer invasion, are likely generated from complex interactions between tumor cells and stromal cells infiltrating from adjacent host connective tissue. In fact, *in vitro* HA synthesis was synergistically increased in co-cultures of human lung tumor cells with fibroblasts (Knudson et al., 1984; 1989), and similar synergistic effects have been demonstrated with the combination of fibroblasts and other tumor cell types (Merrilees and Finlay, 1985).

Although the main roles of tumor-associated stromal cells in modulating tumor cell behavior is well-established, critical questions still remain as to the molecular mechanisms underlying communication carcinoma and stromal cells and regulating stromal cell recruitment within tumor tissues.

NOVEL FUNCTION OF HA IN STROMAL CELL RECRUITMENT

Genetic evidence supporting the role of tumor-derived HA in stromal cell recruitment has recently come from our experiments using HAS-2 Tg mice; ectopic expression of HAS-2 in mammary tumor cells leads to a marked recruitment of stromal cells inside tumors followed by formation of intratumoral stroma (Koyama et al., 2007). To date, considerable efforts have been made to purify the stromal cell chemotactic factors produced by tumor cells, but only a few factors, such as PDGF, have been identified (Dong et al., 2004). The above notion therefore strongly suggests the function of an HA-rich ECM as a stromal cell chemotactic factor.

Several complex and multifaceted mechanisms can be considered for understanding how tumor-derived HA intratumorally recruits stromal cells during tumor formation. Extracellular accumulation of highly hydrated HA provides microenvironments amenable to easy fibroblast-penetration by increasing turgidity (Laurent and Fraser, 1992), and the fact that forced expression of HAS-2 impairs the intercellular adhesion machinery of tumor cells. This also explains how HA-rich matrices provide an environment favorable for fibroblast-infiltration (Itano et al., 2002; Zoltan-Jones et al., 2003).

HA-induced signaling pathways govern the migratory phenotypes of stromal cells via interaction with HA receptors (Turley et al., 2002). For instance, CD44 and RHAMM, both typical HA receptors, have been implicated in the HA-dependent cell migration and invasion of stromal

fibroblasts. Additionally, the interaction of HA and CD44 can activate receptor tyrosine kinases, which in turn induce the activation of downstream Ras/MAPK and PI3K/Akt signaling pathways (Turley et al., 2002). HA binding to CD44 also activates Rac1 signals, which regulate actin assembly associated with membrane ruffling and cell motility (Bourguignon et al., 2000). Thus, HA appears to promote cell motility by acting on intracellular signaling pathways and controlling the assembly of the actin cytoskeleton. Alternatively, HA–CD44 interactions may recruit mesenchymal stem cells (MSCs) (Zhu et al., 2006); in a mouse model of acute renal failure, MSCs injected into mice migrated to the injured kidney, where HA is abundant (Herrera et al., 2007). Renal localization of MSCs is blocked by preincubation with CD44 blocking antibodies or soluble HA. Likewise, MSCs derived from CD44 knockout mice do not localize to the injured kidney, but are rescued by transfection with cDNA encoding CD44. This same mechanism may participate in the recruitment of MSCs to HA-rich tumors.

Lastly for consideration of HA-mediated stromal cell recruitment is the action of HA-binding molecules. Versican (also called PG-M), an HA-binding proteoglycan, is highly expressed in tissue compartments undergoing active cell proliferation and migration (Wight, 2002) and participates in the formation of an HA-rich ECM (Fig. 10.3). In the peripheral invasive areas of infiltrating ductal carcinomas, the most intense staining by a versican-specific antibody is visualized in the mesenchymal

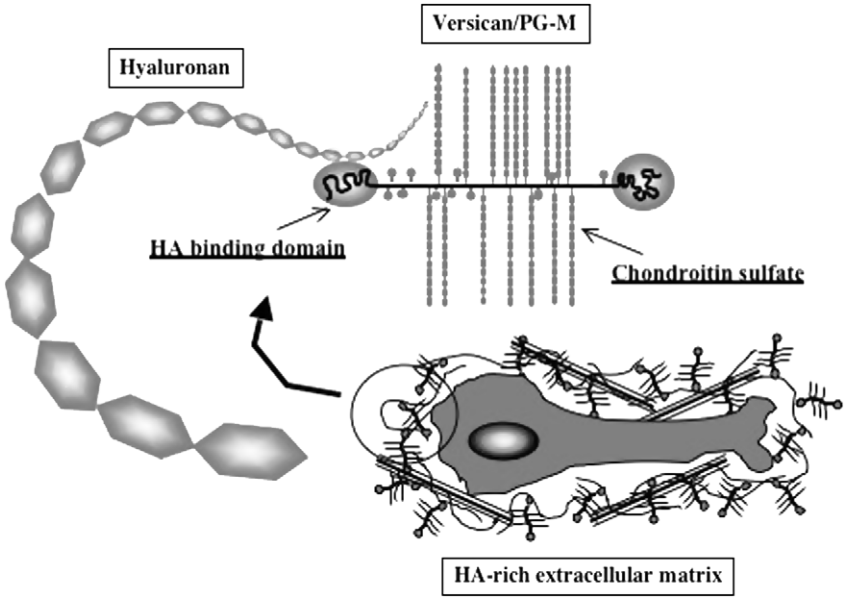

FIGURE 10.3 Scheme of HA-rich extracellular matrix and its constituents.

tissues between carcinoma cell clumps and surrounding tissues, where HA can be demonstrated histochemically (Nara et al., 1997). This cooperative action of HA and versican in mobilizing stromal cells has been demonstrated by the Matrigel plug assay (Koyama et al., 2007). When Matrigel plugs are subcutaneously implanted into nude mice, only trace numbers of stromal cells migrate into the Matrigel plugs containing high molecular weight (HMW) HA alone. However, the infiltration of stromal cells is markedly increased in the presence of HA/versican aggregates. In tumor xenograft models, exogenously added HA/versican aggregates also significantly promote the infiltration of stromal cells within tumors (Koyama et al., personal communication). In concert with versican, HA may therefore allow cells to prepare for migration by enhancing cell detachment from the ECM (Fig. 10.3). The anti-adhesive and motility-promoting effects of versican is evident by combining an earlier observation that versican can inhibit cancer cell attachment to fibronectin, together with the recent finding that formation of an HA/versican pericellular matrix promotes prostate cancer cell motility (Yamagata and Kimata, 1994; Ricciardelli et al., 2007). Current studies have also implied a role of versican in enhancement of cell motility in the assembly of cytoskeletal machinery and transmitting signals.

EPITHELIAL–MESENCHYMAL TRANSITION CAUSED BY HA OVERPRODUCTION

Epithelial–mesenchymal transition (EMT) is the process whereby epithelial cells convert into mesenchymal cells (Thiery and Sleeman, 2006). Following a series of events, epithelial cells lose their epithelial polarity and characteristics while simultaneously acquiring the mesenchymal phenotype. Typically, EMT switches gene expression characteristics from epithelial E-cadherin and cytokeratin to mesenchymal vimentin and α-smooth muscle actin (α-SMA).

Recent advances have fostered a more detailed understanding of the molecular machinery and networks governing EMT. TGF-β and related growth factors mainly influence the process of EMT and receptor tyrosine kinases, which induce the activation of downstream Ras/MAPK and PI3K/Akt signaling pathways, govern EMT in cooperation with growth factors. The nuclear translocation of β-catenin is a key downstream signal that triggers EMT, and all of these pathways crosstalk with each other and transmit signals towards a common endpoint to promote EMT. ECM molecules and degrading enzymes can also convert epithelial cells into mesenchymal cells by triggering EMT, and recent studies using gene-targeted mice have revealed that HA plays a central role in EMT as well (Camenisch et al., 2000); in one report, HAS-2 null mice showed severe

cardiac and vascular abnormalities, and died during midgestation due to a lack of transformation of cardiac endothelial cells to mesenchyme.

EMT was originally defined as a morphological conversion during normal development, but has recently gained attention as a central mechanism for carcinoma progression and metastasis (Huber et al., 2005). The progression of carcinoma cells to metastatic cells frequently involves an EMT-like epithelial change towards a migratory fibroblastic phenotype, particularly at the invasive front of tumors. During tumor progression, downregulation of E-cadherin, a hallmark of EMT, aids tumor cells in spreading. In one study, infection of recombinant HAS-2 adenoviral vectors converted normal Madin-Darby canine kidney and MCF-10A human mammary epithelial cells to mesenchyme as assessed by upregulation of vimentin, dispersion of cytokeratin, and loss of E-cadherin at intercellular boundaries (Zoltan-Jones et al., 2003). All of this suggests that increased HA production appears to be sufficient to induce EMT. Our recent observation using HAS-2 conditional Tg mice has also revealed that overproduction of HA in mammary carcinoma cells results in the suppression of E-cadherin expression and nuclear translocation of β-catenin, further providing evidence for HA-mediated promotion of EMT (Koyama et al., 2007). Carcinoma cells having undergone EMT may then participate in the formation of intratumoral stroma observed in HA overproducing tumors, such as those seen in human breast cancers (Petersen et al., 2003).

ROLES OF HA-RICH ECM IN TUMOR ANGIOGENESIS

Angiogenesis, the formation of new capillaries from preexisting vessels, is an absolute requirement for tumor growth and metastasis (Carmeliet, 2003) and is controlled by the aberrant production of angiogenic factors expressed by malignant tumor cells, host cells, or both. Among such factors, vascular endothelial growth factor (VEGF) has emerged as a central regulator. In addition, the local composition of the ECM surrounding the vasculature can affect angiogenesis either positively or negatively (Sottile, 2004), and HA oligosaccharides have been implicated in the promotion of angiogenesis (West et al., 1985). Studies in chick chorioallantoic membranes and rat skin have demonstrated that HA degradation products of specific size (3–10 disaccharide units) have the potential to induce neovascularization (Sattar et al., 1994; Slevin et al., 1998). Furthermore, HA oligosaccharides, together with angiogenic factors such as VEGF and basic fibroblast growth factor (bFGF), synergistically stimulate endothelial cell proliferation, migration, and capillary formation *in vitro*. The angiogenic activity of HA depends on its molecular mass; HMW native HA is antiangiogenic by inhibiting endothelial cell proliferation and migration and capillary formation in a three-dimensional matrix (Feinberg and Beebe,

1983). Because angiogenesis is the result of complex interactions between positive and negative regulators of angiogenesis (Slevin et al., 1998), the balance of regulatory HMW HA and effecter HA oligosaccharides may be important for controlling the angiogenic response.

HAS gene manipulation provides an opportunity to assess the role of HA during angiogenesis *in vivo*. The significance of HA has been highlighted in HAS-2 deficient mice having vascular defects, implicating a critical function of HA in embryonic vasculogenesis (Camenisch et al., 2000). HAS-2 Tg mouse models of breast cancer have also shown that overproduction of HA in tumor cells accelerates formation of intratumoral neovasculature (Koyama et al., 2007). This altered formation in genetically manipulated mice may be explained by the well-known fact that HA degradation products induce an angiogenic response. Indeed, HAS-2-overexpressing tumors contained significant amounts of small HA oligosaccharides, as assessed by gel filtration chromatography of tumor homogenates, whereas control tumors contained mostly HMW HA (Koyama et al., 2007). This supports the conventional notion that HA oligosaccharides influence tumor-induced angiogenesis. Although the physiological significance of HA oligosaccharides in the promotion of angiogenesis is well-established, it is still open to debate whether the ECM consisting of HMW HA and HA-binding molecules has any role in angiogenesis. Interestingly, in HAS-2-overexpressing mammary tumors, most neovasculature is predominantly found penetrating into the intratumoral stroma where HA is abundant as a constituent of ECM (Koyama et al., 2007). The constituents of stromal ECM therefore likely provide a supporting framework for easy penetration of endothelial cells and subsequent neovascularization.

Versican is abundant in both the perivascular elastic tissues of blood vessels and stromal ECM (Nara et al., 1997). In our recent study, administration of HA-versican aggregates, but not native HMW HA alone, promote the infiltration of endothelial cells within Matrigel plugs containing angiogenic bFGF (Koyama et al., 2007), suggesting the potency of HMW HA to accelerate angiogenesis in the presence of versican. Currently, one can only speculate as to the function of HA/versican aggregates in the context of angiogenesis (Fig. 10.4). Since HA/versican complexes can stimulate cell migration, their possible role would be one that enhances migration and invasion of endothelial cells. As an alternative explanation, the HA-rich matrix can be proposed to serve as a reservoir for various growth factors involved in vessel development; degradation of the matrix results in the release of various growth factors sequestered within, which in turn promotes an angiogenic response (Fig. 10.4). Further investigation is being conducted to clarify both the functional aspect of HA/versican aggregates in angiogenesis, as well as the relationship between such aggregates and HA oligosaccharides.

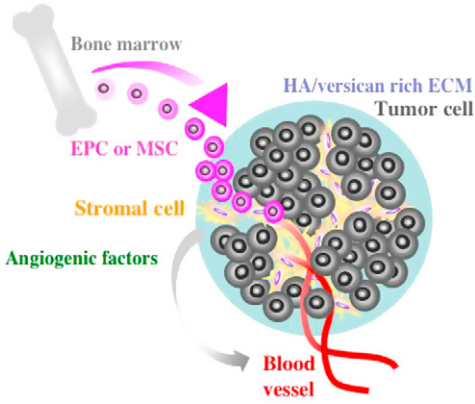

FIGURE 10.4 A model of stroma-induced angiogenesis.

Until recently, new blood vessels in adults were thought to grow exclusively through the sprouting of preexisting vasculature (Hillen and Griffioen, 2007). However, emerging evidence suggests that bone marrow-derived endothelial progenitor cells (EPCs) contribute to tissue vascularization during both embryonic and pathogenetic conditions; circulating bone marrow-derived EPCs are mobilized from the bone marrow and recruited to the foci of neovascularization where they form new *in situ* blood vessels through vasculogenesis. However, the homing process of EPCs remains unclear. Similarly to the recruitment of MSCs, HA-rich matrices may provide a stem cell niche for recruitment and retention of circulating EPCs (Fig. 10.4). In the future, a greater understanding of the mechanisms regulating selective cell movement and recruitment will lead to the development of novel anticancer therapeutic agents targeting reparative progenitor cells.

CONCLUSION AND PERSPECTIVES

This review focused on the role of HA in cancer progression with respect to its biosynthesis and function. A wealth of data has been accumulated on HA function in the promotion of malignancies, showing that enhanced cancer invasion and dissemination may be partly dependent on the mesenchymal conversion of cancer cells by HA overexpression. Furthermore, recent studies have enabled postulation of reliable mechanisms by which HA influences tumor growth and invasion by modulating the tumor microenvironment to recruit stromal cells and vasculature. Although the angiogenic function of HA oligosaccharides has been

well-established, the anti-angiogenicity of HMW HA being modulated by HA-binding molecules needs further clarification and study.

The roles of HA in cancer progression may differ according to the HAS isoforms expressed, meaning cancer cells at different stages may differentially utilize the three HAS isoforms to maximize their survival. Studies are now in progress to identify exactly which HAS proteins are associated with cancer progression. This will provide an opportunity to develop new strategies for cancer therapy targeting specific cancer-associated HAS species.

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CHAPTER

11

Hyaluronidase: Both a Tumor Promoter and Suppressor

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INTRODUCTION

Originally termed as the ''spreading factor,'' hyaluronidases (HAases) are present in a variety of toxins and venoms. For example, HAase is the virulent factor of β-hemolytic *Streptococci* and it is also present in the venoms of snake, bee, wasp, scorpion, etc, where it aids in the spread of

these venoms in the body (Markovic-Housley et al., 2000; Girish and Kemparaju, 2006; Morey et al., 2006; Nagaraju et al., 2006; Skove et al., 2006). In mammals, testicular HAase present in the sperm acrosome is necessary for the fertilization of the ovum (Gould and Bernstein, 1975). Despite a lot of work on bacterial, invertebrate and testicular HAases, a connection between HAase and cancer was unequivocally established just over a decade ago and the functional significance of HAases in cancer was demonstrated just about a year ago (Lokeshwar et al., 1999; 2005a, b; Jacobsen et al., 2002; Simpson, 2006). In this part of the review, we will focus on the recent advances in our understanding of the role of HAases in cancer.

HYALURONIDASES

HAases are a class of enzymes that predominantly degrade hyaluronic acid (HA). However, HAases can also degrade chondroitin sulfate and chondroitin, albeit at a slower rate (Stern and Jedrzejas, 2006). HAases are endoglycosidases, as they degrade the β-*N*-acetyl-D-glucosaminidic linkages in the HA polymer. Six HAase genes are present in the human genome and these occur in two linked triplates. HYAL-1, -2 and -3 genes are clustered in the chromosome 3p21.3 locus, whereas HYAL-4, HYAL-P1 and PH-20 (encodes testicular HAase) reside in the chromosome 7q31.3 locus (Csoka et al., 2001). It is likely that the six mammalian HAase genes must have arisen through gene duplication events, since they share a significant amino acid identity. For example, HYAL-1, -2, -3, -4 and PH-20 share ~40% amino acid identity (Stern and Jedrzejas, 2006). Based on their pH activity profiles, HAases are divided into two categories. HYAL-1, -2, and -3 are considered as acidic HAases because they are active at acidic pH. For example, HYAL-1 has a pH optimum around 4.0–4.2 and the enzyme is inactive above pH 5.0 (Lokeshwar et al., 2001). On the contrary, PH-20 is a neutral active HAase as it is active at pH 7.0 (pH activity profile 3.0–9.0) (Franzmann et al., 2003).

Among the six mammalian HAases, HYAL-1, -2 and PH-20 are well-characterized. As described above, PH-20 is necessary for ovum fertilization and several natural and synthetic HAase inhibitors have been tested for their use as contraceptives (Hardy et al., 2004; Suri, 2004; Garg et al., 2005a, b). PH-20, as well as HYAL-2 are glycosyl phosphatidyl-inositol (GPI)-linked proteins. HYAL-2 degrades HA into ~20 kDa oligosaccharides (~25 disaccharide units). HYAL-1 is the serum HAase and is expressed in several somatic tissues (Lepperdinger et al., 2001; Miller, 2003; Chow and Knudson, 2005; Stern and Jedrzejas, 2006). HYAL-1 has also been purified from human urine, where it is expressed as two molecular forms (Csoka et al., 1997). Although HYAL-1 has high specific activity for

degrading HA, its concentration in human serum is low (60 ng/ml) (Stern and Jedrzejas, 2006).

Site directed mutagenesis of PH-20, identification of naturally occurring mutations in HYAL-1 and alternatively spliced variants of HYAL-1 and HYAL-3, crystal structure of bee venom HAase and 3-D X-ray structure of bovine PH-20 have revealed the catalytic site of HAases involved in HA degradation (Markovic-Housley et al., 2000; Triggs-Raine et al., 1999; Arming et al., 1997; Lokeshwar et al., 2002; Botzski et al., 2004). The crystal structure of the bee HAase and X-ray structure of bovine PH-20 show that HAases have a classical $(\beta/\alpha)_8$ TIM barrel structure. The dominant feature of the HAase structure is a large grove that extends perpendicular to the barrel axis. In bee HAase, the loops following the β strands 2, 3, and 4 form one wall of the groove, and those of 1, 5, 7 and 7 form the other wall. This groove is large enough to accommodate a hexasaccharide. In bee HAase, the catalytic site that cleaves the glycosaminidic bond between *N*-acetyl-D-glucosamine and D-glucuronic acid lies in amino acid residues Asp^{111} and Glu^{113} (Stern and Jedrzejas, 2006). In a substrate-assisted acid-base catalytic mechanism Glu^{113} acts as the proton donor, and the *N*-acetyl group of the substrate acts as the nucleophile. In all six mammalian HAases, this Glu residue is conserved along with the Asp and is believed to be responsible for the substrate cleavage. For example, site directed mutagenesis has identified Glu^{148} and Asp^{146} in human PH-20 as the important residues involved in the actual catalysis of the glucosaminidic linkage. In HYAL-1 the equivalent residues are Glu^{131} and Asp^{129}. In addition to the active site, a 30 amino acid sequence that is conserved in all six mammalian HAases and also in the bee HAase, appears to be necessary for HAase activity (Lokeshwar et al., 2002). In HYAL-1, this sequence appears in amino acids 301 to 330. Based on the bee HAase crystal structure, the 30 amino acid sequence (amino acid 313 to 342 in the bee HAase sequence), forms β sheets 6 and 7, α-helix 8 and the loops in between (Markovik-Housley et al., 2000). Thus, this 30 amino acid sequence is an integral part of one of the walls of the substrate binding groove. In addition, in this 30 amino acid sequence, a Trp residue (Trp^{333}, bee HAase, Trp321 HYAL-1) is conserved in all mammalian and bee HAases and in chitinolytic enzymes and is involved in hydrophobic interaction with the *N*-acetyl side chain (Markovik-Housley et al., 2000). It is noteworthy that in HYAL-1 and HYAL-3 transcripts, this 30 amino acid sequence is encoded by a separate exon that is alternatively spliced (Lokeshwar et al., 2002).

Among the six mammalian HAases, HYAL-1 is the major tumor-derived HAase and is expressed by a variety of tumor cells. HYAL-1 was initially purified from the urine of patients with high-grade bladder cancer and was shown to be expressed in epithelial cells of bladder and prostate tumors and in head and neck squamous cell carcinoma cells (Lokeshwar et al., 1999; Franzmann et al., 2003).

HAase EXPRESSION IN TUMOR CELLS

Detection and measurement of HAase activity in tissues, body fluids, and cell conditioned media became possible because of an HAase ELISA-like assay developed by Stern and Stern (1992). A modified version of this assay was used by Lokeshwar et al. to measure HAase levels in prostate and bladder carcinoma tissues, cells, and in the urine of bladder cancer patients (Lokeshwar et al., 1996; 1999; 2000; 2001; 2002; Pham et al., 1997; Franzmann et al., 2003; Hautmann et al., 2004; Schroeder et al., 2004). The modified HAase ELISA-like assay is called the HAase test, which involves incubation of tissue extracts, urine, or cell conditioned media on HA-coated microtiter well plates in an HAase assay buffer. Following incubation at 37°C for ~16 hours, the degraded HA is washed off and the HA remaining on the HA-coated plate is detected using a biotinylated bovine nasal cartilage HA binding protein. The HAase present in biological specimens is determined from a standard graph, plotted as HAase (mU/ml) versus $O.D._{405nm}$. The HAase activity is then normalized to total protein concentration (mg/ml) or to cell number (if assaying cell conditioned media). Using the HAase test and also a substrate (HA)-gel assay, Lokeshwar et al. found that HAase levels are elevated in prostate cancer tissues, when compared to normal prostate and benign prostatic hyperplasia tissues (Lokeshwar et al., 1996). This study also linked for the first time, HAase levels to tumor progression. In that study, HAase levels were found to be elevated 3–7-fold in high-grade (Gleason $\geq$7) prostate cancer tissues when compared to low-grade (Gleason 5–7) prostate cancer tissues. Metastatic prostate cancer lesions were found to have even higher HAase levels than the high-grade primary tumor (Lokeshwar et al., 1996; 2001). HAase levels are also elevated in high-grade bladder tumor tissues and in the urine of patients with high-grade bladder cancer. HAase levels in low-grade bladder tumor tissues and urine are comparable to those found in normal bladder tissues and urine (Pham et al., 1997; Lokeshwar et al., 2000; 2002; Hautmann et al., 2004; Schroeder et al., 2004). These studies have established a link between HAase and the tumor invasive/metastatic phenotype. In addition to bladder and prostate carcinomas, HAase levels have also been shown to be elevated in the urine of children with Wilms' tumor (Stern et al., 1991). In addition to genito-urinary tumors, HAase levels are elevated in head and neck squamous cell carcinoma, breast tumors, metastatic tumors and glioma cells (Bertrand et al., 1997; Madan et al., 1999; Victor et al., 1999; Godin et al., 2000; Beech et al., 2002; Delpech et al., 2002; Enegd et al., 2002; Franzmann et al., 2003; Junker et al., 2003; Bertrand et al., 2005; Udabage et al., 2005; Paiva et al., 2005; Christopolous et al., 2006).

RT-PCR and cDNA cloning, protein purification, immunoblotting, pH activity profile, and immunohistochemistry have revealed that HYAL-1 is

the major tumor-derived HAase expressed in prostate and bladder carcinoma cells. HYAL-1 is an ~55–60 kDa protein consisting of 435 amino acids. In fact HYAL-1 was the first HAase to be recognized as being expressed by tumor cells and its expression correlates with their invasive/metastatic potential (Lokeshwar et al., 1999; 2001). No HYAL-1 expression is observed in the tumor-associated stroma, although HYAL-1 expression appears to correlate and perhaps induce HA production in the tumor-associated stroma (Lokeshwar et al., 2005a, b).

Patients with head and neck squamous cell carcinomas have been shown to have elevated HAase levels in their saliva and HYAL-1 is the major HAase that is expressed in these tumor tissues (Franzmann et al., 2003). However, in addition to HYAL-1, RT-PCR analysis has revealed PH-20 expression in head and neck carcinoma, especially laryngeal carcinoma (Victor et al., 1999; Christopolous et al., 2006; Godin et al., 2000). Interestingly, the pH activity profile of the HAase activity expressed in tumor tissues is similar to that of HYAL-1, and not PH-20 (Franzmann et al., 2003; Christopoulos et al., 2006). HAase levels are also shown to be elevated in breast tumors and RT-PCR analysis has detected the expression of PH-20, HYAL-2 and HYAL-3 in breast cancer tissues (Junker et al., 2003; Udabage et al., 2005). As in the case of prostate and bladder carcinomas, HAase levels in metastatic breast tumors are found to be 4-fold higher than those expressed in primary tumors (Bertrand et al., 1997). Similarly, HAase levels were higher in brain metastatic lesions of carcinomas other than primary glioblastomas (Delpech et al., 2002). Furthermore, there is some evidence that while less invasive breast cancer cells express HAS-3 and HYAL-3, highly invasive cells express HAS-2 and HYAL-2 (Udabage et al., 2005). However, how and why the HA production by HAS-2 and HA degradation by HYAL-2 promote tumor cell invasion, but HA production by HAS-3 and HA degradation by HYAL-3 associates with low-invasive phenotype is unclear. It is noteworthy that in these studies, the expression of HAS and HYAL isoforms was studied only at the transcript level by real time RT-PCR. Given that functionally inactive splice variants of HYAL-1 and HYAL-3 are previously reported (as discussed below), the expression of HYAL genes at the transcript level does not necessarily translate into HAase activity produced by breast cancer or any other cell type. Similar observations regarding HYAL-2 and HYAL-3 expression were reported for endometrial carcinoma. In a relatively small number of endometrial carcinoma specimens ($n = 13$), HYAL-2 and HYAL-3 mRNA expression, determined by real time RT-PCR was found to be >1000- and >30-fold more than HYAL-1, respectively (Paiva et al., 2005).

Contrary to the findings regarding elevated expression of one or more HAases in tumors, it has been shown that the chromosome locus 3p21.3, where HYAL-1, -2 and -3 genes are clustered, is deleted in lung and some breast carcinomas at a higher frequency; however, the tumor suppressor

gene in this region is RASSF1 and not a HAase gene (Junker et al., 2003; Csoka et al., 1998; Ji et al., 2002). Nonetheless, it was previously believed that HYAL-1 is a tumor suppressor gene (Csoka et al., 1998; Frost et al., 2000; Stern, 2005). Interestingly, again based on the real time RT-PCR studies, Bertrand et al. reported that HYAL-2 expression correlates with lymphoma diagnosis, but the expression actually decreases in high-grade lymphomas, when compared to low-grade lymphomas (Bertrand et al., 2005).

Taken together, HAase expression appears to be elevated in many carcinomas and the expression correlates with tumor invasiveness. However, in some carcinomas HAase expression depends on the status of the chromosome 3p21.3 locus and may inversely correlate with tumor grade.

HAase FUNCTIONS IN GENITO-URINARY TUMORS

HAase as a Tumor Promoter

Extensive digestion of HA by HAase generates tetrasaccharides, whereas limited digestion generates HA fragments, some of which are angiogenic (3–25 disaccharide units). HA fragments of 10–15 disaccharide units have been shown to stimulate endothelial cell proliferation, adhesion and capillary formation (Lokeshwar and Selzer, 2000; Takahashi et al., 2005). Such angiogenic HA fragments are found in the urine of patients with high-grade bladder cancer, in the tissue extracts of high-grade prostate tumors, and in the saliva of patients with head and neck squamous cell carcinoma, suggesting that the HA–HAase system is active in high-grade invasive tumors (Lokeshwar et al., 1997; 2001; Franzmann et al., 2003).

Recent evidence based on cDNA transfection studies shows that HYAL-1 is involved in tumor growth, muscle infiltration by tumor, and tumor angiogenesis (Lokeshwar et al., 2005a, b; Simpson et al., 2006). Lokeshwar et al. have shown that blocking HYAL-1 expression in bladder and prostate cancer cells decreases tumor cell proliferation by ~4-fold, due to cell cycle arrest in the G2-M phase and decreases their invasive activity. In xenografts, inhibition of HYAL-1 expression resulted in a decrease in tumor growth by 9–17-fold. While HYAL-1 expressing tumors infiltrated muscle and blood vessels, tumors lacking HYAL-1 expression resembled benign neoplasm and had 4–9-fold less microvessel density and smaller capillaries (Lokeshwar et al., 2005a, b). The contribution of HYAL-1 expression to muscle invasion by a bladder tumor has been observed in bladder cancer patients. Aboughalia has shown that HYAL-1 expression in tumor cells exfoliated in urine correlates with tumor invasion into the bladder muscle and beyond (Aboughalia, 2006). It is noteworthy that patients with muscle invasive bladder cancer have poor prognosis, as 60%

of the patients with muscle invasive bladder cancer will have metastasis within 2 years and two-thirds will die within 5 years. Interestingly, HA production by the tumor stroma correlates with HYAL-1 levels in tumor cells, suggesting crosstalk between the tumor and the tumor-associated stroma (Lokeshwar et al., 2001; 2005a, b). Such crosstalk between HA and HYAL-1, with respect to tumor growth and angiogenesis, was recently confirmed by Simpson who tested tumor growth and angiogenesis following the expression of HAS-2 and HYAL-1, either individually or together, in a non-invasive prostate cancer cell line. While HAS-2 or HYAL-1 when expressed individually in a prostate cancer cell line, increased tumor growth and angiogenesis, their co-expression had a synergistic effect on this increase (Simpson, 2006). Expression of HYAL-1 in a human prostate cancer cell line also causes a slight increase in its ability to form lung metastasis in xenograft (Patel et al., 2002).

HAase as a Tumor Suppressor

Contrary to the tumor promoting effects of HYAL-1, a prevalent concept has been that, in general, HAases are tumor suppressors (Csoka et al., 1998; Frost et al., 2000; Stern, 2005). The origin of this concept lies in the observation that in some epithelial carcinomas, the 3p21.3 locus is deleted and although the tumor suppressor gene in this locus was shown not to be an HYAL gene (i.e., HYAL-1, -2, or -3), the concept continued (Csoka et al., 1998; Junker et al., 2003; Stern, 2005; Stern and Jedrzejas, 2006). Perhaps this concept became popular because HA is known to promote tumor metastasis, and therefore, conceptually it was easier to explain that an enzyme that degrades HA was a tumor suppressor. In support of this concept, Jacobson et al. reported that while HAS-2 expression in a rat colon carcinoma line promoted tumor growth, the overexpression of HYAL-1, at levels (220–360 mu/10^6 cells) that are not found in tumor tissues and tumor cells, inhibited tumor growth and generated necrotic tumors (Jacobson et al., 2002). Furthermore, Shuster et al. showed that administration of super high concentrations of bovine testicular HAase (300 units) caused an $\sim$50% regression in breast tumor xenografts (Shuster et al., 2002). The controversy whether HAase is a tumor promoter or a suppressor was recently resolved, when Lokeshwar et al. showed that while HYAL-1 levels that are expressed in tumor tissues and cells promote tumor growth, invasion, and angiogenesis, HAase levels exceeding 100 milliunits/10^6 cells), i.e., at levels that are not naturally expressed by tumor cells, significantly reduce tumor incidence and growth due to induction of apoptosis (Lokeshwar et al., 2005a). Therefore, the function of HAase as a tumor promoter or a suppressor is a concentration-dependent phenomenon, but in tumor tissues, the tumor cell-derived HAase acts mainly as a tumor promoter.

Regulation of HAase Activity

One of the mechanisms to control cellular HAase expression is the loss of the chromosome 3p21.3 locus, which occurs at a higher frequency in some epithelial tumors (Marsit et al., 2004; Hilbe et al., 2006; Pizzi et al., 2005). Alternative mRNA splicing is another mechanism by which HAase activity is regulated. A common internal splicing event occurs in the 5′ untranslated region present in exon 1 (Junker et al., 2003; Frost et al., 2000). This splicing event joins nucleotides 109 and 597. Frost et al. and Junker et al. reported that HYAL-1 protein levels and HAase activity in tumor cells correlate with a HYAL-1 transcript in which this 5′ untranslated region is spliced. Furthermore, HYAL-1 protein is not detected in tumor cells which express a HYAL-1 transcript that retains the 5′ untranslated region. Based on these findings, Frost et al. and Junker et al. concluded that the HYAL-1 transcript containing the 5′ untranslated region is not translated (Frost et al., 2000; Junker et al., 2003). However, it is unclear how and why the 5′-untranslated region in the HYAL-1 mRNA prevents translation. Using normal and bladder tumor tissues and bladder and prostate cancer cells, Lokeshwar et al. have reported several alternatively spliced variants of HYAL-1 and HYAL-3 transcripts. These variants are generated by alternative splicing occurring in the coding regions of HYAL-1 and HYAL-3 transcripts which encode truncated proteins that lack HAase activity (Lokeshwar et al., 2002). For example, five alternatively spliced variants of the HYAL-1 transcript that affect the coding region have been reported. HYAL-1-v1 protein lacks a 30 amino acid stretch between amino acids 300 and 3001 and is generated by alternative splicing of exon 2. The HYAL-1-v2 protein sequence from amino acids 183 to 435 is identical to HYAL-1 and the HYAL-1-v3 protein contains the first 207 amino acids of the HYAL-1 wild type protein. HYAL-1-v4 and HYAL-1-v5 proteins consist of amino acids 260–435 and 340–435, respectively, that are present in the wild type protein. Among the HYAL-3 splice variants, HYAL-3-v1 lacks a 30 amino acid sequence present in the wild type protein and this truncation joins amino acid 298 to 329. HYAL-3-v1 is generated by alternative splicing of exon 3. HYAL-3-v2 encodes a 168 amino acid protein, and this is identical to amino acids 249–417 in the HYAL-3 wild type protein. HYAL-3-v3 protein encodes a 138 amino acid protein that is 100% identical to amino acids 249–417 except that it also lacks the 30 amino acid sequence from 299 to 328. As discussed above, although various splicing events maintain the open reading frame of the HYAL-1 and HYAL-3 proteins, none of these variants are functionally active (Lokeshwar et al., 2002).

Recent data on one of the HYAL-1 variants, HYAL-1-v1, show that the expression of HYAL-1-v1 is higher in normal bladder tissues than in bladder tumor tissues. Furthermore, HYAL-1-v1 expression reduces HAase activity secreted by bladder cancer cells because of a complex formation

between HYAL-1 and HYAL-1-v1. HYAL-1-v1 expression induces apoptosis in bladder cancer cells and reduces tumor growth, infiltration and angiogenesis (Lokeshwar et al., 2006). This suggests that a critical balance between the levels of HYAL-1 and HYAL-1 variants may regulate HYAL-1 function in cancer.

REGULATION OF HAase GENE EXPRESSION

Although HAases are important, the regulation of HAase gene expression in normal and cancer cells was unknown until recently. The minimal promoter region for HYAL-2 has been identified; however, the regulation of HYAL-2 expression is unknown (Chow and Knudson, 2005). By deletion analyses Chow and Knudson demonstrated that the region between nucleotides +959 and +1158 (within intron-1) contains the basal promoter elements and the region between nucleotides +224 and +958 contain negative elements that may control the basal expression level of HYAL-2. HYAL-2 promoter lacks a TATA-binding site but contains a GATA-binding region. Recently, Lokeshwar et al. mapped the minimal promoter region of HYAL-1 and showed that HYAL-1 expression in normal and tumor cells is regulated by promoter methylation (Lokeshwar et al., 2008). They identified the minimal promoter region between nucleotides −93 and −38 upstream of the transcription start site. HYAL-1 promoter contains a TACAAA sequence and nucleotides −73 to −50, which contain overlapping binding consensus sites for SP1, Egr-1, and AP-2, are important for promoter activity. In addition C^{-71}pG and C^{-59}pG dinucleotides and a NFκB binding site (at position −15), also appear to be necessary for promoter activity. Although, HYAL-1 promoter lacks a CpG island, methylation at C^{-71} and C^{-59} and differential binding of SP1 to the methylated promoter and Egr-1/Ap-2 binding to the unmethylated promoter appear to regulate HYAL-1 promoter activity. Specifically in non-HYAL-1 expressing cells both C^{-71} and C^{-59} are methylated and SP1 binds to the promoter. But in HYAL-1 expressing cells, C^{-71} and C^{-59} are unmethylated and Egr-1/AP-2 binds to the Regulation of HYAL-1 promoter activity by methylation raises an interesting question about cancer therapeutics involving DNA demethylating agents. Hypermethylation of tumor suppressor genes has been extensively investigated for developing cancer markers and therapeutics. However, if DNA hypomethylation turns on the genes such as HYAL-1 (and also heparanase, uPA, MMP-2, [Ehrlich, 2002]) that function in tumor growth and metastasis, then DNA hypo-methylation-inducing therapies may only have short-term efficacy, as they could speed up the progression of surviving cancer cells (Ehrlich, 2002).

HAase AND SIGNALING

HAase and Cell Cycle Progression

As discussed above, blocking HYAL-1 expression in bladder and prostate cancer cells induces cell cycle arrest in the G2-M phase. G2-M arrest results from the down regulation of the positive regulators of G2-M transition. For example, stable HYAL-1 anti-sense transfectants show down regulation of cdc25c, cyclin B1, and cdk1 levels, as well as, cdk1 kinase activity (Lokeshwar et al., 2005a, b). In HSC3 oral carcinoma cells, HYAL-1 expression caused a 145% increase in the S-phase fraction, with a concomitant decrease in the G0-G1 phase (Lin and Stern, 2001).

The mechanism by which HYAL-1-induces cell cycle transition and up-regulates the levels of positive regulators of G2-M transition is unknown. However, testicular HAase has been shown to induce phosphorylation of c-jun N-terminal kinases (JNK)-1 and -2 and p44/42 ERK in murine fibroblasts cells L929 (Chang, 2001). ERK is required for G2-M and G1-S transitions (Liu et al., 2004). Lokeshwar et al. have previously shown that cell surface interaction between HA oligosaccharides and RHAMM stimulates phosphorylation of p42/p44 ERK (activated p42/44ERK) and focal adhesion kinase in human endothelial cells (Lokeshwar and Selzer, 2000). RHAMM co-immunoprecipitates with src and ERK and contains recognition sequences for these kinases, suggesting a direct interaction (Zhang et al., 1988; Hall et al., 1996). Activated FAK also activates ERK through Grb2 and Shc and PI3 kinase through a direct interaction (McLean et al., 2005; Mitra et al., 2005). It is noteworthy that angiogenic HA fragments are detected in high-grade tumor tissues and in body fluids (e.g., urine and saliva) of cancer patients (Lokeshwar et al., 1994; 2001; Franzmann et al., 2003). In addition to ERK activity, transient activation of JNKs is required for G2-M transition. For example, activated JNK may phosphorylate cdc25c and modulate its activity (Mingo-Sion et al., 2004). Furthermore, activated JNKs phosphorylate c-jun, which then increases cdc2 expression (Goss et al., 2003). However, at the present time it is unknown whether hyaluronidase-mediated regulation of the cell cycle involves JNK and/or ERK pathways.

HAase and Apoptosis

As discussed above, the super high expression of HYAL-1 induces apoptosis in prostate cancer cells. The apoptosis induction by HYAL-1 involves mitochondrial depolarization and induction of a pro-apoptotic protein, WOX1. WOX1 is a ww-domain containing oxidoreductase that contains a nuclear localization signal, a mitochondrial localization signal, and an alcohol dehydrogenase domain (Chang, 2002). Chang has shown

that transient transfection of the murine fibroblast line L929, by HYAL-1 or HYAL-2 cDNA or ectopic addition of bovine testicular HAase (100 U/ml) enhances TNF-induced cytotoxicity, which is mediated by increased WOX1 expression and prolonged NK_{KB} activation (Chang, 2001; Chang et al., 2001; 2003). WOX1 is known to induce apoptosis in a p53 independent manner, which involves WOX1 activation (i.e. WOX1-^{P}Tyr33), its translocation to mitochondria and down regulation of anti-apoptotic proteins bcl2 and $bclx_L$ (Chang et al., 2003). Although the kinase, which phosphorylates WOX1, is unknown, JNK1 directly interacts with WOX1 (Chang et al., 2003). JNK is also associated with the mitochondria-mediated apoptotic pathway, as it phosphorylates bcl-2 and bcl_{XL}, and suppresses their anti-apoptotic activity (Basu and Haldar, 2003; Deng et al., 2001).

Recently, Lokeshwar et al. have shown that the expression of HYAL-1-v1 in bladder cancer cells, that express wild-type HYAL-1, induces G2-M arrest and apoptosis. HYAL-1 and HYAL-1-v1 form a non-covalent complex, which is enzymatically inactive. The HYAL-1-v1 induced apoptosis involves the extrinsic pathway, since HYAL-1-v1 expression induces activation of caspases -8, -9 and -3, Fas and FADD (Fas associated death domain) upregulation, and BID activation. Moreover, inhibition of Fas expression by Fas siRNA inhibits HYAL-1-v1 induced apoptosis (Ehrlich, 2002). These reports suggest that HYAL-1 and its variants are capable of inducing apoptotic pathways, the understanding of which has only recently begun.

HAase as a Diagnostic and Prognostic Indicator

The diagnostic potential of HAase, either alone or together with HA has been extensively explored in bladder cancer. For example, urinary HAase levels, measured using the HAase test, have been shown to be 3–7-fold elevated among patients with intermediate (G2) and high (G3)-grade bladder cancer when compared to normal individuals, patients with one of the many benign urologic conditions, patients with a history of bladder cancer, and patients with low-grade bladder cancer (Pham et al., 1997). In a study of 513 urine specimens, the HAase test had 81.5% sensitivity, 83.8% specificity, and 82.9% accuracy to detect G2/G3 patients. When the HAase test was combined with the HA test, which measures urinary HA levels, the combined HA–HAase test had higher sensitivity (91.2%) and accuracy (88.3%), and comparable specificity (84.4%) to detect bladder cancer, regardless of the tumor grade and stage (Lokeshwar et al., 2000). In another study, where 70 bladder cancer patients were prospectively followed for a period of 4 years to monitor bladder cancer recurrence, the HA–HAase test had 91% sensitivity and 70% specificity to detect bladder cancer recurrence (Lokeshwar et al., 2002b). More importantly, a patient

with a false-positive HA–HAase test had a 10-fold increased risk for developing bladder cancer within 5 months. In a side-by-side comparison, the HA–HAase test was also superior to a variety of FDA-approved bladder tumor markers (Hautmann et al., 2004; Schroeder et al., 2004). Hautmann et al. have shown a correlation between increased tumor-associated HYAL-1 and HA in tumor tissues and a positive HA–HAase test (Chang et al., 2001). This suggests that tumor-associated HYAL-1 and HA are released into the urine when it comes in contact with a tumor in the bladder. In addition to urinary HAase levels, measurement of HYAL-1 mRNA levels in exfoliated cells found in urine also appears to be a marker for bladder cancer. For example, Eissa et al. found that HYAL-1 mRNA expression determined by RT-PCR has >90% accuracy in detecting bladder cancer (Eissa et al., 2005). Furthermore, HYAL-1 mRNA levels measured in exfoliated cells are elevated in patients with invasive and poorly differentiated carcinoma (Aboughalia, 2006). These studies show that HAase is a highly accurate marker for detecting high-grade bladder cancer, and when it is combined with HA, it detects both low-grade and high-grade bladder cancer with ~90% accuracy.

The prognostic potential of HYAL-1 has been explored in prostate cancer. Standard clinical and pathological parameters provide very limited information to clinicians regarding which prostate cancers will progress, and/or have a poor prognosis, and as a result, it is difficult to predict which patients need aggressive treatment, from those for whom watchful waiting would be sufficient. By performing immunohistochemistry on radical prostatectomy specimens, on whom there was a minimum 5-year follow-up, Posey et al. and Ekici et al. found that HYAL-1 is highly expressed in specimens from patients who later had a biochemical recurrence (Posey et al., 2003; Ekici et al., 2004). Biochemical recurrence is defined as increasing serum prostate specific antigen (PSA) levels following radical prostatectomy and is an indicator of disease progression (i.e. either local recurrence or metastasis to distant sites). HYAL-1 staining in radical prostatectomy specimens appears to be an independent predictor of biochemical recurrence. Furthermore, HYAL-1 staining when combined with HA staining has 87% accuracy in predicting disease progression (Ekici et al., 2004). It is noteworthy that in prostate cancer specimens while HYAL-1 is exclusively expressed by tumor cells, HA is mostly expressed by the tumor-associated stroma (Posey et al., 2003). These results show that consistent with the function of HYAL-1 in tumor growth, infiltration, and angiogenesis, it is most likely a prognostic indicator for disease progression.

In a limited number of studies, hyaluronidase expression has also been studied in other carcinomas. For example, there is some evidence that HYAL-1 may be an accurate marker for head and neck squamous cell carcinomas and that salivary HAase levels are elevated in head and neck

cancer patients (Franzmann et al., 2003). In addition to HYAL-1, PH-20 mRNA levels have been shown to be elevated in primary and lymph node metastatic lesions of laryngeal carcinoma when compared to normal laryngeal tissues (Christopoulos et al., 2006; Godin et al., 2000; Victor et al., 1999). In contrast to the observations in many other carcinomas, increased HYAL-2 expression inversely correlates with invasion in B-cell lymphomas and may serve as a prognostic indicator (Bertrand et al., 2005).

HAase and Cancer Therapeutics

Testicular HAase has been added in cancer chemotherapy regimens to improve drug penetration. Tumor cells growing in 3-dimensional multicellular masses, such as spheroids *in vitro* and solid tumors acquire resistance to chemotherapeutic drugs (i.e. multicellular resistance) (Green et al., 2004). The resistance of multicellular spheroids of EMT-6 to 4-hydroperoxycyclophosphamide (4-HC) can be abolished by treatment of these spheroids by HAase (St Croix et al., 1988; Kerbel et al., 1996; Croix et al., 1996). Consistent with the findings that HAase is necessary for cell cycle progression (Lokeshwar et al., 2005a, b; Lin and Stern, 2001), HAase treatment increases recruitment of disaggregated cells into the cycling pool, and thus renders them more sensitive to a cell cycle dependent drug (St Croix et al., 1998; Croix et al., 1996; Kerbel et al., 1996). In limited clinical studies, HAase has been used to enhance the efficacy of vinblastin in the treatment of malignant melanoma and Kaposi's sarcoma (Spruss et al., 1995; Smith et al., 1997), boron neutron therapy of glioma (Haselberger et al., 1998; Hobart et al., 1992), intravesical mitomycin treatment for bladder cancer (Hobart et al., 1992; Maier and Baumgartner, 1989), and chemotherapy involving cisplatin and vindesine in the treatment of head and neck squamous cell carcinoma (Klocker et al., 1994; 1998). It is noteworthy that the HAase concentrations ($1 \times 10^5 - 2 \times 10^5$ IU) used in these clinical studies far exceed the amount of HAase present in tumor tissues, and therefore, it is unlikely that at these concentrations the infused HAase will act as a tumor promoter.

In summary, HAase is an endoglycosidase that functions in tumor growth, infiltration, and angiogenesis. At concentrations that are present in tumor tissues, HAase acts as a tumor promoter. However, artificially increasing these concentrations, results in HAase functioning as a tumor suppressor. HYAL-1 type HAase regulates cell cycle progression and apoptosis, and therefore, may regulate tumor growth and angiogenesis. The regulation of HAase in cancer appears to be controlled at the transcription level. HAases either alone, or together with HA are potentially accurate diagnostic and prognostic indicators for cancer detection and tumor metastasis. We are only beginning to understand the complex role that this enzyme plays in cancer. In the future because of its role in tumor

growth and progression, this enzyme may be targeted for developing novel cancer therapeutics and diagnostics.

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CHAPTER

12

Hyaluronidases in Cancer Biology

Robert Stern

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INTRODUCTION

Given the constant synthesis and degradation of hyaluronan (HA) in tissues, it is remarkable that the body maintains precise levels of HA as tightly as it does. Surprisingly, elevated amounts of HA correlate with several types of malignancies. Levels of HA within cancer cells, on the surface of tumor cells, and in their surrounding stroma can increase with malignant progression. On the other hand, levels of hyaluronidases, the

enzymes that degrade HA, are variable in cancer; in some cases elevated and in others suppressed, relative to normal tissues.

A relatively simple explanation is that the mere presence of a lysosomal hyaluronidase enzyme is not sufficient for it to degrade its substrate. Older studies on lysosomal hyaluronidases in fetal development conclude that the substrate must be presented to the enzyme in an acidic environment (<pH 4.5) in order to support enzymatic depolymerization. While the presence of a lysosomal hyaluronidase such as HYAL-1 is found in circulation at higher levels than most tissues (4–6 U/ml) it is clear that this is insufficient to tip the balance for local HA catabolism. Thus, a fundamental question remains towards just what level of acid active enzyme production is necessary to modulate local HA catabolism, and what additional tools exist to modulate HA levels, such as HA synthase (HAS) levels, receptor activation, the presence of enzyme inhibitors, or the immobilization of extracellular HA with aggregating proteoglycans.

While studies in mice have clearly demonstrated that an HAS-2 is the *sine qua non* of the fetal HA system based upon embryonic lethality, no such smoking gun has been identified on the catabolic side with regard to the confirmed human hyaluronidases, HYAL-1, HYAL-2 and PH-20. Rather, these enzyme knockouts are all viable with phenotypes that do not explain the high rate of turnover of HA. The mechanisms involved in HA catabolism in development homeostasis and malignancy are just beginning to be uncovered.

The hyaluronidase enzymes can correlate with cancer progression, but can also function as tumor suppressors. This anomaly can be explained in several ways. The HA exists in a high molecular weight form as well as in a myriad of lower molecular size intra- and extracellular sizes. While high molecular weight HA is a reflection of intact, healthy tissues, the fragmented forms, indicators of distress signals, occur in abundance in malignancies. They promote angiogenesis, stimulate production of inflammatory cytokines, and activate signaling pathways that are critical for cancer progression. These fragments may be truncated products of the synthetic reaction, but may also be the result of hyaluronidase activities. How the levels of these enzymes are modulated is not known. Free, unfettered hyaluronidase activity would create great havoc in tissues and cells. It is obvious that such activities must be finely controlled. But the means by which this is accomplished is not known. Potent hyaluronidase inhibitory activities have been detected in tissue extracts, as well as inhibitors that are unique to cancer. Regulation of enzyme transcription, translation, including the production of various splices variants, and the competition between these various forms are additional sites of potential control. A review of the various aspects of this important class of enzymes, including what is known of their modulation, is presented here.

HYALURONIDASES AND THEIR STRUCTURE

The HYAL enzymes degrade predominantly HA. The term is a misnomer, since they have the limited ability to also degrade chondroitin and chondroitin sulfates. In vertebrate tissues, they are present in exceedingly low concentrations, occurring, e.g. at 60 ng/ml in human serum, but possess extraordinarily high specific activities. The first somatic hyaluronidase to be isolated was purified from out-dated human plasma, and was named HYAL-1 (Frost et al., 1997). From the EST (expressed sequence tag) database, it was established that there are six such sequences in the human genome, three at chromosome 3p21.3, and another three at 7q31.3 (Csoka et al., 1999; 2001). Intriguingly, both of these loci occur at sites of putative tumor suppressor genes (TSGs) (Csoka et al., 1998; Edelson et al., 1997; Mateo et al., 1999). That at position 3p21.3 was located initially by positional cloning. The one termed HYAL-1 had identity to a sequence previously termed LuCa1 (Lung Cancer 1), so named because of loss of heterozygosity or homozygous deletion that occur in most lung cancers. But subsequent work documents that the HYAL's are not the critical or the only tumor suppressor gene products at that locus (Zabarovsky et al., 2002). Multiple 3p21.3 TSGs occur there, and tumor acquired promoter DNA methylation is an epigenetic mechanism for inactivating the expression of many of these genes in human malignancy. Thus, both genetic and epigenetic abnormalities of several genes residing in chromosome region 3p21.3 are important for the development of cancer, but it is still obscure how many of them exist and which of the numerous candidate TSGs are the key players in cancer pathogenesis.

Loss of hyaluronidase does provide the cancer cell, however, with an HA-rich environment that can stimulate growth and motility, and facilitate metastatic spread. The HYAL-1 protein as well as the mRNA exists in multiple forms, and these various isomers may compete with each other in the net expression of activity. The protein can exist in two major isoforms, one being the product of two endoproteolytic cleavage reactions that eliminate 99 amino acids from the molecule (Csoka et al., 1997).

Two species of HYAL-1 transcripts can be identified by RT-PCR when primers are used that include the 5' untranslated region. The predominant mRNA species does not correlate with protein translation and contains a retained intron. The second spliced form lacking this intron occurs that produces HYAL-1 protein. Inactivation of HYAL-1 in those cases is a result of incomplete splicing of its pre-mRNA that appears to be epigenetic in nature. Multiple stop codons are contained in the retained intron that prevent translation. Both isoforms occurs in most tissues, but the relative ratios between them vary widely. This inactivation of HYAL-1 occurs at the RNA level, indicating that not all TSGs are of DNA origin (Frost et al., 2000; Junker et al., 2003).

Lokeshwar and colleagues (2002) report several additional alternatively spliced variants of both HYAL-1 and HYAL-3 occurring in the coding region, including proteins that lack enzymatic activity. All of these enzyme protein variants may be competing with each other for effective expression of activity.

Several mammalian species are reported to lack HYAL-1 activity (Fiszer-Szarfarz et al., 1990). This would suggest that HYAL-1 is not an important enzyme, and that other hyaluronidases may be able to substitute for the enzyme. However, an inactive higher molecular weight form can be observed in the circulation of such animals by immuno-staining on SPAGE using HYAL-1 specific polyclonal antibodies (R. Stern, unpublished observations). This suggests that incomplete processing of circulating enzyme occurs in some mammalian species. Intracellular expression in endothelium rather than liver secretion has also been reported in these species (borine, rabbit, etc.)

A CATABOLIC SCHEME FOR HYALURONAN

Hyaluronan has an extraordinarily high rate of turnover in vertebrates. In the human, 5 g of the 15 g of total body HA turnover daily, mostly the result of the HYAL enzymes, through three catabolic pathways. At the cellular level, the two predominant HYALs are HYAL-1 and HYAL-2, degrading the HA to progressively smaller fragments. Hyaluronan deposition and turnover is even more abundant and more rapid in malignant tissues. The proportion of low molecular weight (LMW) fragments of HA is greater in tumors and tumor patients than in the normal (Kumar et al., 1989; Lokeshwar et al., 1997).

A putative cellular scheme for HA catabolism was recently formulated (Stern, 2003; 2004a). A very simplified version of this scheme is presented in Fig. 12.1, provided originally in the Glycoforum website, Science of Hyaluronan www.glycoforum.gr.jp (Stern, 2004b).

The polymer is degraded in a series of steps generating ever-smaller fragments. The variously sized fragments have a wide and occasionally opposing spectrum of biological activities. Cell surface HYAL-2 makes an initial cut of high molecular weight (HMW) matrix HA generating fragments of 50 to 100 saccharides. These intermediate-sized fragments enter early endosomes, become lysosomal, and degrade further to predominantly tetrasaccharides by lysosomal, acid-active, HYAL-1. The controls that permit accumulation of particular sized fragments are entirely unknown.

THE WARBURG EFFECT

The ability of cancer cells to use anaerobic metabolism and to generate lactate, even in the presence of adequate oxygen, is known as the Warburg

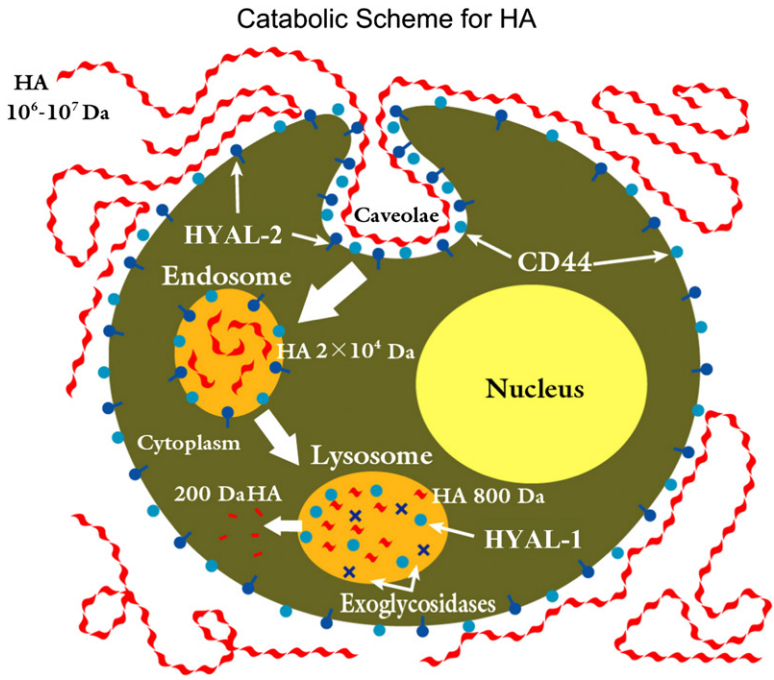

FIGURE 12.1 High molecular weight HA from the extracellular matrix (ECM) is bound to cell surfaces by the combined effects of HYAL-2 and CD44. The clustered complex is guided into caveolae-rich lipid rafts. There, in association with Na^+-H^+ Exchange Protein-1 (not shown), the HA is internalized, and the HA is cleaved to LMW fragments. Such fragments are delivered into early endosomes, and finally to lysosomes. The combined effects of the acid-active β-endoglycosidase HYAL-1, and the two acid-active β-exoglycosidases, β-glucuronidase and β-N-acetylglucosaminidase degrade the HA to tetrasaccharides, and ultimately to individual sugars. Such sugars can then exit lysosomes to enter the cytoplasm where they participate in other cellular reactions. Considering that 5 g of HA are turned over daily in the average 70 Kg individual, the amounts of sugar so processed are not trivial. It is not clear at what point the HA from the ECM becomes intracellular. Additionally, a certain amount of synthesized HA remains intracellular without ever becoming a component of the ECM. How this second HA compartments catabolized is unknown. (Courtesy of Glycoforum website. Science of Hyaluronan. www.glycoforum.gr.jp)

effect (Warburg et al., 1924; Warburg, 1954). Lactate added to cultured fibroblasts increases their HA production (Stern et al., 2002). The phenomenon is dose-dependent at physiological levels, between zero and 10 mM lactate. Lactate also increases expression of CD44, a transmembrane glycoprotein, and the predominant HA receptor on cell surfaces.

The stroma that surrounds carcinomas has increased HA, providing an environment that promotes the growth and motility of cancer cells. Levels of HA often correlate with degree of malignancy. The lactate

produced by tumor cells is a mechanism by which they recruit the host's stroma, in a strategy that promotes their invasiveness, metastatic spread, and survival.

Lactate-sensitive response elements in genes involved in HA metabolism have been identified. The HA is tethered to cell surfaces by CD44 contained in caveolin-rich lipid rafts, and cleaved initially by HYAL-2 into fragments that are highly angiogenic and inflammatory. The HA is internalized, and further degraded by HYAL-1. Sequence analysis of the promoter regions of genes for CD44, HYAL-2, HYAL-1, and caveolin revels multiple AP-1 and ets-1 response elements. Lactate exposure increases the RNA for c-fos, c-jun, c-ets-1, HYAL-1, HYAL-2, CD44, and caveolin-1, as indicted by RT-PCR (Stern et al., 2002; Formby and Stern, 2003). This may be one of the mechanisms by which the Warburg effect promotes carcinogenicity, and one of the ways that cancer cells are able to commandeer the surrounding normal stroma to participate in malignant progression.

HYALURONIDASE INHIBITORS

Hyaluronidase inhibitors are a class of biologicals about which very little is known. Cells in culture secrete enzymes into the culture medium away from the cells, but this does not occur in tissues. The production of unopposed hyaluronidase activity would be very destructive in tissues. Potent hyaluronidase inhibitors can be detected in tissue extracts. These are obvious during the process of enzyme purification, the apparent number of total units of activity rising significantly after the initial steps of isolation. The hyaluronidase inhibitors may parallel the careful control of the matrix metalloproteinases (MMPs), another class of matrix-degrading enzymes, by their tissue inhibitors (TIMPs) (Nagase and Visse, 2006). The hyaluronidase inhibitors were first detected over 60 years ago in blood (Haas, 1946; Dorfman et al., 1948). These activities are magnesium dependent, are acute phase substances synthesized by the liver, some of which are members of the Kunitz type of inter-alpha-inhibitor family (Mio and Stern, 2000; Mio et al., 2000).

An ever-present inhibitor of HYAL-2 activity on cell surfaces would have to be invoked in normal, healthy tissues, to preserve ECM integrity. A combination of CD44, HYAL-2, and the Na^+ H^- exchanger 1 (NHE1) are complexed within lipid rafts on cell surfaces for binding HMW HA (Bourguignon et al., 2004). Alternatively, a block of any one of these components could function as a potential inhibitor, for preserving HA integrity.

An entirely different class of inhibitors of hyaluronidases is found in the circulation of cancer patients (Kiriluk et al., 1950; Kolárová, 1975), activities

that are independent of magnesium (Fiszer-Szafarz, 1968). They have not been further characterized, but are clearly important for cancer progression. Using the theoretical scheme for HA metabolism, invoking an inhibitor of HYAL-1 would permit accumulation of intermediate-sized HA fragments, the products of HYAL-2 cleavage. These stimulate the angiogenesis necessary for cancer viability after a primary tumor or its metastases have grown past a critical stage. Such fragments also induce the inflammatory reaction that facilitates tumor growth (Coussens and Werb, 2002).

HYALURONIDASE MODULATES CD44 EXPRESSION

CD44 has multiple isoforms generated by alternative exon splicing of a single gene. CD44 and many of its variants are expressed on cancer cells, but the mechanisms by which splice variant exons are selected are unknown. The presence of HA in the environment of the cell appears to influence that selection process. The expression of particular splice variants of CD44 as well as the simultaneous presence of HA is important for motility, invasion, and the metastatic spread of some tumors. The influence of hyaluronidase digestion on the expression of CD44 was examined in a number of human cancer cell lines. CD44 isoforms containing alternatively spliced exons are sensitive to hyaluronidase digestion in all the cell lines examined. However expression of CD44s, the standard form, is resistant to digestion in most cancer cell lines. A tentative model proposes that CD44 isoform variants are unstable, requiring the continuous presence of the HA ligand for expression. On the other hand CD44s are relatively more stable, not requiring the continuous presence of HA or its expression (Stern et al., 2001). A diagrammatic presentation is given in the accompanying figure. Evidence indicates that CD44 variant expression and stabilization requires phosphorylation events catalyzed by casein kinase II (Stern et al., 2001). Stabilization of CD44 occurs through phosphorylation of ser 323 and 325 on the intracellular portion of the transmembrane molecule (Formby and Stern, 1998).

HYALURONAN FRAGMENTATION

Hyaluronan exists not only in an HMW form but also in a number of discreet LMW sizes that have a wide variety of biological activities (Stern, 2006). Tumor cells secrete abundant levels of HYAL-1. The enzyme generates HA fragments that in turn, induce proteases that cleave the tumor cells own CD44 while also stimulating motility. This is an autocrine, paracrine process that increases the tumor cells own malignant potential (Sugahara et al., 2003; 2006). This re-enforces the concept that HA

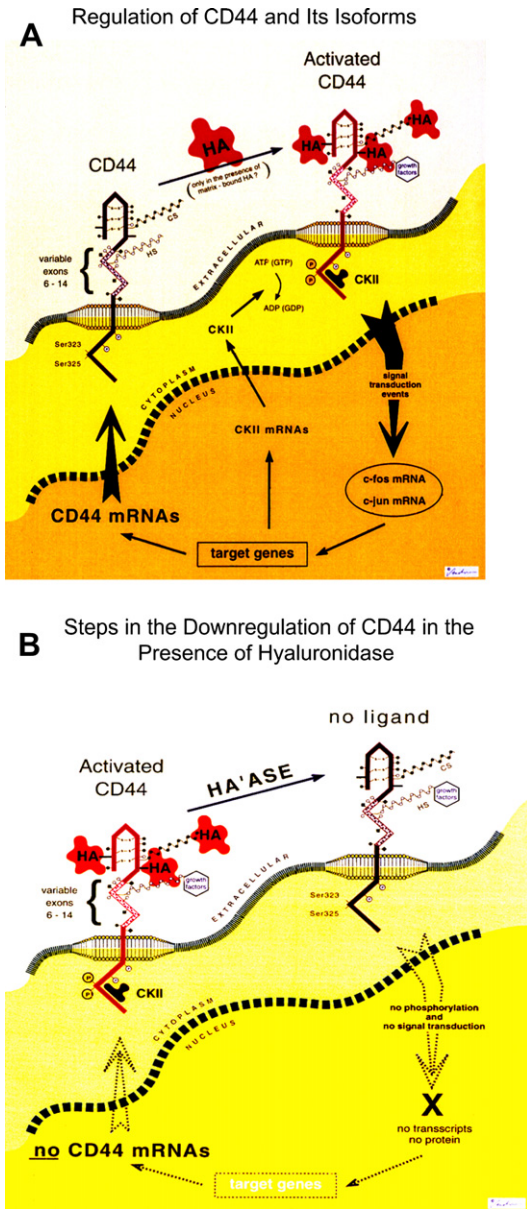

FIGURE 12.2 CD44 exists in a great number of isofoms, in part, the result of expression of ten variable exons within the single *CD44* gene. CD44s, the standard form which contains none of the variable exons, is a stable isoform that does not require the continuous presence of extracellular HA for expression. Other CD44 isoforms, and in particular some that are cancer-associated, are highly unstable transcripts requiring the continuous presence of HA for their expression. Ser-323 and Ser-325 of the intracellular component of transmembrane CD44 are sites of phosphorylation (Neame and Isacke, 1992). Such phosphorylation is catalyzed in part by casein kinase II. Additional kinases have also been detected (B. Formby and R. Stern, unpublished observations). Signal transduction events lead to increased levels of transcripts for c-fos, c-jun, and c-ets mRNAs (A).
In the presence of hyaluronidase activity, HA of the ECM is degraded. The unstable CD44 isoforms requiring the continuous presence of HA for their expression become downregulated when HA is absent or degraded. There is no phosphorylation of the Ser-323 or Ser-325 sites, no signal transduction or transcription of target genes, including the genes for certain CD44 transcripts containing critical exon variants (B).

fragments facilitate cancer progression. Additionally, the autocrine mechanism described above indicates that this malignant progression is self-stimulating without the necessity of external signals.

The size range of HA oligosaccharides that induce cleavage in this cultured pancreatic tumor cell system was carefully investigated. Added

exogenously, 6–14 saccharides induced maximal cleavage in a dose-dependent manner. The CD44 theoretically released into the circulation might be part of a strategy for the cancer to become independent of CD44-related control mechanisms, providing a circulating CD44 that would compete with cell-bound CD44. Enhanced CD44 cleavage has been demonstrated in gliomas, breast, colon, and ovarian cancers, and in non-small cell carcinomas of the lung (Okamoto et al., 2002).

Human tumors inoculated into immunodeficient (SCID) mice regress when treated with PH-20. The chromosomal loci at 3p21.3 and 7q31.3 are both TSG sites. It is tempting to assume that HYALs are TSG products, and must be eliminated for tumor progression. Together with the observation that 100% of human small cell (oat cell) carcinomas, and 80% of bronchogenic carcinomas carry deletions at the 3p21.3 site makes the argument all the more attractive. However, 3p21.3 is a gene-rich locus, and more recent data indicate that the HYALs are not the identity of the TSG products as was assumed initially (Wong et al., 2006; Oh et al., 2007).

Highly invasive bladder cancers generate HA fragments in the 30–50 saccharide range, sizes that are highly mitogenic for endothelial cells, and therefore very angiogenic (Lokeshwar, 1997). This may be the mechanism that supports their invasiveness. Similar size fragments also activate tumor integrins that enhance cell binding (Fujisaki et al., 1999).

By contrast, the very small HA oligosaccharides have the ability to inhibit a variety of tumors. In the 6–7 saccharide range, they inhibit anchorage-independent growth of tumor cells (Ghatak et al., 2002). Even the smallest tetrasaccharides, the limit product of hyaluronidase digestion (Stern and Jedrzejas, 2006), have biological functions. They induce heat shock proteins, and prevent apoptosis (Xu et al., 2002). The very small oligomers appear to ameliorate the effect of intermediate-size HA fragments, and may even be a mechanism of host defense against the tumor. Oligosaccharides of HA thus appear to be able to either promote or inhibit tumor progression, depending on size.

HYALURONIDASES AS ANTI-CANCER CHEMOTHERAPEUTIC AGENTS

Hyaluronidases have long been added to anti-cancer regimens, particularly in Europe. Tumors previously resistant to chemotherapy become sensitive when hyaluronidase is added (Klocker et al., 1998; Baumgartner et al., 1998) (Baumgartner and Hamilton, this volume). The enzyme may decrease intratumoral pressure, permitting drugs to penetrate the malignancy. However, studies are available suggesting that hyaluronidase has intrinsic anti-tumor activity.

Anti-Cancer Properties of Hyaluronidase

Evidence for the anti-cancer effects of hyaluronidase come from experimental model systems. The enzyme enhances the anti-cancer effects of adriamycin *in vitro* (Beckenlehner et al., 1992). Human cancers grown in SCID mice regress dramatically following administration of purified testicular hyaluronidase (PH-20) (Shuster et al., 2002). Over expression of HYAL-1 suppresses tumorigenicity in a model for colon carcinoma (Jacobson et al, 2002). Hyaluronidase administration delays the appearance of carcinogen-induced tumors (Pawlowski et al., 1979). Hyaluronidase treatment also prevents lymph node invasion in a murine model for T-cell lymphoma (Zahalka et al., 1995; Naor et al., this volume). The mouse has several alleles for HYAL-1, while there is only one in the human. The murine alleles have different levels of circulating hyaluronidase activity. The growth rates of murine malignancies correlate inversely with enzyme levels (DeMaeyer and DeMaeyer-Guignard, 1992). Hyaluronidase also blocks TNF-mediated cancer cell death, reverses multidrug resistance (Chang, 1998), and alters cell cycle kinetics of chemo-resistant carcinomas (St. Croix et al., 1996).

HYALURONIDASES IN CANCER PROGRESSION

However, data have also accumulated that hyaluronidase correlates with tumor progression. This has been particularly documented in tumors of the male genito-urinary tract, in prostate (Lokeshwar et al., 1996; Madan et al., 1999a; Kovar et al., 2006) and urinary bladder cancers (Lokeshwar et al., 2000; Lokeshwar et al., 2005). Aggressiveness of other human cancers also correlates with hyaluronidase, including breast (Madan et al., 1999b; Beech et al., 2002) and laryngeal cancer (Godin et al., 2000). At the same time levels of hyaluronidase sufficient to remove HA are reportedly anti-tumorigenic in prostate tumors. Whether this dose-dependent effect is a result of reaching a threshold of enzyme necessary to remove HA is still not clear. On the other, overexpression of HYAL-2 in murine astrocytoma cells accelerates tumor formation (Novak et al., 1999). The inconsistencies that abound suggest that additional work must be performed, before comprehensive understanding emerges.

THE CONUNDRUMS INVOLVING HYALURONIDASES IN MALIGNANCY

The confusion and inconsistencies of HA and the HYALs in tumor biology can be attributed to the concept that different tumors do different things, and the same tumor can do different things at different times. The

phenomenon of the angiogenic switch (Folkman, 2002) may be applicable here. Early in the course of a malignancy, HMW HA may be required to open up tissue spaces, for the flow of nutrients at the primary site. But when simple diffusion no longer suffices, the action of hyaluronidase, particularly HYAL-2, can provide the HA fragments that induce angiogenesis and the neo-vascularization to support the malignancy (West et al., 1985). There is also a dose effect, as outlined by Lokeshwar and Selzer in this volume, in which opposite effects of hyaluronidase enzymes are achieved at different doses. However, HA has a very rapid rate of turnover in vertebrate tissues, and increased amounts of HA in human cancers may merely reflect simultaneous enhanced synthetic and degradative enzyme levels (Jacobson et al., 2002; Simpson, 2006).

Instability of the tumor genome, and the constant pressure of the Darwinian selection process of metastatic tumor cells underscore the resilience and ingenuity of malignant cells in their ability to survive and thrive. This appears to be applicable also to the cancer cells ability to generate different size of HA fragments at various points in the life of the cancer, with profoundly different effects achieved by large, intermediate, and small HA fragments.

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CHAPTER

13

Hyaluronan Fragments: Informational Polymers Commandeered by Cancers

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INTRODUCTION

Hyaluronan (HA), a high molecular weight polymer ($\sim 10^7$ Da) consisting of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine (Laurent and Fraser, 1992), was first isolated from the

vitreous body of the eye by Karl Meyer in 1934 (Meyer and Palmer, 1934), and was shown to act as a viscous space-filler of the extracellular cavity due to its high capacity to hold water (Meyer et al., 1939). The involvement of HA in inflammatory diseases related to synovial joints, such as rheumatoid arthritis (RA), was widely investigated in the first few decades after its discovery (Balazs et al., 1967; Ragan and Meyer, 1949). In the 1950s and 1960s, it was reported that a high amount of HA is present in the body fluids of patients bearing tumors such as mesothelioma (Blix, 1951; Truedsson, 1951) and sarcomas (Marcante, 1965), and that HA is produced by Rous sarcoma *in vitro* (Grossfeld, 1962). In the late 1970s and 1980s, it became clear that HA plays a critical role in the development and the remodeling of tissues (Toole and Gross, 1971). It was found that not only the accumulation of HA in such tissues was critical for the events, but also the degradation and removal of the HA that accumulated in the tissues, were crucial for the following maturation steps (Feinberg and Beebe, 1983; Toole and Gross, 1971). The findings by West et al. in 1985 added a twist to these understandings (West et al., 1985). They demonstrated that the degraded forms of HA mainly consisting of HA 8–50-mers, but not the native high molecular weight HA (HMW-HA), induced angiogenesis in a chick chorioallantoic membrane (CAM) and in a porcine heart. They called the HA fragments ''angiogenic oligosaccharides.'' This finding supported the profound significance of the degradation of HA during tissue remodeling, and also showed that even the degradation products had an active role in such events.

Accumulating evidence suggests that such degraded forms of HA have distinct and diverse functions from HMW-HA, and are involved in the pathology of cancer and inflammation (Stern et al., 2006; Sugahara et al., 2004; Toole, 2004). It is suggested that these HA fragments are generated by direct synthesis or enzymatic degradation of HMW-HA under such conditions. The HA fragments interact with various HA binding proteins such as CD44 (Aruffo et al., 1990), receptor for HA-mediated motility (RHAMM) (Hardwick et al., 1992), and toll-like receptors (TLRs) (Akira et al., 2006) that mediate their signals. The functions of HA fragments with respect to their receptors will be discussed here in detail.

PRODUCTION OF HA FRAGMENTS

A variety of reports demonstrates the accumulation of low molecular weight HA fragments *in vivo* when cancer or inflammation is present, suggesting that such HA fragments contribute to the pathology of the diseases. For instance, HA fragments similar to the ''angiogenic oligosaccharides'' in size have been found in tumor tissues and body fluids of patients with cancer (Kumar et al., 1989; Lokeshwar et al., 1997; Lokeshwar

et al., 2001). A high amount of low polymerized HA was found in the synovial fluids of patients with RA (Balazs et al., 1967; Ragan and Meyer, 1949). HA fragments ranging from 5.5–146 kDa (approximately 28–760-mers) were found in transgenic mice that produce a high amount of HA in the tumor tissues while developing spontaneous breast tumors (Koyama et al., 2007). Intermediate-sized HA fragments ranging from 20–160 kDa (approximately 100–830-mers) were present in mice with non-infectious pulmonary inflammation (Teder et al., 2002). Various tumor cells were shown to produce HA fragments similar in size as the ''angiogenic oligosaccharides'' (Lokeshwar et al., 2001; Sugahara et al., 2006).

Two mechanisms can be considered for the production of HA fragments. One is the direct synthesis of HA fragments by HA synthases (HAS), and the other is the degradation of HMW-HA by enzymes and other means. The three synthases known in mammals, HAS-1, HAS-2, and HAS-3 (Itano et al., 1999b), have slightly different features from each other. HAS-2 and HAS-3 have higher abilities to synthesize HA than HAS-1 (Itano et al., 1999b). HAS-1 and HAS-3 synthesize and secrete HA of broad size distributions ranging from 200–2000 kDa (approximately 1000–10,000-mers), while HAS-2 synthesizes extremely large HA mainly over 2000 kDa (approximately 10,000-mers) (Itano et al., 1999b). Therefore, theoretically, intermediate-sized HA fragments around 200 kDa (approximately 1000-mers) can be synthesized by HAS-1 and HAS-3. A number of reports demonstrate the correlation between high HAS-1 expression and progression of tumors such as endometrial cancer (Yabushita et al., 2005), mesothelioma (Kanomata et al., 2005), ovarian cancer (Yabushita et al., 2004), and colon cancer (Yamada et al., 2004). It has also been shown experimentally that HAS-1 has an important role in tumor progression and metastases. For example, stable transfection of HAS-1 in a mouse mammary carcinoma cell line defective in HA synthesis rescued the ability of the cancer cells to metastasize to the lungs (Itano et al., 1999a), and a HAS-1 stable transfectant of transformed 3Y1 rat fibroblast showed growth promotion *in vitro* and *in vivo* (Itano et al., 2004).

Besides the contribution of HA synthases in the generation of HA fragments, it is very likely that the degradation of HA takes place in this process as well, especially when HA fragments of small sizes are produced. Such degradation can be achieved by various means including hyaluronidases and reactive oxygen species (ROS) (Stern, 2004; Stern et al., 2006). While ROS-directed degradation of HA is considered to be the major pathway to generate HA fragments in inflammatory and autoimmune diseases such as RA and osteoarthritis, hyaluronidase-mediated HA degradation is often involved in tumor progression (Stern, 2004; Stern et al., 2006). Among the five known human hyaluronidases, HYAL-1 and HYAL-2 play a central role in HA catabolism (Stern, 2004). HYAL-2 first degrades HMW-HA tethered on cell surfaces by HA receptors such as

CD44 into intermediate-sized HA fragments around 20 kDa (approximately 100-mers). Then, HYAL-1 further degrades the 100-mer fragments into 4-mers in lysosomes. These hyaluronidases are often found to be active in tumors. For instance, HYAL-1 was present in high-grade human prostate cancer tissues that also contained biologically active HA fragments ranging from 20–30-mers (Lokeshwar et al., 2001). Because the expression levels of HYAL-1 correlate with the stages and metastases of human bladder cancer, it has been suggested that HYAL-1 can be used as a tumor marker for bladder cancer (Lokeshwar et al., 2005). We have found that both HYAL-1 and HYAL-2 were expressed in pancreatic tumor cells and were secreted into the culture supernatant (Sugahara et al., 2006). The supernatant was active in degrading HA, and contained HA fragments ranging from approximately 10–40-mers.

Although it is becoming clear that HA synthases and hyaluronidases contribute to tumor growth and metastases, it is often unclear how their products come into the stories except for the cases in which the sizes and biological functions of the actual HA found in the systems were analyzed (Koyama et al., 2007; Lokeshwar et al., 1997; Lokeshwar et al., 2001; Sugahara et al., 2006). Since there must be a tight connection and a balance between HA synthesis and degradation as part of the HA turnover process, it is very likely that there are large quantities of HA fragments of various sizes in tumors that over express HA synthases, hyaluronidases, or both. Therefore, it is of high importance to understand the HA profiles in such tumors and to dissect out the principal actor(s) in each event. In addition, the receptors that mediate the signals of such HA fragments have to be investigated as well.

RECEPTORS OF HA FRAGMENTS

Various molecules have been reported to function as the receptor for HA fragments (Stern et al., 2006). Such receptors, and the sizes of HA that they are able to recognize, are summarized in Table 13.1. In the text, three major molecules will be discussed.

CD44

CD44 is a widely distributed transmembrane cell adhesion molecule that is probably the most well-characterized receptor for HA (Aruffo et al., 1990). CD44–HA interaction is involved in various biological events, such as lymphocyte rolling, tumor cell migration, and invasion (Toole, 2004). Recently, CD44 has regained attention because it is now recognized as one of the most important markers for cancer stem cells in various tumors such as prostate, colon, breast, and pancreatic cancers (Ailles and Weissman, 2007).

TABLE 13.1 HA Binding Proteins and the Sizes of HA Fragments That Interact with Them

HA binding proteins	Smallest size of HA that efficiently binds to the HA binding proteins	Additional information	References
CD44	6–10-mers 10-mers (keratinocytes)	HA 4-mers interacted with CD44, but with a much lower affinity than 6-mers.	Lesley et al., 2000 (Tammi et al., 1998)
RHAMM	14-mers	HA 2–4-mers and 4–6-mers did not interact with RHAMM.	Manzaranes et al., 2007 Lokeshwar et al., 2000
TLR2	135–200 kDa	HA 4–16-mers did not interact with TLR2.	Jiang et al., 2005 Tesar et al., 2006 Termeer et al., 2002
TLR4	4–6-mers	HA 4–6-mers up to HMW-HA interacted with TLR4.	Voelcker et al., 2008 See text for further references
TSG-6	6–8-mers	HA 6–16-mers bound with a similar affinity. HA 4-mers did not bind to TSG-6.	Kahmann et al., 2000
SHAP	10-mers	HA 8-mers competed less with HA. HA 6-mers failed to do so.	Yoneda et al., 1990
Aggrecan	10-mers	HA 4–8-mers weakly interacted with aggrecan.	Hascall and Heinegård, 1974
Versican	10-mers	HA 6–8-mers competed less with HA. HA 24-mers induced a ternary complex.	Seyfried et al., 2005
Link protein	10-mers	HA 6–8-mers competed less with HA. HA 24-mers induced a ternary complex.	Seyfried et al., 2005

Refer to text for abbreviations.

The interaction between CD44 and HA is of very low affinity as described by Kd ranging from 5–150 μM (Lesley et al., 2000). When a CD44-expressing cell attaches to an HA substrate, multiple receptor-

ligand bonds are formed, resulting in an increase in the avidity of the interaction. This is enabled by the repeating disaccharide structure and the huge size (up to 10^7 Da) of HA. Therefore, when a CD44-expressing cell binds an HA of a much smaller size, it is not surprising that the avidity is much weaker than the interaction with native HMW-HA.

Lesley et al. showed that HA 6-mer was the minimum oligomer size required for efficiently occupying the HA binding site of CD44 (Lesley et al., 2000). In addition, they showed that beginning at HA 20-mers, there was a dramatic and progressive increase in the avidity with increasing oligomer size up to 38-mers, suggesting that HA 20-mers or larger can occupy more than one HA binding site of CD44 (Lesley et al., 2000). This differential binding pattern of CD44 and HA might partially explain why HA fragments ranging from 6–36-mers exhibit distinct effects compared to intermediate-sized HA or native HMW-HA (refer to ''HA fragments of 6–36-mers'' later in this chapter). Additional speculations can also be made to explain the distinct functions of HA fragments of this size range. For example, it is possible that these HA fragments, especially those larger than 20-mers, have the capability to induce cross-linking of CD44 molecules, an event that initiates CD44-mediated intracellular signaling (Fujii et al., 2001). It is also possible that due to the small size, these HA fragments have a high accessibility to the HA binding sites of CD44, or are able to tickle a specific epitope within the HA binding site. These HA fragments might be internalized much more easily than HMW-HA (Thankamony and Knudson, 2006) and alter cellular functions.

Takeda et al. observed an interesting phenomenon when CD44 interacts with HA fragments (Takeda et al., 2006). They found that the HA binding domain of CD44 showed a rearrangement of the β-strands in the extended lobe when the HA binding domain interacted with HA 6-mers. In addition, they observed that the C-terminal region of the HA binding domain became disordered and was released from the structural domain upon interaction with HA fragments, and that this structural change resulted in a higher susceptibility to proteolytic cleavage of the CD44 molecule by trypsin (Figure 13.1). These findings suggest that HA fragments may directly affect

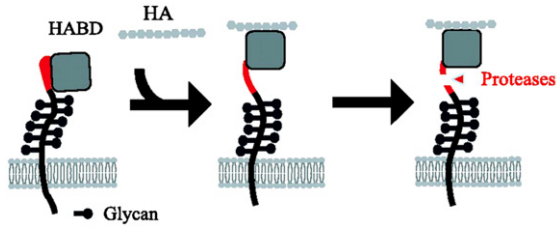

FIGURE 13.1 Proposed schematic model for the structural change-induced CD44 proteolytic cleavage upon HA binding. Binding of HA 6-mers to CD44 induces a conformational change in CD44 that makes it more susceptible to protease cleavage. HABD stands for hyaluronan binding domain. Figure adopted from Takeda et al., 2006.

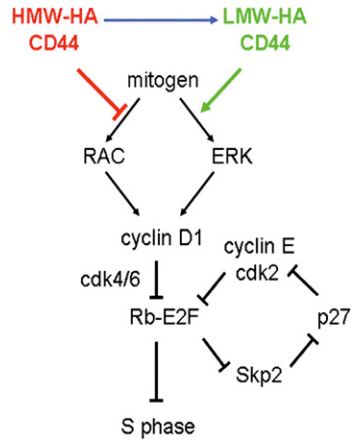

FIGURE 13.2 Differential regulation of cell proliferation signals by HMW-HA and HA fragments (LMW-HA; low molecular weight hyaluronan). HMW-HA suppresses cell proliferation by inhibiting Rac dependent signaling to cyclin D1, whereas HA fragments activate ERK and stimulate cell proliferation. Figure adopted from Kothapalli et al., 2008.

the structure of CD44 that might lead to proteolytic cleavage of the molecule. It is well-accepted that CD44 cleavage is deeply related to the migration of tumor cells (Okamoto et al., 1999; Sugahara et al., 2003; Sugahara et al., 2006), tumor progression (Okamoto et al., 2002), and the turnover of CD44 molecules (Okamoto et al., 2001). Therefore, it is an attractive hypothesis that HA fragments affect tumor cell functions by modifying the conformation of CD44 and altering cellular functions such as the up-regulation of CD44 cleavage on cell surfaces and cell motility. Opposing effects of HMW-HA and HA fragments on cell proliferation through CD44 appear to be strictly regulated by signaling pathways (Kothapalli et al., 2008). After binding to CD44 on vascular smooth muscle cells, HMW-HA suppresses cell cycle progression, while HA fragments stimulate it. This difference results from a differential regulation of signaling pathways to cyclin D1, the common signaling target for both HMW-HA and HA fragments. HMW-HA suppresses Rac dependent signaling to cyclin D1 by inhibiting GTP loading of Rac, whereas HA fragments activate ERK and stimulate ERK-dependent cyclin D1 gene expression (Figure 13.2). Similar differential regulation of intracellular signaling may be expected for other effects induced by HA fragments but not by HMA-HA.

Receptor for HA-Mediated Motility (RHAMM)

RHAMM was cloned as an HA binding protein required for cell locomotion regulated by a *ras*-oncogene (Hardwick et al., 1992). It exists in almost all cell types such as fibroblasts, macrophages, endothelial cells,

and breast carcinoma cells (Entwistle et al., 1996), and in multiple cellular components including the cell surface, cytoskeleton, mitochondria, and the nucleus (Turley et al., 2002). Since it does not possess any signal proteins or transmembrane domains, the cell surface RHAMM initiates intracellular signaling by modifying the functions of other receptors such as platelet-derived growth factor receptor (Turley et al., 2002) upon interaction with HA. In addition, intracellular RHAMM interacts with molecules such as extra cellular signal-regulated kinase (ERK) and Src, leading to the regulation of signaling pathways including the ras-ERK pathways (Slevin et al., 2007; Turley et al., 2002).

The interaction between RHAMM and HA fragments has not been studied as extensively as that of CD44. However, reports cover a wide range of HA fragment size that interact with RHAMM, thus allowing one to speculate the mode of interaction. For example, it was demonstrated that native HA and HA 20–30-mers induced mitogenic responses in human pulmonary artery endothelial cells (ECs) and human lung microvessel ECs, while HA 4–6-mers or HA 2–4-mers did not (Lokeshwar and Selzer, 2000). Both native HA and HA 20–30-mers were able to induce tyrosine phosphorylation of molecules in the ECs, although for an unknown reason, HA 20–30-mers had a stronger effect than the native HA (Lokeshwar and Selzer, 2000). In addition, it was shown that an HA preparation of an average molecular mass of 200 kDa (approximately 1000-mers) stimulated ciliary activity in airway epithelial cells through an RHAMM-dependent manner (Forteza et al., 2001), and that HA 14-mers and 20–30 kDa HA (approximately 100–150-mers) were able to do so, whereas HA larger than 500 kDa (approximately 2600-mers) were not (Manzanares et al., 2007). Therefore, this information may suggest that the minimum HA size required to bind to RHAMM is larger than HA 6-mers, and that the size of the HA fragments that can efficiently induce an effect through RHAMM might range somewhere from 8–14-mers up to 200 kDa (approximately 1000-mers) and less than 500 kDa (approximately 2600-mers). Although this is not a fair assumption because the systems and cell types used in the experiments were different, some reports support this notion. For example, it has been reported that the up-regulation of RHAMM-mediated cell motility is induced by HA fragments ranging from 250–300 kDa (approximately 1300–1500-mers) (Hamilton et al., 2007) and a mixture of 10 kDa HA (approximately 50-mers) and 276.7 kDa HA (approximately 1440-mers) (Tolg et al., 2006).

Collectively, RHAMM seems to favor a certain size range of HA fragments when mediating effects, as does CD44. Although the mechanism of such regulation is ill-defined as is with CD44–HA fragment interaction, several reports provide us clues to understand the mechanism. It was demonstrated that RHAMM, CD44, and ERK1,2 formed a complex in invasive breast cancer cell lines and such complex formation sustained the

high motility of the cancer cells (Hamilton et al., 2007). It has also been shown that RHAMM was required for cell surface display of CD44 and the formation of CD44-ERK1,2 complex (Tolg et al., 2006). These reports clearly show that RHAMM and CD44 co-operate to mediate the signals of HA fragments in tumor cells. Investigating the role of HA size in inducing such complex formation might guide us to the mechanism of the differential function of HA fragments mediated by CD44 and RHAMM.

Toll-Like Receptor (TLR)

TLRs are pattern-recognition receptors that regulate innate immunity (Akira et al., 2006). They are type I integral membrane glycoproteins with extracellular domains containing leucine-rich repeats and cytoplasmic domains homologous to that of the interleukin 1 (IL-1) receptor (Akira et al., 2006). So far, 12 members of TLRs have been identified in mammals. TLR1, TLR2, and TLR6 mainly recognize lipids, while TLR7, TLR8, and TLR9 recognize nucleic acids (Akira et al., 2006). TLR4 is unusual in a way that it recognizes structurally unrelated ligands including lipopolysaccharides (LPS), virus glycoproteins, and fibronectin (Akira et al., 2006).

Among the members of the TLR family, TLR2 and TLR4 have been shown to serve as the receptors for HA (Chang et al., 2007; Jiang et al., 2005; Scheibner et al., 2006; Taylor et al., 2004; Termeer et al., 2002; Tesar et al., 2006). Although they both are able to recognize HA fragments, they appear to have different tastes in the size of the fragments, judging from published reports. For example, TLR4 has been shown to mediate signals of HA 4–6-mers (Voelcker et al., 2008), HA 4–8-mers (Taylor et al., 2004), HA 4–16-mers (Termeer et al., 2002), HA 30-mers, 23 kDa (approximately 120-mers), and 230 kDa (approximately 1200-mers) (Chang et al., 2007), 135–200 kDa HA (approximately 700–1000-mers) (Jiang et al., 2005; Tesar et al., 2006), 500–800 kDa HA (approximately 2600–4200-mers) (Wang et al., 2006), and 1300 kDa HA (approximately 6800-mers) and larger (Chang et al., 2007). In contrast, TLR2 has been reported to be involved in events initiated by 135–200 kDa HA (approximately 700–1000-mers) (Jiang et al., 2005; Tesar et al., 2006) and 200 kDa HA (approximately 1000-mers) (Scheibner et al., 2006), and its involvement in mediating signals of HA 4–16-mers was denied (Termeer et al., 2002). Although the optimal HA sizes that TLR4 and TLR2 recognize might possibly differ between cell types, it can be roughly estimated that TLR4 might be able to recognize a broad range of HA sizes beginning from HA 4–6-mers up to HMW-HA, whereas TLR2 prefers intermediate-sized HA fragments. This is in line with the fact that TLR4 recognizes a variety of structurally unrelated pathogen-associated molecular patterns, while TLR2 recognizes limited ligands such as lipids and some glycoproteins (Akira et al., 2006). Structural analyses of TLR4 and

TLR2 with or without association with various sizes of HA fragments will provide us further information to draw a conclusion.

How TLRs recognize such HA fragments is unclear. Recently, Taylor et al. (2007) reported an interesting finding that is similar to what is known about the collaboration between CD44 and RHAMM (Hamilton et al., 2007; Tolg et al., 2006). When they treated macrophages with HA, they found that TLR4 formed a complex with CD44 and MD-2, an adapter molecule required for TLR4 signaling, and that this complex was responsible for HA recognition. In addition, this complex formation was not observed under LPS treatment, suggesting that the recognition pattern of HA and LPS by TLR4 is different. Considering the reports regarding the complex formation between CD44 and RHAMM (Hamilton et al., 2007; Tolg et al., 2006), it is possible that different sizes of HA fragments are able to tie up different receptors resulting in unique receptor complexes depending on the cell types.

It is now becoming clearer that TLRs serve as sensors on macrophages and dendritic cells (DCs) that mediate danger signals in response to HA fragments under non-infectious inflammations (Akira et al., 2006; Jiang et al., 2007). A similar scenario can be envisaged under tumor-bearing conditions because of the accumulation of HA fragments (Lokeshwar et al., 1997; Sugahara et al., 2006) and the presence of tumor-associated macrophages and DCs in such conditions (Chang et al., 2007). However, an emerging concept demonstrates that various immune cells within the tumor microenvironment such as macrophages (Giraudo et al., 2004) and neutrophils (Pahler et al., 2008) co-operate with the tumor cells to help them grow. Therefore, the interaction of HA fragments with the immune cells through TLRs may be a "double-sided blade" that can evoke a danger signal to the host, but helps the tumor cells to make their comfortable beds at the same time. In addition, TLRs appear to be directly involved in tumor cell functions (Voelcker et al., 2008). It has been reported that a series of TLRs are expressed on various tumor cells such as colon cancer, breast cancer, prostate cancer, lung cancer, and melanoma, and that TLR4 expressed on colon cancer cells mediates the suppression of T cell proliferation and natural killer cell activity enabling the cancer cells to evade immune surveillance (Huang et al., 2005). Therefore, TLRs might not only be regulating innate immunity, but also are involved in cellular functions of non-immune cells including tumor cells when they interact with certain ligands such as HA fragments.

DIFFERENT SIZES OF HA HAVE DIFFERENT BIOLOGICAL FUNCTIONS

The various functions of HA fragments are discussed below in three groups according to the sizes of the HA fragments (Tables 13.2–13.4).

TABLE 13.2 Biological Functions of HA 4–6-mers

MW (kDa)	Number of saccharides	Biological functions	Responsible receptors	References
1–4	N.D.	Blood flow was increased in cryoinjured grafts. Number of blood vessels was increased in the grafts.	N.D	Lees et al., 1995**
N.D.	4-mers	Heat shock protein 72 expression was upregulated.	CD44*	Xu et al., 2002
N.D.	4–6 mers	The internalization and intracellular catabolism of HA by cartilage explant cultures were inhibited.	N.D.	Ng et al., 1995**
		Proliferation of melanoma cells enhanced by HMW-HA was inhibited.	CD44	Ahrens et al., 2001**
		Mmp-9 and *Mmp-13* expression was enhanced in tumor cells.	CD44* RHAMM* TLR*	Fieber et al., 2004
		NF-B, MMP-2, and IL-8 expression and cell migration was enhanced in melanoma cells *via* TLR4.	TLR4	Voelcker et al., 2008
N.D.	6-mers	Transcription factors were activated in articular chondrocytes. Expression of MMP-3 and collagen II was upregulated.	N.D.	Ohno et al., 2005
		MMP-13 expression was induced in articular chondrocytes through activation of NF-B and p38 MAPK.	CD44 and U.R.	Ohno et al., 2006
		Concormational change in the HA binding domain of CD44 was induced.	CD44	Takeda et al., 2006

The abbreviations used are: N.D., not described; U.R., unknown receptor. Refer to text for other abbreviations. Receptors with asterisks indicate those that were found to be NOT responsible for the events. References that do not appear in the text (double asterisks) are as follows.

Ahrens, T., Assmann, V., Fieber, C., et al. (2001). CD44 is the principal mediator of hyaluronic-acid-induced melanoma cell proliferation. *J Invest Dermatol* **116**, 93–101.

Lees, V.C., Fan, T.P., and West, D.C. (1995) Angiogenesis in a delayed revascularization model is accelerated by angiogenic oligosaccharides of hyaluronan. *Lab Invest* **73**, 259–266.

Ng, C.K., Handley, C.J., Preston, B.N. et al. (1995). Effect of exogenous hyaluronan and hyaluronan oligosaccharides on hyaluronan and aggrecan synthesis and catabolism in adult articular cartilage explants. *Arch Biochem Biophys* **316**, 596–606.

TABLE 13.3 Biological Functions of HA 6–36-mers (Approximately 1.35–6.9 kDa)

MW (kDa)	Saccharide units	Biological functions	Responsible receptors	References
N.D.	4–8-mers	IL-8 release from human ECs was enhanced. MIP-2 and KC levels were increased in mouse serum.	TLR4	Taylor et al., 2004
		NO production was increased through the activation of NO synthase in articular chondrocytes.	CD44	Iacob and Kundson, 2006**
N.D. PIP	4–14-mers	PIP_3 production and Akt phosphorylation levels were reduced. Apotosis of T lymphoma cells lines were induced.	N.D.	Alaniz et al., 2006**
		Drug resistant T cell lymphoma cells were sensitized to vincristine.	CD44	Cordo Russo et al., 2008
N.D.	4–16-mers	Maturation of human blood derived DCs was induced. IL-1 β, TNF- α and IL-12 production from DCs was increased.	CD44* RHAMM*	Termeer et al., 2000
		Human and murine DCs were activated *via* TLR4 but not TLR2.	TLR4 TLR2*	Termeer et al., 2002
1.35–4.5	6–20-mer	HA fragments were found in the sera of patients with Wilms' tumors and bone metastaszing renal tumors of childhood.	N.D.	Kumar et al., 1989
		Angiogenesis was induced (chick CAM assay). Sythesis of collagen type I and type VIII was promoted.	N.D.	Rooney et al., 1993

TABLE 13.3 (*continued*)

MW (kDa)	Saccharide units	Biological functions	Responsible receptors	References
		Blood vessel numbers were increased in rat skin. EC migration was promoted.	N.D.	Sattar et al., 1994
		Angiogenesis was induced synergistically with VEGF. Components of the PA-plasmin system were activated.	N.D.	Montesano et al., 1996
		Gene expression was upregulated in ECs. (*c-fos, c-jun, jun-B, Krox-20, Krox-24*).	CD44	Deed et al., 1997**
		CD44 phosphorylation was induced leading to increased tyrosine phosphorylation.	CD44	Slevin et al., 1998
		Phosphorylation and translocation of PLC wound recovery, ERK1/2 activation were induced.	N.D.	Selvin et al., 2002**
		PI3K activity and Akt phosphorylation were inhibited. PTEN expression was stimulated in tumor cells.	CD44	Ghatak et al., 2002
		HA oligosaccharides were found in the post-mortem tissues and serum of patients with acute-stage stroke injury.	N.D.	A'Qteishat et al., 2006**
N.D.	6–24-mers	Growth of B16F10 melanoma cells was inhibited.	CD44(?)	Zeng et al., 1998
N.D.	6–32-mers	Proliferation of bovine aortic ECs was induced.	N.D.	West and Kumar, 1989

(*continued*)

TABLE 13.3 (*continued*)

MW (kDa)	Saccharide units	Biological functions	Responsible receptors	References
1.35–6.9	6–36-mers	CD44 cleavage was induced in tumor cells. CD44-dependent tumor cell migration was promoted.	CD44	Sugahara et al., 2003
~500	6-mers~	Chemokine gene expression was induced in murine alveolar ECs. (*crg-2, MCP-1, RANTES, MIP-1 a, MIP-1 β*).	CD44	Mckee et al., 1996
N.D.	8-mers	Tumor cell motility and invasion were inhibited *in vitro.* Number of lung metastases was decreased *in vivo.*	CD44	Hosono et al., 2007
N.D.	8–50-mers	Angiogenesis was induced (chick CAM assay).	N.D.	West et al., 1985
N.D.	~10–40-mers	HA oligosaccharides generated by tumor cells enhanced. CD44 cleavage and tumor cell motility.	CD44	Sugahara et al., 2006
N.D.	12-mers	Tube formation of brain ECs was induced in collagen gels.	CD44 (?)	Rahmanian et al., 1997
		Ec differentiation and CXCL1 upregulation were induced.	CD44	Takahashi et al., 2005
2.675 and 20	14-mers and N.D.	CBF was stimulated in tracheal epithelial cells.	RHAMM	Manzanares et al., 2007
N.D.	20–30-mers	Found in high-grade TCC patient urine specimens. Mitogenic respose in HMVEC-L was induced.	N.D.	Lokeshwar et al., 1997

TABLE 13.3 (*continued*)

MW (kDa)	Saccharide units	Biological functions	Responsible receptors	References
		Tyrosine phosphorylation of p 125 ^{FAK}, paxillin, and p42/44 ERK was induced in HPAEC and HMVEC-L.	RHAMM	Lokeshwar and Selzer, 2000
		Found in high-grade human prostate cancer specimens.	N.D.	Lokeshwar et al., 2001
5.5–146	N.D.	HA fragments were detected in HAS-2 overexpressing breast tumor transgenic mice.	N.D.	Koyama et al., 2007
N.D.–230	30-mers–N.D.	Osteoclastogenesis was induced in bone marrow Mø.	TLR4 (?)	Chang et al., 2007
6.8	32-mers	bFGF-induced *in vivo* angiogenesis was promoted.	N.D.	Koyama et al., 2007
		Erk 1/2 phosphorylation and migration of aortic smooth muscle cellls were inhibited.	CD44	Vigetti et al., 2007**
6.9	36-mers	LFA-1 was activated in tumor cells. Tumor cell binding to ICAM-1 was enhanced.	CD44 (?)	Fujisaki et al., 1999**
		Fas expression was enhanced. Fas-mediated early apoptotic change of RA synovial cells was increased.	CD44	Fujii et al., 2001

The abbreviations used are: N.D., not described; KC, keratinocute chemoattractan; NO, nitric oxide; PIP3, phosphatidylinositol-3,4,5-triphosphate; PA, plasminogen activator; PLC 1, phospholipase C 1; PTEN, phosphatase and tensin homolog deleted on chromosome 10; CBF, ciliary beat frequency; HMVEC-L, human lung microvessel endothelial cell; HPAEC, human pulmonary artey EC; Mø, macrophage; LFA-1, lymphocyte function-associated antigen-1. Refer to text for other abbreviations. Receptors with asterisks indicate those that were found to be resposible for the events.(?) indicates the receptors that were suggested to be involved in the events but not experimentally proved.

References that do not appear in the text (double asterisks) are as follows.
Alaniz, L., Garcia, M. G., Gallo-Rodriguez, C., et al. (2006). Hyaluronan oligosaccharides induce cell death through PI3-K/Akt pathway independently of NF-kappaB transcription factor. *Glycobiology* **16**, 359–367.
A′Qteishat, A., Gaffney, J., Krupinski, J., et al. (2006). Changes in hyaluronan production and metablism following ischaemic stroke in man. *Brain* **1229**, 2158–2176.
Deed, R., Rooney, P., Kumar, P., et al. (1997). Early-response gene signalling is induced by angiogenic oligosaccharides of hyaluronan in endothelial cells. Inhibition by non-angiogenic, high-molecular-weight hyaluronan. *Int J Cancer.* **71**, 251–256.
Fujisaki, T., Tanaka, Y., Fujii, K., et al. (1999). CD44 stimulation induces integrin-mediated adhesion of colon cancer cell lines to endothlial cells by up-regulation of integrins and c-Met and activation of integrins. *Cancer Res* **59**, 4427–4434.
Iacob, S. and Knudson, C.B. (2006). Hyaluronan fragments activate nitric oxide synthase and the production of nitric oxide by articular chondrocytes. *Int J Biochem Cell Biol* **38**, 123–133.
Slevin, M., Kumar, S. and Gaffney, J. (2002). Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wond healing responses. *J Biol Chem* **277**, 41046–41059.
Vigetti, D., Viola, M., Karousou, E., et al. (2008). Hyaluronan-CD44-ERK1/2 regulate human aortic smooth muscle cell motility during aging. *J Biol Chem* **283**, 4448–4458.

HA Fragments of 4–6-mers (≈1.35 kDa) (Table 13.2)

HA fragments as small as 4–6-mers are special in a sense that they do not efficiently bind to known HA binding proteins such as RHAMM (Lokeshwar and Selzer, 2000), TSG-6 (Kahmann et al., 2000), serum-derived HA-associated protein (SHAP) (Yoneda et al., 1990), aggrecan (Hascall and Heinegård, 1974), versican (Seyfried et al., 2005), and link protein (Seyfried et al., 2005) (refer to ''Receptors of HA fragments'' and Table 13.1). The functions of HA 6-mers can be partially explained through its interaction with CD44 because it is able to bind to CD44, although with a relatively low affinity (Lesley et al., 2000). For instance, HA 6-mers up-regulated transcription factors and several mRNAs including *matrix metalloprotease 3* (*Mmp3*) (Ohno et al., 2005), and also MMP13 protein through a CD44-dependent pathway as well as through a CD44-independent pathway (Ohno et al., 2006). However, regarding HA 4-mers, it is difficult to explain its mode of function through conventional HA receptors. It was reported that HA 4-mers up-regulated the expression of heat shock protein 72 in a chronic myelogeneous leukemia cell line, K562, while HA 2-mers and 6–12-mers did not, and that CD44 was not the responsible receptor for these events (Xu et al., 2002). Then, what can be the receptor for these very small HA fragments? One receptor that should be considered as a candidate is TLR4. It has been demonstrated that HA 4–6-mers activate NF-κB and enhance the expression of IL-8 and MMP2 in melanoma cells through interaction with TLR4 (Voelcker et al., 2008). However, some reports exclude the involvement of TLR4 in mediating the signals of HA 4–6-mers, suggesting the presence of a yet-unknown HA fragment receptor. HA 4–6-mers but not 60–200 kDa HA (approximately 300–1000-mers) or HA larger

TABLE 13.4 Biological Functions of HA Fragments of Approximately 100-mers (20 kDa) or Larger

MW (kDa)	Saccharide units	Biological functions	Responsible receptors	References
<10	N.D.	Receptor-mediated uptake of DNA/PEI was facilitated.	CD44	Hornof et al., 2008
10 + 276.7	N.D.	Random motility of fibroblasts was stimulated.	RHAMM	Tolg et al., 2006
~20–160	N.D.	Accumulation of HA fragments was observed in bleomycin-induced lung injury in CD44-/- mice.	CD44	Teder et al., 2002
40–80	N.D.	*IL-1 β*, *TNF α*, *IGF-1* expression in murine bone marrow-derived Mø was induced.	CD44	Nobel et al., 1993
80–600	N.D.	ICAM-1 and VCAM-1 expression through activation of NF- B and AP-1 was up-regulated.	N.D.	Oertli et al., 1998
		COX-2 expression and TXA production was increased in renal tubular epithelial cells and ^{2}Mø.	N.D.	Sun et al., 2001
80–800	N.D.	MCP-1 expression was up-regulated in cortical tubular cells. Chemokine production was stimulated in Mø.	CD44	Beck-Schimmer et al., 1998
135–200	N.D.	An inflammatory response was initiated to promote recovery in acute lung injury.	TLR2 TLR4	Jiang et al., 2005
		DC maturation was induced and alloimmunity was initiated.	TLR4 TLR2	Tesar et al., 2006

(*continued*)

TABLE 13.4 (*continued*)

MW (kDa)	Saccharide units	Biological functions	Responsible receptors	References
200	N.D.	iNOS mRNA and protein expression was induced, alone or synergistically with IFN- in murine Mø.	CD44	Mckee et al., 1997
		IP-10 mRNA and protein expression was induced synergistically with IFN- in murine Mø.	N.D.	Horton et al., 1998
		IL-10 and IFN-independently inhibited HA fragment-induced chemokine gene expression in murine Mø.	N.D.	Horton et al., 1998**
		iNOS expression was induced *via* the NF-κB pathway in rat sinusoidal ECs and Kupffer cells.	N.D.	Rockey et al., 1998
		MME expression and enzyme activity were upregulated in alveolar Mø.	CD44*	Horton et al., 1999
		HA fragment-induced VCAM-1 expression was inhibited by a PKC inhibitor.	N.D.	Schwalder et al., 1999
		PAI activity was augmented and uPA was inhibited in alveolar Mø.	N.D.	Horton et al., 2000
		CBF in tracheal epithelial cells was stimulated. RHAMM.		Forteza et al., 2001
		Innate immune response was activated *via* TLR-2.	TLR2 CD44* TLR4*	Scheibner et al., 2006
250–300	N.D.	Motility of MDA-MB231 but not MCF-7 was stimulated. RHAMM.		Hamilton et al., 2007

TABLE 13.4 (*continued*)

MW (kDa)	Saccharide units	Biological functions	Responsible receptors	References
267–513	N.D.	*NF- B/I- B*[a] system was activated in murine Mø.	N.D.	Noble et al., 1996
280	N.D.	RANTES, MIP-1 a, and MIP-1 ß secretion and active IL-12 production were induced in elicited Mø.	CD44	Hodge-Dufour et al., 1997
400	N.D.	Activities of collagenase, gelatinase, and IL-8 production in human myometrial tissue cultures were stimulated.	N.D.	EI Maradny et al., 1997**
500–800	N.D.	TNF- a production was elevated in Mø.	CD44 RHAMM TLR4	Wang et al., 2006

The abbreviations used are: N.D., not described; Mø, macrophage. Refer to text for other abbreviations. Receptors with asterisks indicate those that were found to be NOT responsible for the events.

References that do not appear in the text (double asterisks) are as follows.

Horton, M.R., Burdick, M.D., Strieter, R.M., Bao, C and Noble, P.W. (1998). Regulation of hyaluronan-induced chemokine gene expression by IL-10 and IFN-gamma in mouse macrophages. *J Immunol* **160**, 3023–3030.

El Maradny, E., Kanayama, N., Kobayashi, H., et al. (1997). The role of hyaluronic acid as a mediator and regulator of cervical ripening. *Hum Reprod* **12**, 1080–1088.

than 600 kDa (approximately 3000-mers) induced the expression of *Mmp9* and *Mmp13* mRNA through the activation of nuclear factor (NF)-κB in 3LL Lewis lung carcinoma cells and normal murine embryonic fibroblasts (Fieber et al., 2004). In these events, CD44, RHAMM, and also TLR4 were all experimentally excluded as candidates for the responsible receptor.

Collectively, although the pathways are not fully uncovered, it is clear that even these very small HA fragments are capable of initiating signals such as the activation of NF-κB that leads to increased expression of various MMPs. These effects can promote tumor development, as it is well-established that MMPs are crucially involved in migration of tumor cells, tissue remodeling, and cell–cell communication (McCawley and Matrisian, 2001). However, it is also possible that such HA fragments act as a tumor suppressor because MMPs may also participate in tumor suppression (López-Otín and Matrisian, 2007) and the HA fragments might affect the immune system as well as

tumor cell functions through interaction with TLR4. In any case, identification of the receptor for HA 4–6-mers will allow us to understand further the characteristics of these very small HA fragments.

HA Fragments of 6–36-mers (≈1.35–6.9 kDa) (Table 13.3)

Most of the reports that deal with the biological functions of HA fragments fall into this category. Biological functions of the HA fragments of this size include their roles in angiogenesis, tumor cell biology, and the immune system.

Angiogenesis

As discussed earlier, the discovery of ''angiogenic oligosaccharides'' by West et al. (1985) opened the door to the research field of HA fragments. Later, they supported their own findings by demonstrating that HA 6–32-mers stimulated the proliferation of bovine aortic ECs but not fibroblasts or smooth muscle cells (West and Kumar, 1989). They also found that HMW-HA but not HA 6–32-mers disrupted a confluent monolayer of the ECs (West and Kumar, 1989) suggesting the importance of HA fragments not only in angiogenesis but also in maintaining vascular integrity. A number of reports followed this discovery further supporting their notion. For example, HA 6–20-mers induced angiogenesis and also the deposition of collagen type I and VIII at the sites of angiogenesis (Rooney et al., 1993), and HA 12-mers induced tube formation of brain capillary ECs (Rahmanian et al., 1997; Takahashi et al., 2005). The angiogenic effects of HA 6–20-mers were also observed *in vivo* by injecting the HA fragments into the skin of rats (Sattar et al., 1994).

HA fragments appear to evoke such responses by regulating several key molecules involved in angiogenesis. It was shown that HA fragments induce the expression of vascular endothelial growth factor (VEGF), one of the key molecules that promote angiogenesis (Adams and Alitalo, 2007) in endocardial cushion cultures (Rodgers et al., 2006), and synergistically cooperate with VEGF in bovine microvascular ECs to induce angiogenesis (Montesano et al., 1996). HA 6–20-mers up-regulated the expression levels of active urokinase type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1) (Montesano et al., 1996) that might contribute to the invasiveness of the ECs. In addition, HA fragments were able to initiate signaling cascades that include molecules such as ERK and protein kinase C (PKC) (Slevin et al., 1998) that might lead to the proliferation of the ECs. These signals induced by HA fragments are often mediated by CD44 (Slevin et al., 1998), but some reports show that RHAMM is also involved in these events. It was demonstrated that HA 20–30-mers bound to human pulmonary artery ECs, human lung microvessel ECs, and human umbilical vein ECs through RHAMM, and such

interaction lead to tyrosine phosphorylation of $p125^{FAK}$, paxillin, and p42/44 ERK (Lokeshwar and Selzer, 2000).

These reports are in line with the notion that HA fragments ranging from approximately 6–20-mers induce angiogenesis while HMW-HA does not. However, Koyama et al. (2007) appear to challenge this concept by demonstrating that not only HA oligosaccharides but also certain forms of HMW-HA are able to induce angiogenesis. They crossed a line of conditional transgenic mice expressing murine HAS-2 with the mouse mammary tumor virus-Neu transgenic model of spontaneous breast cancer to make a spontaneous breast tumor model that produces high amount of HA ($HAS\text{-}2^{\Delta Neo}$). They found that the breast tumors of $HAS\text{-}2^{\Delta Neo}$ showed an increased number of angiogenic blood vessels, and that degraded forms of HA ranging from 5.5–146 kDa (approximately 28–760-mers) were present in the tumors suggesting that these HA fragments contributed to the angiogenic response. Indeed, they were able to confirm the well-accepted notion that HA fragments but not HMW-HA induce angiogenesis in a matrigel plug assay using HA 32-mers and HMW-HA derived from *Streptococcus*, but surprisingly, when they tested the effect of HMW-HA from human umbilical cord, they noticed that the HMW-HA was able to induce angiogenic responses as well as HA 32-mers. In attempt to explain the discrepancy, they found a high amount of versican enriched in the human umbilical cord HA preparation forming an aggregate with HMW-HA. This aggregate induced angiogenesis in the matrigel plug assay while HMW-HA alone had no effect. The receptor responsible for this effect has not been revealed yet, but CD44 seems to be excluded because the angiogenic response still persisted in CD44-deficient mice. These results suggest that HA behaves distinctively by changing its size and also by interacting with molecules that co-exist with it in the microenvironment, adding a new concept to understand the functions of HA *in vivo*.

Recently, Koyama et al. found another interesting phenomenon induced by HA using the $HAS\text{-}2^{\Delta Neo}$ mice (Koyama et al., 2008). In the tumors of the transgenic mice, they found an increased number of lymphangiogenic vessels that were accompanied by an increased expression of VEGF-C and VEGF-D, suggesting the role of HA not only in angiogenesis but also in lymphangiogenesis. They confirmed in a xenograft breast tumor model that again HA-versican aggregates induced this response. In the report, the size-dependent effects of HA in lymphangiogenesis were not examined, but since $HAS\text{-}2^{\Delta Neo}$ produces degraded forms of HA (Koyama et al., 2007), it is possible that HA fragments as well as HA-versican aggregates contributed to the induction of lymphangiogenesis.

Effects on Tumor Cells

Similar to what Koyama et al. observed in the $HAS\text{-}2^{\Delta Neo}$ mice (Koyama et al., 2007), a variety of reports demonstrate the presence of HA

fragments in tumor tissues or in body fluids of cancer patients. Kumar et al. found "angiogenic oligosaccharides" in the sera of patients with Wilms' tumors and bone metastasizing renal tumors of the childhood (Kumar et al., 1989). Lokeshwar et al. found that urine from healthy people contained a small fraction of intermediate-sized HA, while a large amount of HA that contained both HMW-HA and HA 20–30-mers was detected in the urine specimens of patients with transient cell carcinoma (TCC) of the bladder (Lokeshwar et al., 1997). The HA 20–30-mers isolated from the urine of high-grade TCC patients were shown to have mitogenic responses in human lung microvessel ECs, suggesting the active role of HA fragments in transient cell carcinoma progression. They also found a similar HA profile in human prostate cancer tissues (Lokeshwar et al., 2001). These findings point to the fact that HA fragments are generated under tumor-bearing conditions, and somehow contribute to tumor progression.

Our findings substantiate this notion and add one way to explain the mechanism of HA fragment-induced tumor progression. We found that tumor cells produce HA fragments at cellular levels, and that such HA fragments activate the tumor cells through a CD44-dependent mechanism (Sugahara et al., 2003; 2006). The cell surface CD44 is proteolytically cleaved at the extracellular domain by proteases such as membrane type 1-MMP (Kajita et al., 2001), a disintegrin and metalloproteinase (ADAM)-10 and ADAM-17 (Nagano et al., 2004), followed by further cleavage at the intracellular domain that leads to increased expression of CD44 (Okamoto et al., 2001). Elevated CD44 cleavage is observed in various human tumors such as glioma, breast carcinoma, non-small cell lung carcinoma, colon carcinoma, and ovarian carcinoma (Okamoto et al., 2002) suggesting its critical role in tumor progression, and has been experimentally shown to be involved in tumor cell migration along extracellular matrices (Kajita et al., 2001; Okamoto et al., 1999). We found that HA 6–12-mers and a 6.9 kDa HA preparation that mainly contained HA 36-mers enhanced CD44 cleavage in tumor cells, while larger HA preparations such as 36 kDa, 200 kDa, and 1000 kDa HA did not (Sugahara et al., 2003). This phenomenon was induced by the interaction of the HA fragments and the cell surface CD44 molecules as demonstrated by the inhibition of the HA-fragment-induced CD44 cleavage by an anti-CD44 blocking antibody. The 6.9 kDa HA enhanced not only CD44 cleavage but also the migration of tumor cells in a CD44-dependent manner. We further found that MIA PaCa-2 human pancreatic cancer cell line expressed and secreted at least two hyaluronidases, HYAL-1 and HYAL-2, and that the culture supernatant of MIA PaCa-2 was able to degrade HA (Sugahara et al., 2006). Indeed, fragmented HA ranging from approximately 10–40-mers was found in the culture supernatant of tumor cells such as MIA PaCa-2. These purified tumor-associated HA fragments successively enhanced CD44 cleavage and cell migration in MIA PaCa-2 cells through a CD44-dependent

manner. Collectively, these results suggest the presence of an autocrine/paracrine activation mechanism in tumor cells which is mediated by HA fragments produced by the tumor cells themselves.

In contrast to the reports that demonstrate the contribution of HA fragments in tumor progression, some claim that the fragments counteract tumor development. Ghatak et al. reported that the *in vivo* tumor growth of LX-1 human lung carcinoma and TA3/St murine mammary carcinoma was inhibited and that their anchorage-dependent growth was inhibited as well by HA 6–20-mers (Ghatak et al., 2002). They further demonstrated that the survival pathway necessary for anchorage-independent growth characterized by the activity of phosphoinositide 3-kinase (PI3K) and the phosphorylation of Akt, was inhibited by the fragments. Such growth inhibitory effects of HA fragments were also observed in B16F10 murine melanoma subcutaneous tumors (Zeng et al., 1998). In addition, it has been reported that HA fragments exert pro-apoptotic effects on tumor cells (Cordo Russo et al., 2008; Hosono et al., 2007). Two possibilities can be considered to explain the suppressive effects of the HA fragments in tumor development. First, it is possible that the HA fragments actively initiate signals in the tumor cells that negatively regulate the cellular functions. Another possibility is that the effects observed (Cordo Russo et al., 2008; Ghatak et al., 2002; Hosono et al., 2007; Zeng et al., 1998) may have been due to the perturbation of the interaction between HMW-HA and CD44 expressed on the tumor cells, as discussed by Ghatak et al. (Ghatak et al., 2002). In fact, the latter possibility is valid because the HA fragments used in these reports include HA 20-mers (Cordo Russo et al., 2008; Ghatak et al., 2002; Hosono et al., 2007; Zeng et al., 1998) that is able to occupy more than one HA binding site of CD44 and efficiently inhibit the interaction between HA and CD44 (Lesley et al., 2000) that is essential to maintain the signaling cascade for tumor cell survival (Kosaki et al., 1999). In either explanation, it is not surprising even if HA fragments of a same size showed both tumor-promoting and tumor-regressing effects because of the differential binding patterns of such HA fragments (refer to ''CD44'' under ''Receptors for HA fragments''). The simple repeating disaccharide structure of HA enables such complicated behavior which is an important and unique aspect of HA.

Immune Response and Inflammation

Termeer et al. reported an important observation in which they showed for the first time that a member of the TLR family serves as one of the receptors for HA fragments. They demonstrated that HA fragments ranging from 6–16-mers induced the maturation of both human (Termeer et al., 2000) and murine (Termeer et al., 2002) DCs. This was accompanied by the production of various cytokines from the DCs, and was mediated by the activation of the mitogen-activated protein (MAP) kinase pathway and NF-κB (Termeer et al., 2002). Upon treatment of the DCs with an anti-TLR4

blocking antibody prior to the HA fragment treatment, the DC maturation did not occur, suggesting that the HA fragments induced the maturation by interacting with TLR4. This was further confirmed by using bone marrow-derived DCs from TLR4-deficient mice. In contrast, treatment of TLR2-deficient DCs with the HA fragments still resulted in the maturation of the DCs, indicating that TLR4 but not TLR2 serves as the receptor for the HA fragments in DCs. Similarly, it was shown in human microvessel dermal ECs that HA fragments up-regulate IL-8 through TLR4 (Taylor et al., 2004). These reports suggest that certain sizes of HA fragments initiate a systemic self-defensive response by eliciting innate immunity under certain conditions and also a local inflammation perhaps at the sites of injuries by interacting with ECs to promote wound healing.

Opposed to such protective functions, HA fragments appear to cause an undesired consequence in some cases by inducing immunological reactions. For instance, it was demonstrated that HA 36-mers up-regulate Fas expression on synovial cells and augment Fas-mediated early apoptosis of the cells which might be one of the mechanisms of the pathogenesis of RA. Indeed, it was already noticed about 50 years ago that in spite of the elevated concentration of HA, the viscosity of the synovial fluid of RA patients was lower than that of normal synovial fluids (Ragan and Meyer, 1949), and that it was due to the low degree of polymerization of HA (Balazs et al., 1967; Ragan and Meyer, 1949). Perhaps, HA fragments actively participate in the pathogenesis of immunological disorders and inflammatory diseases as well.

HA Fragments of 100-mers or Larger (≈20 kDa) (Table 13.4)

HA fragments of this size range may be described as intermediate-sized HA fragments. They have been shown to be capable of up-regulating the expression of adhesion molecules such as intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Oertli et al., 1998; Schawalder et al., 1999), enhancing ciliary functions in tracheal epithelial cells (Forteza et al., 2001), and elevating random motility of tumor cells (Hamilton et al., 2007) and fibroblasts (Tolg et al., 2006). Among the various functions of intermediate-sized HA fragments, their roles in regulating macrophages and DCs are most well-characterized. For example, sonicated HA preparations mainly consisting of HA fragments of 438 kDa (approximately HA 2300-mers), 474 kDa (approximately HA 2500-mers), and 513 kDa (approximately HA 2700-mers) in size induced the expression of I-κBα mRNA in mouse alveolar macrophages while native HMW-HA of 6000 kDa (approximately HA 31,000-mers) did not (Noble et al., 1996). HA fragments within a similar size range as the sonicated HA such as 40–80 kDa (approximately HA 200–400-mers) (Noble et al., 1993), those smaller than 500 kDa (approximately HA 2600-mers) (McKee et al., 1996), and 200 kDa (approximately HA 1000-mers)

(Hodge-Dufour et al., 1997; Horton et al., 1998) were also biologically active and able to induce the expression of chemokines. Such effects are often mediated by NF-κB (Noble et al., 1993; Noble et al., 1996; Rockey et al., 1998). In addition, a 280 kDa HA preparation (approximately HA 1500-mers) was demonstrated to be much more potent than HA 6-mers in inducing chemokines such as macrophage inflammatory protein (MIP)-1α, MIP-1β, cytokine-responsive gene (crg)-2, regulated on activation normal T cell expressed and secreted (RANTES), and monocyte chemoattractant protein (MCP)-1 (McKee et al., 1996). These findings suggest that HA fragments within the size range of approximately 40–500 kDa (approximately HA 200–2600-mers) are particularly potent in activating macrophages.

HA fragments of such size range also induce key molecules in inflammatory responses other than chemokines. HA fragments of 200 kDa (approximately HA 1000-mers) but not HMW-HA enhanced the expression of cytokines such as IL-12 that induce a Th1-type immune response in macrophages (Hodge-Dufour et al., 1997). Such HA fragments also increased the expression of inducible nitric oxide synthase (iNOS) in macrophages (McKee et al., 1997), Kupffer cells (Rockey et al., 1998), and ECs in the liver (Rockey et al., 1998), and cyclooxygenase type 2 (COX-2) and thromboxane A2 (TXA2) in macrophages and renal tubular cells (Sun et al., 2001). In addition, they induced the expression of macrophage metalloelastase (MME), an MMP that degrades various extracellular matrix (ECM) components such as elastin, collagen, and fibronectin, and plays an integral role in the development of emphysema (Horton et al., 1999). They were even shown to modulate the balance of fibrinolysis. The 200 kDa HA preparation (approximately HA 1000-mers) but not HA 2-mers or HA 12–16-mers augmented the expression and inhibitory activity of PAI-1 and diminished the expression and activity of uPA in macrophages, tilting the balance of fibrinolysis to a lesser extent (Horton et al., 2000).

Many of the effects of HA fragments discussed above were found to be mediated by CD44 (Beck-Schimmer et al., 1998; Hodge-Dufour et al., 1997; McKee et al., 1997; Noble et al., 1993; Wang et al., 2006). The involvement of CD44 in HA fragment-induced inflammatory responses was verified *in vivo* by Teder et al. (2002). They found that CD44-deficient mice succumb to unremitting pulmonary inflammation after bleomycin treatment. It was characterized by the accumulation of HA fragments ranging from 20–160 kDa in size (approximately HA 100–830-mers) and a decreased activity of transforming growth factor-β (TGF-β). The survival defect and the histologic changes were significantly reversed by reconstitution with CD44-positive bone marrow, indicating that CD44 plays a crucial role in resolving inflammatory responses. Although it is clear that CD44 is involved in the clearance of the HA fragments under such inflammatory conditions, one question rises from this report. How did the HA fragments

that accumulated in the injured lungs impact the inflammation under a CD44-negative circumstance, and what was the responsible receptor? The findings reported by Jiang et al. give a possible answer to this question (Jiang et al., 2005). These authors demonstrated that a 135 kDa HA preparation (approximately HA 700-mers) and HA fragments isolated from the serum of patients with acute lung injury (approximately HA 1000-mers) induced MIP-2 expression in macrophages through both TLR2 and TLR4 by using macrophages that lack TLR2, TLR4, or both. The involvement of CD44, TLR1, TLR3, TLR5, and TLR9 in these events was excluded. In contrast to roles of intermediate-sized HA fragments in eliciting inflammatory responses, they found that HMW-HA was protective against acute lung injury. Therefore, they concluded that HA degradation products generated after tissue injury initiate inflammatory responses by stimulating macrophages while HMW-HA maintain epithelial integrity, and that these effects are mediated by TLR-2 and TLR-4.

Although it is clear that TLR2 and TLR4 mediate the signals induced by intermediate-sized HA that initiate inflammatory responses, further discussion is required to understand their division of labor in these events. Similar to the findings by Jiang et al. (2005), it was shown that 135 kDa HA (approximately HA 700-mers) activated bone marrow-derived DCs mainly through TLR4 and partial synergy with TLR2 (Tesar et al., 2006). On the other hand, it was reported that 200 kDa HA (approximately HA 1000-mers) but not HMW-HA was able to induce a series of inflammatory chemokines, cytokines, and enzymes in thioglycollate-elicited peritoneal macrophages and MIP-1α in bone marrow-derived DCs through TLR2 but not TLR4, CD44, IL-1R, or IL-10R (Scheibner et al., 2006). It is currently difficult to explain how TLR2 and TLR4 co-operate. However, taking these reports into account, it appears that both TLR2 and TLR4 mediate the inflammatory responses induced by intermediate-sized HA fragments, and under some conditions, TLR2 and TLR4 compensate each other. It is of particular interest if different TLRs recognize different sizes of HA, or different cell types respond to HA fragments using different TLRs depending on their location and type of inflammation even if they were categorized as the same cell type such as macrophages.

CONCLUSIONS AND FUTURE PROSPECTS

HA fragments of various sizes have a variety of functions on different cell types, such as endothelial cells, tumor cells, and immune cells. The effects are mediated by different receptors including CD44, RHAMM, and TLRs, and also by unknown molecules. The effects look diverse, however have tight links to each other. From tumor biology point of view, perhaps such effects can be summarized as shown in Fig. 13.3. HA fragments, most

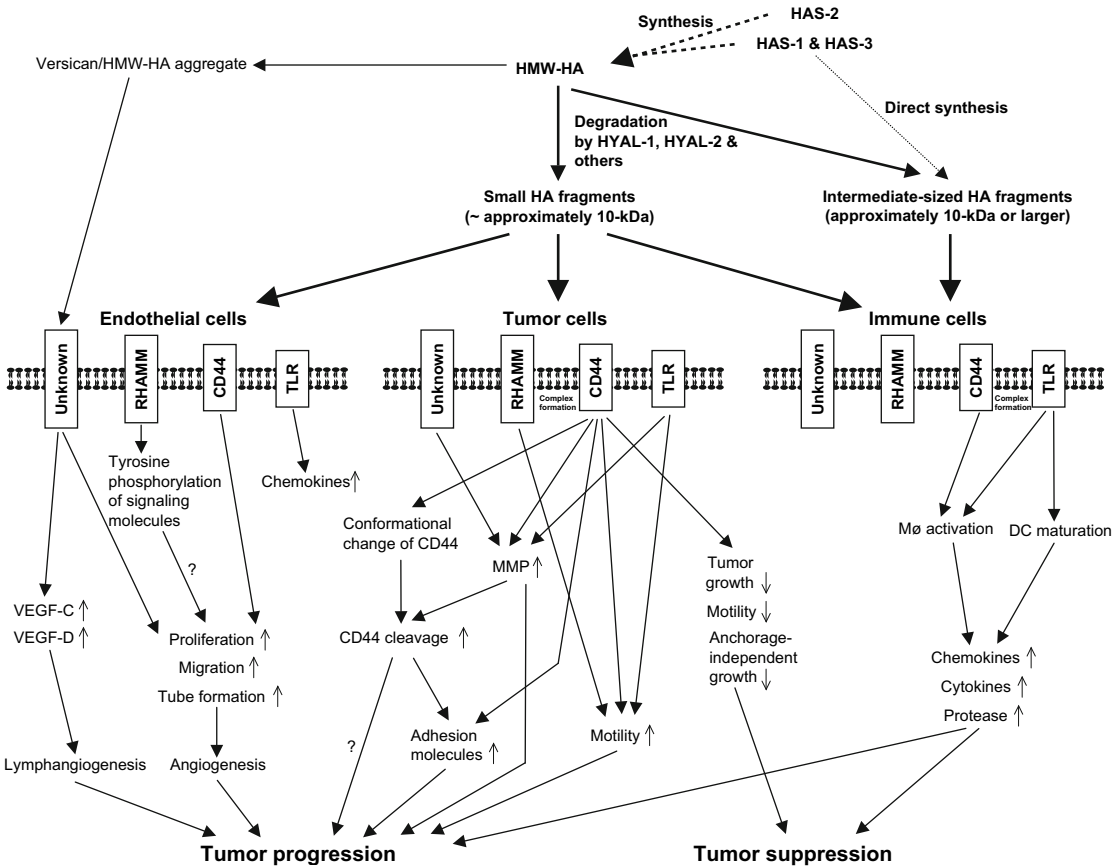

FIGURE 13.3 Effects of HA fragments on tumor development. HA fragments are generated through direct synthesis by HA synthases and/or degradation of HMW-HA by hyaluronidases and other means. The HA fragments cause various changes in the cellular functions of endothelial cells, tumor cells, and immune cells through receptors such as CD44, RHAMM, TLR, and a yet unknown receptor(s) that lead to tumor progression or suppression. "?" indicates connections that have been suggested but not experimentally proven. Refer to text for abbreviations.

of them within the range of 6–36-mers, promote angiogenesis through interaction with CD44 and RHAMM. Not only HA fragments, but also HMW-HA that formed an aggregate with versican up-regulates angiogenesis as well as lymphangiogenesis through a yet unknown receptor. These effects seem to positively regulate tumor progression since it is widely accepted that angiogenesis and lymphangiogenesis are crucial events for tumors growth (Adams and Alitalo, 2007). HA fragments of a wide size range directly influence tumor cell behavior. By interacting with CD44, they alter the conformation of CD44 that might lead to CD44 cleavage which is considered to play an important role in tumor progression. The interaction of HA fragments with CD44 as well as with RHAMM and TLR4 result in the up-regulation of tumor cell motility. In contrast, they sometimes inhibit tumor growth, tumor cell motility, and anchorage-independent growth of tumor cells, perhaps by interfering with HMW–HA–CD44 interaction, leading to suppression of tumor development. HA fragments generated under tumor-bearing conditions must be able to exert an influence on immune cells as well. Through TLRs and/or CD44, they induce the activation of macrophages and maturation of DCs that lead to the production of various chemokines, cytokines, and proteases. These effects might act as ''danger signals'' for the host so that the host can fight against tumors. However, it is likely that such effects on immune cells are ''double-sided blades'' that help tumor cells to make their own beds in the host tissue at the same time. Because tumors are considered as ''complex tissues'' that contain various components other than the tumor cells including the tumor ECM such as HA, infiltrating immune cells, and fibroblasts (Hanahan and Weinberg, 2000), it is not difficult to accept that each cellular and acellular component affect each other to create a perfect tumor microenvironment.

HA is indeed a major component of the ECM, though there are still various molecules that organize the matrix. Even only within glycosaminoglycans, there are various members such as keratan sulfate, chondroitin sulfates, and heparan sulfates. It is possible that the effects of HA fragments just reflect a tip of the iceberg, and that other members of the ECM possess similar functions as HA fragments do. For example, it has been shown that keratan sulfate disaccharides but not tetrasaccharides or native keratan sulfate chains inhibit IL-12 production in macrophages (Xu et al., 2005). Recently, we have also identified that a member of chondroitin sulfates is highly over-expressed in number of tumors and that pancreatic tumors contain degraded forms of the CS. Such small fragments of the chondroitin sulfate chain enhance CD44 cleavage and tumor cell motility in a fashion similar to HA fragments (Sugahara et al., 2008). Therefore, it is tempting to think that, as tumors develop, tumor-associated ECM becomes degraded and some of the degradation products within the tumor microenvironment start to send signals to the surrounding cells.

By understanding such mechanisms, it is possible to make use of them. Some attempts are already made with HA fragments. For instance, different sizes of HA fragments were compared for their ability to enhance elastin matrix synthesis and generate a scaffolding material for the regeneration of vascular elastin matrices (Ibrahim et al., 2007; Joddar and Ramamurthi, 2006). In addition, HA fragments were tested for use to facilitate DNA uptake by cells (Hornof et al., 2008). It was demonstrated that when DNA/polyethylenimine (PEI) was coated with HA fragments smaller than 10 kDa (approximately HA 50-mers), the specific uptake of DNA by CD44 was enhanced in human corneal epithelial cells, whereas coating with larger HA polymers did not allow such enhancement. In addition to the use of HA fragments themselves, hyaluronidases that generate HA fragments are being tested as tumor markers in bladder cancers (Lokeshwar and Selzer, 2006). Such utilization of our knowledge regarding HA fragments in diagnostic and therapeutic technologies must be the next step that we should take to fight against tumors and inflammatory diseases.

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CHAPTER

14

Hyaluronan in Human Tumors: Importance of Stromal and Cancer Cell-Associated Hyaluronan

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HYALURONAN ACCUMULATION IN MALIGNANT TUMORS

Hyaluronan, a huge linear sugar polymer, composed of alternating N-acetyl-glucosamine (GlcNAc) and glucuronic acid (GlcUA) units, is common in many vertebrate extracellular matrices (Fraser et al., 1997). The number of the repeating disaccharide units varies, but can reach at least 25,000, corresponding to a molecular mass of 10 million Daltons, and an extended length of 22.5 μm. Its unique physicochemical properties, like the capacity to bind large amounts of water and form viscous gels at relatively low concentrations, has been suggested to provide a pliable matrix for tissue remodeling during embryonic development, and wound healing (Toole, 1997). It also acts like a filter, facilitating the diffusion of small, but excluding large molecules (Fraser et al., 1997). In contrast to other glycosaminoglycans, hyaluronan does not contain sulfate groups, is not covalently attached to a core protein, but can bind to proteoglycans and other proteins to organize pericellular and extracellular matrix. More recently, its capacity to activate various intracellular signaling routes through specific plasma membrane receptors has gained wide interest. Hyaluronan has turned out to be an active regulator of cell behavior, rather than solely an inert extracellular matrix component (see sections II and III in this volume).

The high concentration of hyaluronan in many embryonic tissues correlates with their rates of cell migration and proliferation. In addition, hyaluronan accumulation signals for epithelial to mesenchymal transformation, for example at the future valve sites of the embryonic heart tube (Camenisch et al., 2000). Because of the similarities between developing and tumor tissues, it was suggested decades ago that hyaluronan might be important for cancer growth. In line with this idea, biochemical analyses of glycosaminoglycans have shown accumulation of hyaluronan in tumors originating from connective tissues but also in those of epithelial origin, like carcinomas of breast, head and neck, ventricle, colon, and pancreas (reviewed by Knudson et al., 1989 and references in this paper, and Theocharis et al., 2003; Garcia et al., 2000; Llaneza et al., 2000).

The question whether hyaluronan resided in the tumor parenchyma, or the peritumoral stroma, remained obscure, as it could not be addressed with biochemical assays, and no suitable histological methods were available. During the last ten years, taking advantage of the high affinity binding between hyaluronan and the cartilage proteoglycan aggrecan (Hardingham and Muir, 1972), using either whole aggrecan (Knudson and Toole, 1985) or its isolated hyaluronan binding region (Ripellino et al., 1985) has enabled the acquisition of detailed information on the localization and relative content of hyaluronan in the different compartments of

tumor tissues (Wang et al., 1996). Today, such analyses performed on a number of patient materials and tumor types allows us to recognize distinct patterns and trends. Overall, it is now well-established that hyaluronan levels both in the cancer epithelium and peritumoral stroma relate to tumor progression, and often indicate an unfavorable outcome of the disease. Moreover, recent reports suggest that hyaluronan is not only a prognostic indicator, but an active participant in the disease, and a novel target of therapy (Nakazawa et al., 2006). Therefore, in the future we may see hyaluronan inhibitors as one asset in the battle against cancer.

HYALURONAN ASSOCIATED WITH CANCER CELLS

There is a large body of evidence from experimental animals and *in vitro* models suggesting that the production of hyaluronan by tumor cells is important for their malignant behavior. Carcinoma cells isolated from different human or animal tumors produce variable amounts of hyaluronan under *in vitro* conditions, and although not universally true, high hyaluronan synthesis rate correlates with an aggressive, metastatic behavior in many cancer cell types (Kimata et al., 1983; van Muijen et al., 1995). Experimental overexpression or underexpression of hyaluronan synthases (HASes) supports the hypothesis that hyaluronan production by malignant cells enhances tumor spreading. Thus, HAS overexpression enhances local tumor growth (Kosaki et al., 1999; Liu et al., 2002; Jacobson et al., 2002; Itano et al., 2004), and distant metastases (Itano et al., 1999; Simpson et al., 2002), while antisense inhibition of HAS expression reportedly inhibits the growth of the primary tumor (Udabage et al., 2005) and distant metastasis (Simpson et al., 2002). Analyses of clinical materials has supported the relevance of the experimental studies cited above, by showing that hyaluronan on cancer cells may indeed contribute to high spreading potential of human tumors *in vivo*. However, opposite trends are seen in tumors arising from simple or stratified epithelia as described below.

Hyaluronan in Tumor Cells Arising from Simple Epithelia

The normal simple epithelia, and the transitional epithelium of the bladder show either a low signal for hyaluronan staining, or none at all (Fig. 14.1) (de la Torre et al., 1993; Wang et al., 1996; Auvinen et al., 1997; Pirinen et al., 1998; Lokeshwar et al., 2001; Hautmann et al., 2001), while cancer cells arising from these epithelia are often decorated by hyaluronan. Neoplastic growth starting from simple epithelia is thus associated with acquisition of hyaluronan expression (Fig. 14.1, Table 14.1). In most cases hyaluronan is localized on the plasma membranes, but sometimes also in the cytoplasm, and occasionally even in the nucleus (Ropponen et al., 1998;

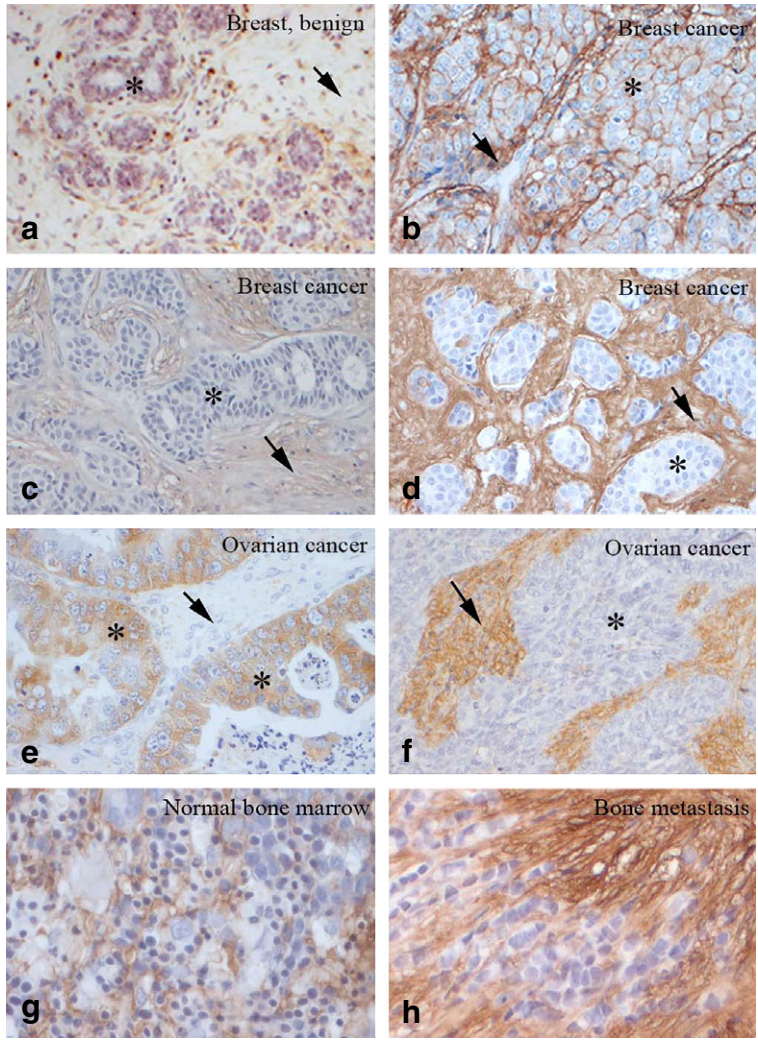

FIGURE 14.1 Stromal hyaluronan versus cancer cell-associated hyaluronan. Tumors archived in paraffin were sectioned and stained with a probe specific for hyaluronan (brown), nuclei were visualized with hematoxylin (blue). In benign breast tissue the normal tubular cells are hyaluronan negative and the stromal component is faintly hyaluronan-positive (**a**). In invasive breast carcinomas (**b–d**) cancer-cell-associated hyaluronan is sometimes present close to plasma membrane (**b**) and the stromal staining varies from faint (**c**), to strong (**d**). An ovarian carcinoma with tumor cell-associated hyaluronan and very low stromal staining is shown in (**e**), and another case with strong stromal hyaluronan signal without epithelial staining in (**f**). Bone metastasis of a breast carcinoma shows a strong stromal hyaluronan staining (**h**) while the adjacent normal bone marrow is less intensely stained (**g**). Stars point the tumor epithelial cells and the arrows the stromal compartment. (See Page 6 in Color Section at the back of the book).

Auvinen et al., 2000). Some hyaluronan-positive cells are found in about half of breast and prostate tumors, while in ovarian, colorectal, and gastric cancers more than 80 and 90% of the tumors can be positive (Ropponen et al., 1998; Setälä et al., 1999; Auvinen et al., 2000; Anttila et al., 2000; Lipponen et al., 2001). However, all tumors are internally heterogeneous in the expression of cancer cell-associated hyaluronan, with areas of high and low frequency of positive cells in a single tumor. In general, the number of positive cells is typically higher in colon and gastric cancers than for example in ovarian cancers (Ropponen et al., 1998; Setälä et al., 1999; Anttila et al., 2000). Different histological subtypes of cancers arising from a certain epithelial tissue can display distinct hyaluronan expression profiles, as shown by examples of lung (Pirinen et al., 2001) (Fig. 14.2), gastric (Setälä et al., 1999), salivary gland (Xing et al., 1998), and thyroid cancers (Böhm et al., 2002). Hyaluronan content also correlates with other biological parameters of cancer cells, such as missing estrogen and progesterone receptors in breast cancer cells (Auvinen et al., 2000). This must be considered when comparing tumor sets with different histological subtype distributions.

Several findings support the idea that an ectopic expression of hyaluronan in the malignant cells arising from simple epithelia contributes to an aggressive behavior of these tumors (Table 14.1). Tumor grades, or degrees of cellular dedifferentiation, show direct correlations with the proportion of hyaluronan-positive cancer cells, or the intensity of hyaluronan staining on those cells (Table 14.1). Cell-associated hyaluronan in cancers arising from simple epithelia also correlates with local invasive growth, spreading to local lymph nodes, and even with distant metastasis, as in salivary gland and colorectal carcinomas (Table 14.1). Interestingly, metastases often contain cancer cells with a high level of hyaluronan expression and correlate with those of the original tumors (Anttila et al., 2000; Böhm et al., 2002).

The correlations described above, suggesting that tumors with hyaluronan-positive cells are more aggressive, have been studied further by analyzing patient prognosis. A high level of hyaluronan on tumor cells is a strong indicator of unfavorable outcome, i.e. shortened overall survival (OS) and/or the time of disease free survival (DFS) in breast, gastric, and colorectal cancers (Table 14.1). In colon cancer, only 20% of patients survived if a large fraction of their tumor cells were hyaluronan-positive, while 80% survived when there were just few hyaluronan expressing cells (Ropponen et al., 1998) (Fig. 14.3). In gastric cancer the corresponding survival levels were 25 and 60% in cases with high and low hyaluronan expression, respectively (Setälä et al., 1999), and in breast cancer 55 and 80% (Auvinen et al., 2000). In a Cox multivariate analysis of colon cancer, including all conventional prognostic factors, tumor cell hyaluronan is an independent prognostic factor for both OS and DFS (Ropponen et al., 1998; Köbel et al., 2004).

TABLE 14.1 Cancer Cell-Associated Hyaluronan, Tumor Characterics, and Patient Prognosis

Tumor	HA content	HA correlation with				HA as a prognostic factor				Reference
						Univariate***		Multivariate***		
		Grade	Local invasion	Nodal invasion	Distant metastases	OS	RFS/DFS	OS	RFS/DFS	
Simple epithelia										
Bladder	Up	+								Hautmann et al. (2001)
Breast	Up	+		+		+	−	−	−	Auvinen et al. (2000)
Breast*	Up	+						−	−	Suwiwat et al. (2004)
Colon	Up	+	+			+	+	+	+	Ropponen et al. (1998)
Colon	Up	−		+	+	+		−/+**		Köbel et al. (2004)
Gastric	Up	+	+	−	−	+		−		Setälä et al. (1999)
Lung, adenocarcinoma	Up	+				−				Pirinen et al. (2001)
Ovarian	Up	+				−	−			Anttila et al. (2000)
Prostate	Up		Perineural invasion			−		−		Lipponen et al. (2001)
Salivary	Up				+					Wein et al. (2005)
Thyroid	Up	−				−	−			Böhm et al. (2002)
Stratified epithelia										
Esophagus	Down	+								Wang et al. (1996)
Larynx	Down	+	+		+		+		−	Hirvikoski et al. (1999)
Lung, SCC + adenocarcinoma	Down	−				−	+	−	−	Pirinen et al. (2001)

Melanoma	Down		Breslow thickness			+	+	−	−	Karjalainen et al. (2000)
Oral	Down	+	+		+?	+	+	+	+	Kosunen et al. (2004)
Skin	Down	+								Karvinen et al. (2003a)

OS, overall survival; DFS/RFS, disease free/recurrence free survival.
SCC, squamous cell carcinoma.
*Node negative patients only.
**Combined with CD44v6 expression.
***+ correlates with short OS/RFS, − no correlation.

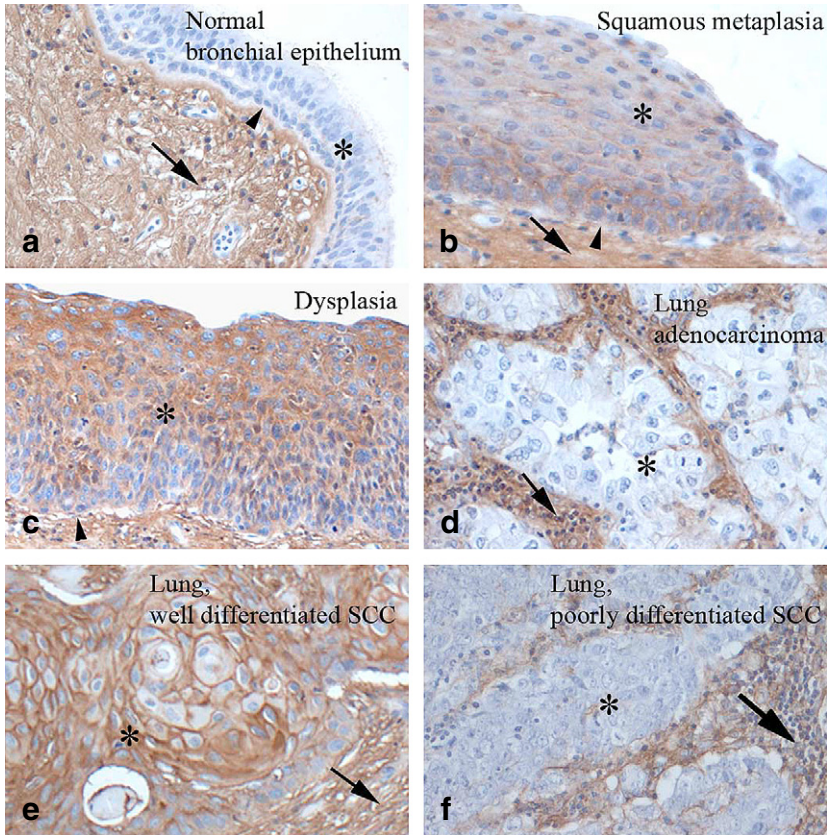

FIGURE 14.2 Association of epithelial hyaluronan expression with squamous cell differentiation. Tumors archived in paraffin were sectioned and stained with a probe specific for hyaluronan (brown), nuclei were visualized with hematoxylin (blue). Basal cells (arrowhead) may show a faint hyaluronan signal, while most of the normal bronchial epithelium does not stain for hyaluronan (**a**). Metaplastic bronchial epithelium is associated with a strong hyaluronan expression, especially in the superficial cell layers (**b**). Epithelial hyaluronan expression is also seen in dysplastic bronchial epithelium (**c**) and in well differentiated SCCs (**e**). In the latter case the staining pattern is very similar to that in cancers originating from squamous epithelia, like skin SCC or oral SCC. In contrast, a poorly differentiated lung SCC shows just a faint hyaluronan staining on the cancer cells (**f**). Well differentiated lung adenocarcinoma shows very little hyaluronan among the cancer cells, but the stroma is intensely stained (**d**). The stars point the (tumor) epithelial cells and the arrows the stromal compartment. (See Page 7 in Color Section at the back of the book).

Furthermore, hyaluronan is the only significant prognostic factor in the Dukes B subgroup of patients (Ropponen et al., 1998). The histological findings are in agreement with radioimmunometric assays of hyaluronan concentration in a large series of colon and gastric carcinomas (Llaneza et al., 2000; Vizoso et al., 2004).

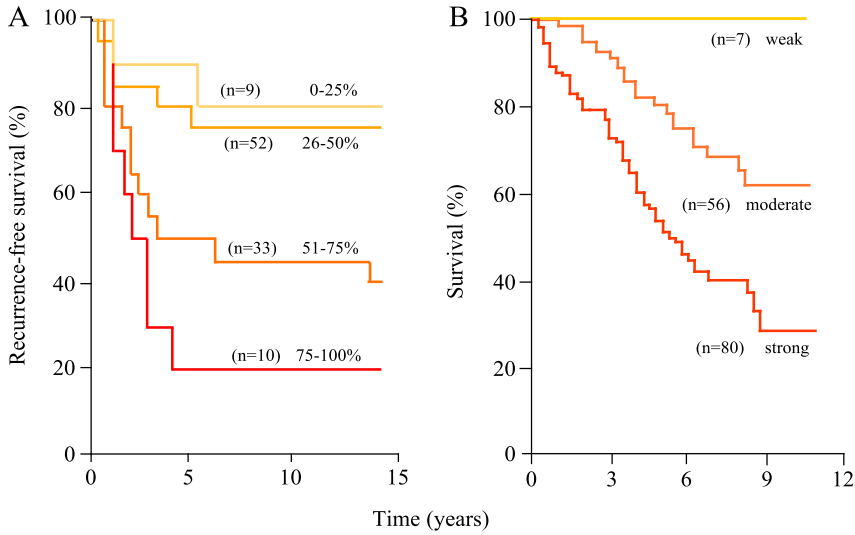

FIGURE 14.3 Prognosis of cancer patients – correlations with cell-associated and stromal hyaluronan. Sections of colorectal carcinoma were stained for hyaluronan with a specific probe, scored for the proportion of tumor cells positive for hyaluronan, and grouped into the indicated ranges of percentages for Kaplan–Maier curves of cumulative recurrence-free survival (**A**). Sections of breast cancer were scored for the intensity of hyaluronan staining in the peritumoral stroma (weak, moderate, strong) to present curves for survival as above (**B**). The numbers of patients in each group are shown in parenthesis. The data have been compiled from Ropponen et al. (1998) and Auvinen et al. (2000), respectively.

Hyaluronan in Tumor Cells Arising from Stratified Epithelia

Stratified epithelial cells display a fair amount of surface hyaluronan already in their native state (Tammi et al., 2004). Therefore, it is difficult to tell whether hyaluronan increases in the early grade neoplasms arising from stratified epithelia, while it is quite obvious that cell-associated hyaluronan is decreased in high-grade as compared to low-grade squamous cancers (Table 14.1). Anyway, several premalignant or early malignant lesions, like dysplasias and the *in situ* carcinomas of oral, laryngeal, esophageal and skin epithelium, all show a tendency to increased hyaluronan expression compared to normal epithelium (Wang et al., 1996; Hirvikoski et al., 1999; Karvinen et al., 2003a; Kosunen et al., 2004). Hyaluronan expression remains high in well-differentiated carcinomas of these tissues, while in high-grade (poorly differentiated) squamous cell carcinomas hyaluronan staining becomes irregular, and its overall intensity is reduced (Table 14.1). Skin melanomas show a hyaluronan expression pattern similar to that of squamous cell carcinomas (Karjalainen et al., 2000). Thus, aggressive melanomas contain less hyaluronan-positive

cells than tumors with lower Breslow thickness and lower Clark's level (Karjalainen et al., 2000). The staining pattern of CD44, the major hyaluronan receptor, closely parallels that of hyaluronan in all the tumors in this group (Wang et al., 1996; Hirvikoski et al., 1999; Karjalainen et al., 2000; Karvinen et al., 2003a; Kosunen et al., 2004).

Interestingly, not only squamous cell carcinomas from stratified epithelia, but also squamous cell carcinomas arising from simple epithelium show the same biphasic pattern in hyaluronan expression (Fig. 14.2). Thus, normal bronchial epithelium has little hyaluronan, while squamous metaplasia shows strong staining of the metaplastic cells, and high-grade squamous cell carcinomas of the lung show an irregular hyaluronan staining pattern and low staining intensity (Pirinen et al., 1998) (Fig. 14.2). Again, high and low CD44 staining patterns parallel to those of hyaluronan (Pirinen et al., 2000).

The reduced hyaluronan in high-grade squamous cancer cells correlates with nodal involvement in laryngeal (Hirvikoski et al., 1999) and oral (Kosunen et al., 2004) squamous carcinomas, and with distant metastasis in laryngeal cancers (Hirvikoski et al., 1999) (Table 14.1). The importance of the loss of hyaluronan in squamous cell carcinoma progression was indicated by the findings that it was associated with poor patient outcome both in laryngeal and oral carcinomas (Hirvikoski et al., 1999; Kosunen et al., 2004), as well as in skin melanomas (Karjalainen et al., 2000). Only 50% of oral carcinoma patients showing irregular and low hyaluronan staining at the time of diagnosis were alive after 5 years, as compared to 80% survival among those with normal hyaluronan staining pattern (Kosunen et al., 2004). The loss of hyaluronan from the oral cancer cells turned out to be an independent prognostic factor in Cox multivariant analysis of (Kosunen et al., 2004) (Table 14.1).

The analyses of tumor biopsies do not tell whether the decline of hyaluronan in the high-grade squamous carcinomas results from reduced synthesis, enhanced rate of degradation, or loss from cell surface due to CD44 depletion. Increased and ectopic hyaluronidase expression (Franzmann et al., 2003; Christopoulos et al., 2006), and increased expression of MT-MMP1 and ADAM proteases, capable of CD44 stripping from cell surface, were reported in head and neck squamous cell carcinomas (Nakamura et al., 2004; Zhai et al., 2005), suggesting that hyaluronan turnover was increased. In addition to hyaluronan and CD44, other adhesion molecules like cadherins and catenins are often downregulated or their function is impaired (Thomas and Speight, 2005). Thus, the decrease of hyaluronan and CD44 may reflect the more general loss of adhesion molecules in poorly differentiated SCCs. Although the cell–cell contacts based on cadherin probably dominate in normal conditions, the loss of the low affinity contacts mediated by CD44 and hyaluronan may become significant in the absence of cadherin.

In summary, cell-associated hyaluronan has a completely different role in the progress of adenocarcinomas and squamous cell carcinomas, originating from monolayered and stratified epithelia, respectively.

STROMAL ACCUMULATION OF HYALURONAN

Even the first histochemical stainings with the hyaluronan probe derived from cartilage aggrecan suggested that stromal hyaluronan is increased in lung and breast tumors (Knudson et al., 1989; Bertrand et al., 1992; Ponting et al., 1993), and that the increase is more pronounced in the invasion front as compared with the central tumor areas (Kudson et al., 1989; Bertrand et al., 1992; Ponting et al., 1993). The increased stromal hyaluronan staining in breast cancer has been later confirmed in several studies, and is also present in prostate, ovarian, bladder, endometrial, and thyroid carcinomas, and lung adenocarcinomas (Table 14.2) (Fig. 14.1). The accumulation of stromal hyaluronan can be drastic in tissues with naturally low hyaluronan concentration. For example, in grade three ovarian cancer the median increase of hyaluronan concentration was 49-fold (Hiltunen et al., 2002) (Fig. 14.1).

Poorly differentiated or high-grade tumors have typically more stromal hyaluronan than well-differentiated tumors, examples including breast, prostate, and ovarian cancers (Table 14.2). Stromal hyaluronan accumulation also associates with cancer cell penetration of capsules, lymph vessels, and nerves in prostate carcinomas, local lymph node infiltration in breast cancers, and with distant metastasis in thyroid, ovarian, and prostate cancers (Table 14.2).

In line with its association with invasion, strong stromal hyaluronan staining indicates poor patient outcome in breast, ovarian, prostate, and thyroid cancers (Table 14.2). In breast cancer, half of the patients showing strong stromal hyaluronan signal were alive after 5 years follow-up, while all patients with low stromal hyaluronan signal survived (Auvinen et al., 2000). In ovarian cancers the corresponding values were 25 and 45% (Anttila et al., 2000). Furthermore, strong hyaluronan signal is an independent prognostic factor in breast and ovarian tumors in Cox multivariate analysis (Table 14.2). In ovarian cancers, which present a rather bleak outlook even in early disease, strong stromal hyaluronan signal is an independent prognostic factor for short DFS and OS also when FIGO I and II stages are examined separately (Anttila et al., 2000). The lack of a similar statistically significant correlation in a node negative subgroup of breast cancers (Suwiwat et al., 2004) with a generally favorable prognosis, may just reflect the low number of cases (disease recurrences or deaths) in these patient groups during a limited follow-up period. Figure 14.1 shows hyaluronan staining of a metastatic lesion of a breast cancer in bone

TABLE 14.2 Stromal Hyaluronan Expression, Tumor Characteristics, and the Patient Prognosis

Tumor	HA content	HA correlation with				HA as a prognostic factor				Reference
						Univariate		Multivariate		
		Grade	Local invasion	Nodal invasion	Distant metastases	OS	RFS/DFS	OS	RFS/DFS	
Bladder	Up	+								Hautmann et al. (2001)
Breast	Up	+		+		+	+	+		Auvinen et al. (2000)
Breast	Up	+	+							Bertrand et al. (1992)
Breast	Up	−	+							Pontig et al. (1993)
Breast*	Up	+						−	−	Suwiwat et al. (2004)
Breast*	Up	+		+				−		Wernicke et al. (2003)
Endometrium	Up									Afify et al. (2005)
Lung, adenocarcinoma	Up	−					+		+	Pirinen et al. (2001)
Ovarian	Up	+			+	+	+	+	+	Anttila et al. (2000)
Ovarian	Up	+								Hiltunen et al. (2002)
Prostate	Up	Gleason	+		+	+		−	−	Lipponen et al. (2001)
Prostate	Up		+						−	Aaltomaa et al. (2002)
Prostate	Up		+							Lokeshwar et al. (2001)

Prostate	Up			+		−		Posey et al. (2003)
Prostate	Up			+		−/+**		Ekici et al. (2004)
Thyroid	Up	−	+	+***	−	−		Böhm et al. (2002)

OS, overall survival; DFS/RFS, disease free/recurrence free survival.
*Node negative patients.
**If HYAL-1 is not included in the model.
***Cancer related mortality.
+ Correlates with short OS/RFS/DFS; − no correlation.

marrow. The strong hyaluronan staining highlights the cancer in the host tissue. Metastases of thyroid and ovarian carcinomas show a similar hyaluronan-rich peritumoral stroma which correlates with the expression level in the original tumor (Anttila et al., 2000; Böhm et al., 2002). Invasive lesions of prostate cancer into adjacent tissues like seminal vesicle often contain a high level of hyaluronan (Lokeshwar et al., 2001). The data suggest that tumors able to induce stromal hyaluronan accumulation have a growth advantage in the new environment as compared to those without this capacity.

The staining intensity of stromal hyaluronan does not associate with the progression of tumors from the gastrointestinal tract (Ropponen et al., 1998; Setälä et al., 1999; Köbel et al., 2004), or those arising from stratified epithelia (Hirvikoski et al., 1999; Karjalainen et al., 2000; Karvinen et al., 2003a), perhaps because hyaluronan is naturally abundant in the stroma of these tissues. The strong prognostic power of stromal hyaluronan in breast and ovarian cancers may be due to the ease of detection of the increased signal in these hyaluronan poor tissues, but probably also because of the more profound change in tissue structure induced by hyaluronan accumulation (de la Torre et al., 1993; Auvinen et al., 1997; Anttila et al., 2000) (Fig. 14.1).

The hyaluronan-rich peritumoral stroma is also enriched in versican, a hyaluronan binding proteoglycan (Voutilainen et al., 2003), the expression level of which can further define the prognostic value of hyaluronan (Suwiwat et al., 2004).

MECHANISMS OF HYALURONAN ACCUMULATION IN TUMORS

The assays on tumor biopsies do not give information about the dynamic processes that control the synthesis and catabolism of hyaluronan. Currently, we have not much data on the relative importance of factors that determine the content of hyaluronan in malignant tissues, but some of the alternatives are discussed below.

Enhanced Synthesis of Hyaluronan in Tumors

In a given cell, hyaluronan is produced by one or more of the three homologous hyaluronan synthases (HAS1, HAS2, and HAS3). Each of these enzymes can take care of all the tasks required for hyaluronan secretion, including initiation of the chain, the alternating transfer of glucuronic acid (GlcUA) and N-Acetyl-glucosamine (GlcNAc) from their UDP-activated forms, holding the growing polysaccharide, and creating a plasma membrane pore for its continuous extrusion (Weigel et al., 1997).

Therefore, the rate of hyaluronan synthesis is mostly determined by the activity of HAS.

While experimental UDP-GlcUA depletion may limit the rate of hyaluronan synthesis in some cases (Kakizaki et al., 2004; Vigetti et al., 2006), there is limited evidence that increasing the cellular UDP-GlcNAc and UDP-GlcUA pool size could markedly stimulate hyaluronan synthesis. Instead, most of the studies currently available indicate that hyaluronan synthesis correlates with the level of HAS mRNA (Pienimäki et al., 2001; Karvinen et al., 2003b; Pasonen-Seppänen et al., 2003), suggesting that transcriptional regulation is a major determinant of the net HAS activity. The importance of HAS transcription as a major determinant of hyaluronan synthesis is in line with the short turnover time of the HAS protein (Rilla et al., 2005), and the rapid and large fluctuations observed in HAS expression (Fülop et al., 1997; Pienimäki et al., 2001). However, most of these studies have been conducted *in vitro*, leaving some uncertainty, for instance, in the HAS protein turnover times *in vivo* (Anggiansah et al., 2003).

The genomic region containing the HAS2 gene is frequently amplified in prostate cancer (Tsuchiya et al., 2002), but it is not known whether such amplifications correlate with Has mRNA levels or hyaluronan synthesis. In fact, it seems more likely that up-regulation of HAS expression is mediated by the frequently mutated signaling proteins such as those of the ErbB family (Normanno et al., 2006) and Ras (Shaw and Cantley, 2006) with downstream targets in the HAS promoter (Saavalainen et al., 2005).

Various cellular signaling pathways are likely to contribute to transcriptional up-regulation of HASes, since growth factor exchange between epithelial and stromal cells is common (Smola et al., 1993). It was suggested long ago that the cancer (epithelial) cells have adopted an ability to stimulate the adjacent stromal cells to constantly change the tissue structure to enable tumor growth, hyaluronan being an important component in this molecular remodeling package (Knudson et al., 1988). The idea of growth factor-induced hyaluronan accumulation in cancer is consistent with the findings that overexpression of EGF and EGFR (Normanno et al., 2006), TGF-beta (Glick, 2004), PDGF (Pietras et al., 2003), IGF-1 (Giovannucci, 2001), and FGF (Kwabi-Addo et al., 2004) is common in cancers, and that HAS2 expression is often increased by the same growth factors (Jacobson et al., 2000; Pienimäki et al., 2001; Karvinen et al., 2003b). PDGF and TGFβ are good candidates for the stimulation of hyaluronan synthesis in stromal cells (Mueller et al., 2004).

To resolve the issue of HAS involvement in hyaluronan accumulations, analyses of both HAS mRNA and protein levels in tissues would be highly desirable, using real-time RT-PCR and Western blotting. Unfortunately, good HAS antibodies have not been generally available and the level of HAS proteins is low, which has delayed progress in the field. Using immunohistochemistry, expression of all three HAS proteins was recently

reported in tissue sections of mesotheliomas (Kanomata et al., 2005), ovarian cancers (Yabushita et al., 2004), and endometrial cancers (Yabushita et al., 2005). However, due to general problems in the immunohistochemical reactions, these findings need confirmation by more specific assays like western blotting, and support from quantitative mRNA analyses of the corresponding HASes.

As discussed above, a reasonable hypothesis is that increased hyaluronan content in the malignant tumors is associated with transcriptional up-regulation of one or more of the HASes. However, at the moment relatively limited data are available on HAS mRNA levels in malignant tumors. HAS1 has been associated with unfavorable prognosis in colon cancer (Yamada et al., 2004) and myeloma (Calabro et al., 2002; Adamia et al., 2005), while our unpublished real-time PCR data indicate that there is hardly any HAS1 mRNA in serous ovarian cancers (Nykopp et al., submitted for publication), and that HAS2 and HAS3 messages are not statistically significantly elevated as compared with normal ovaries and benign ovarian tumors.

Catabolism as a Determinant of Tumor Hyaluronan Content?

The uncertainty concerning biosynthesis as the only or main determinant of hyaluronan accumulation in cancer tissue makes examination of hyaluronan catabolism warranted. Unlike synthesis, which is mostly controlled by HAS activity, the rate of hyaluronan catabolism depends on multiple reaction chains that occur sequentially and in parallel with each other.

Hyaluronan, once released from HAS, may remain bound to cell surface receptors like CD44, or get immobilized in the extracellular matrix due to its large size, enhanced by complex formation with hyaluronan binding proteoglycans like versican and aggrecan (Iozzo, 1998), or specific cross-linking systems like TSG6/IαI (Milner et al., 2006).

It is reasonable to assume that the catabolism of native, high molecular mass hyaluronan can be triggered by a few initial cleavages by hyaluronidases or oxygen free radicals (Ågren et al., 1997; Tammi et al., 2001; Stern, 2005). Hyaluronan thus released may either become endocytosed and degraded by local cells (Tammi et al., 2001; Knudson et al., 2002) or diffuse into lymph vessels which carry it for specific, high capacity uptake receptors in the lymph nodes and liver endothelial cells (Harris et al., 2004).

Except PH-20, the enzyme on the surface of spermatozoa, most hyaluronidases have their pH optima below 4, suggesting that they are only active in lysosomes (Stern and Jedrzejas, 2006). Therefore, it is currently unknown, whether the more commonly expressed HYAL-1-3 hyaluronidases can degrade hyaluronan in the extracellular pH, usually above 6. If hyaluronidases mainly work within endosomes or lysosomes, they are

unlikely to control tissue hyaluronan degradation, which becomes more dependent on the rate of endocytosis and the initial cleavages that facilitate its diffusion into lymphatics.

Increased HYAL-1 expression and hyaluronidase activity occur in prostate and bladder cancers, and form a strong, unfavorable prognostic indicator (Lokeshwar et al., 2001; 2002). On the other hand, hyaluronidase activity is low in advanced ovarian cancer, as compared with normal ovary (Hiltunen et al., 2002), suggesting that in different tumors, hyaluronidase may have opposite effects. Moreover, a recent study suggests that both elevated and lowered levels of HYAL-1 enhance malignant growth in a single cancer cell type (Lokeshwar et al., 2005). In general, the role of hyaluronidases in cancer is contradictory, since by degrading hyaluronan they would counteract the formation of environment favorable for tumor progression, while the hyaluronan oligosaccharides created by its activity stimulate angiogenesis, important for cancer growth (Rooney et al., 1995; Takahashi et al., 2005).

Changes, defects, or allelic imbalance in chromosome 3p21.3 containing HYAL-1, HYAL-2 and HYAL-3 hyaluronidases, are frequent in neoplasms, suggesting tumor suppressor function (Stern, 2005). However, allelic imbalance in this genomic region does not correlate with patient survival or hyaluronan content in ovarian cancer (Tuhkanen et al., 2005), which may reflect the inaccuracy of the LOH technique, and involvement of other genes in the region (Ito et al., 2005). Nevertheless, the expression of HYAL-1 is significantly reduced, and correlates with reduced hyaluronidase activity and increased hyaluronan concentration in ovarian cancer (Nykopp et al., submitted for publication). At this time, it is not known whether chromosomal defects or transcriptional down-regulation account for the reduced HYAL-1 gene expression. Nevertheless, the lower HYAL-1 expression, concomitantly with unchanged expression of HASes, suggests that hyaluronidase activity somehow contributes to hyaluronan accumulation in ovarian cancer (Nykopp et al. submitted for publication).

Genomic Changes in the Tumor Stroma

As described above, tumor stroma is an unusual type of connective tissue with hyaluronan accumulation of as a specific feature. While the factors responsible for the accumulation of stromal hyaluronan remain to be defined, recent analyses of the stromal cells have revealed a novel alternative to explain its distinct nature. Several chromosomal probes revealed frequent loss of heterozygosity (LOH) in the stromal cells adjacent to the malignant tumor epithelium. Some of the defective chromosomal sites were identical, some different from those in the actual cancer cells (Tuhkanen et al., 2004). Therefore, the accumulation of hyaluronan may not be just due to stimulation of the normal stroma of the organ, but reflect

the existence of a novel, transformed connective tissue which serves the needs of the tumor. Peritumoral stromal cells in breast cancer express epithelial keratins, suggesting that they are probably tumor cells that have undergone epithelial-mesenchymal transition (Petersen et al., 2003). While these myofibroblast type cells cannot form tumors on their own, they are crucial in supporting their malignant epithelial companions. The exact origin of these genetically altered stromal cells is puzzling. Have they differentiated from a common ancestral cancer cell? Do they reflect a continuous epithelial-mesenchymal transition in the malignant cells? Are both cell types derived from resident tumor stem cells? Moreover, the role of stromal cells in tumorigenesis was stressed by the recent finding that a genomic defect in the TGF receptor of stromal cells can initiate tumor in the prostate epithelial cells (Bhowmick et al., 2004). At this time, it looks highly likely that many of the most common carcinomas have a transformed stromal cell type that determines the progression of the tumor.

HOW DOES HYALURONAN CONTRIBUTE TO CANCER PROGRESSION?

Hyaluronan Support for Migration and Survival of Malignant Cells

The invasive front of a malignant tumor often shows a very strong hyaluronan signal (Knudson et al., 1989; Bertrand et al., 1992; Ponting et al., 1993), suggesting that hyaluronan is involved in the migration of the cancer cells into the adjacent tissue. Indeed, there is a large body of evidence for the stimulatory effect of hyaluronan on the locomotory activity of cells. Hyaluronan as a gel-forming, extremely hydrophilic polymer may, owing to its swelling pressure, open up extracellular spaces for cells to migrate into.

Mesenchymal type migratory properties are induced in carcinoma cells that gain active hyaluronan synthesis (Zoltan-Jones et al., 2003), perhaps through stimulation of lamellipodial extensions and Rac activity (Bakkers et al., 2004) or microvillous extensions (Kultti et al., 2006) (Fig. 14.4). Cell migration was inhibited by blocking HAS2 expression (Bakkers et al., 2004) or hyaluronan synthesis through UDP-GlcUA depletion (Kakizaki et al., 2004; Rilla et al., 2004). The stimulatory effect of hyaluronan on cell migration is at least partly mediated through its receptors on cell surface, and the activation of signaling pathways within the cells (Thorne et al., 2004). The role of hyaluronan as a pro-invasion agent is supported by the finding that hyaluronan content predicts local recurrence of breast cancer better than any of the common risk factors (Dr. Elda Tagliabue, Milan, Italy, personal communication).

In addition to the signals related to migration, hyaluronan binding to its receptors also activates protein phosphorylation cascades that prevent apoptosis, a threat to cells that have escaped their regular adhesive restraints and endogenous growth restrictions (Misra et al., 2006). This is exemplified by the ability of hyaluronan to stimulate PI-3-kinase/Akt signaling that keeps cancer cell lines alive in the presence of cytotoxic drugs (Misra et al., 2005) and in soft agar (Ghatak et al., 2002), a medium that tests the ability to survive without regular matrix support. These functions of hyaluronan may also operate *in vivo*, as suggested by the ability of hyaluronan oligomers to inhibit the growth of cancer cells inoculated subcutaneously, perhaps by a mechanism involving displacement of the endogenous high molecular mass hyaluronan from its CD44 receptor (Zeng et al., 1998).

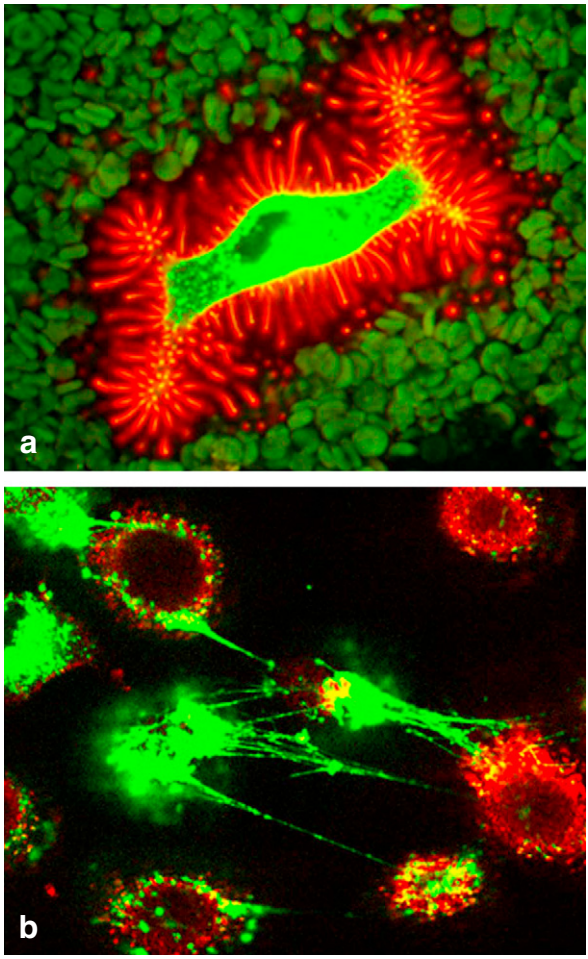

FIGURE 14.4 Hyaluronan dependent microvilli and hyaluronan "cables" on GFP-HAS3 overexpressing breast cancer cells. Cultured breast cancer cells (MCF7) overexpressing GFP-HAS3 show long microvillous extensions (green) on its dorsal cell surface (**a**). These microvilli are covered with extensive hyaluronan coats (red), which exclude the red blood cells from the vicinity of the cells (**a**). A part of the cell-associated hyaluronan extends relatively far out, and is visible as hyaluronan "cables" after methanol fixation (**b**). (See Page 8 in Color Section at the back of the book).

Hyaluronan and Inflammation

The inflammatory reaction has a dual role in malignant tumors. Cells of the immune system can attack and destroy the transformed cells, but they can also enhance tumor growth when inflammation becomes chronic (reviewed in Ben-Baruch, 2006) (Fig. 14.5). The development of cancer at sites of chronic inflammation is well-known in several organs, including skin (reviewed in Ben-Baruch, 2006; Mueller, 2006). On the other hand, a growing malignant tumor often maintains the tissue in an inflammatory state, as demonstrated by the residence of large numbers of leukocytes (Mueller and Fusenig, 2004). The inflammatory cells in tumors are often immunologically suppressed, i.e. unable to destroy the cancer cells (Ben-Baruch, 2006), but still secrete growth factors that contribute to tumor growth (Pollard, 2004).

Hyaluronan deposition in epithelium is a general feature of inflammatory reactions, for instance that associated with skin wounds (Tammi et al., 2005) and the immunological injury of celiac disease (Kemppainen et al., 2005). Recent studies on colon (de la Motte et al., 2003), kidney (Wang and Hascall, 2004), and arterial wall (Wilkinson et al., 2006) indicate that an excess of free hyaluronan, in the form of hyaluronan ''cables,'' is synthesized by the local cells during the inflammatory reaction (de la Motte et al., 2003). This hyaluronan can immobilize monocyte (macrophage) type of cells patrolling in the tissue (Fig. 14.5). Although monocytes may acutely secrete pro-inflammatory signals that may injure cancer cells, it seems that upon binding to high molecular mass hyaluronan, they are deactivated. The deactivation means that they reduce the synthesis of cytokines, start hyaluronan clearance, and increase the synthesis of growth factors and matrix molecules (Day and de la Motte, 2005). Accordingly, the down-regulation of the acute inflammatory reaction and the start of the healing process in experimental lung inflammation are dependent on CD44+ monocytic cells (Teder et al., 2002). It has also been suggested that the deactivation of monocytes and the resulting immunosuppression is mediated via binding of high molecular mass hyaluronan to CD44 on tumor cells (Mytar et al., 2003; del Fresno et al., 2005). On the other hand, long-term stimulation of Toll-like receptors by degradation products of matrix molecules like hyaluronan may cause deactivation of the immune system (reviewed in Tsan, 2006). The binding of leukocytes to hyaluronan ''cables'' may prevent their association with the cancer cell surface, necessary for a proper immune reaction (Day and de la Motte, 2005). This kind of immunological protection had been shown already decades ago in cancer cells surrounded by large hyaluronan coats (Dick et al., 1983). Accumulation of hyaluronan is thus a feature common to inflamed and malignant tissues, and by gathering monocytes hyaluronan may help to catch the monocytes in the first place, but also to suppress their inflammatory activity and facilitate the secretion

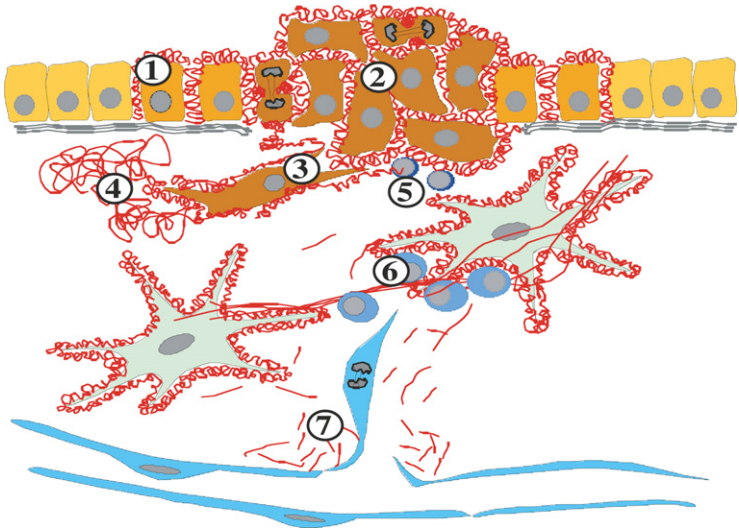

FIGURE 14.5 Localizations and forms of tumor hyaluronan (red), and suggested roles in the growth and spreading of cancers derived from simple epithelia.

1. Hyaluronan is not present in normal simple epithelia, but emerges in epithelial inflammation or injury, presumably to enhance cell proliferation and migration to cover the defect.
2. Hyaluronan is up-regulated when the epithelial cells undergo malignant transformation. It supports cell proliferation, prevents apoptosis, maintains intercellular space to facilitate nutrient diffusion, and enhances cell locomotion which stimulates invasion.
3. Hyaluronan synthesis enhances epithelial to mesenchymal transition in the cancer cell phenotype which releases the cells from their epithelial compartment for invasion.
4. Accumulation of hyaluronan in the stroma opens the fibrillar matrix for cell migration.
5. A coat of hyaluronan on cancer cells shields them from the cytotoxic effects of T-lymphocytes.
6. Free hyaluronan, arranged as "cables" in fixed cell preparations, bind tissue macrophages and modulate their activity to favor tumor growth.
7. Fragments of hyaluronan (oligosaccharides) stimulate endothelial cell proliferation and budding of new capillaries that allow tumor expansion. (See Page 9 in Color Section at the back of the book)

of growth factors and matrix molecules that support the formation of a tumor-friendly host tissue.

Recently, we found that HAS2 and HAS3 overexpressing cells form unique plasma membrane extensions, which form the skeleton of an extensive hyaluronan coat (Fig. 14.4; Kultti et al., 2006). Similar structures are also seen in untransfected cells which secrete large amounts of hyaluronan (Rilla et al., 2008) and bind monocytes (Jokela et al., 2008). It is

tempting to speculate that these HAS-induced extensions with their hyaluronan cover can also act as immunological shields either via mechanical basis or via immunomodulatory actions. A hypothetical scheme summarizing the contribution of hyaluronan to cancer spreading is presented in Fig. 14.5.

CONCLUSIONS

Analyses of clinical patient materials show that alterations in the hyaluronan content, whether on the actual malignant cells or their surrounding stroma are tightly associated with patient prognosis. Tumor progression is highly likely when hyaluronan is abundant on the surface or within tumor cells of gastric or colon carcinoma. Likewise, hyaluronan accumulation in the peritumoral stroma of breast, prostate, ovarian, and lung adenocarcinomas indicate a bleak prognosis for the patient. In contrast, reduced hyaluronan signal on the cells of squamous carcinomas correlates with cancer progression and unfavorable prognosis. There are a number of ways hyaluronan can be involved in the regulation of cancer growth and spreading, as suggested by experiments on animals, and studies *in vitro*. However, understanding the relative importance of the various aspects of hyaluronan functions and metabolism in human cancers *in vivo* is still lacking, and warrants more research on clinical materials. Nevertheless, there is reason to believe that hyaluronan will be an important therapeutic target in several human cancers.

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CHAPTER

15

The Oncofetal Paradigm Revisited: MSF and HA as Contextual Drivers of Cancer Progression

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INTRODUCTION

Cell differentiation during fetal development is driven by the sequential transcription of certain genes and the reciprocal transcriptional silencing of others. Such changes in gene expression are stably transmitted to daughter cells and ultimately result in the emergence of functionally restricted (specialized) cell lineages. Recent technological advances have fueled an explosion in our detailed understanding of the epigenetic mechanisms controlling this process. Changes in gene expression also characterize cancer pathogenesis and are similarly responsible for the emergence of distinct lineages of neoplastic cells. Advances in molecular biology during the latter half of the 20th century made it possible to document the multiple mutational and chromosomal alterations associated with cancer. The impressive accomplishments of this venture have resulted in the now widely accepted view that genetically regulated changes in the expression of oncogenes and tumor suppressor genes provide the necessary and sufficient impetus to drive cancer progression.

Cancer progression is also invariably accompanied by the re-expression of fetal isoforms of various structural and regulatory proteins not normally present in healthy adult tissues. Different models involving both genetic and epigenetic mechanisms have been proposed to account for the observed ''oncofetal'' pattern of gene expression. *Migration stimulating factor* (MSF) is an oncofetal regulatory molecule constitutively expressed by epithelial and stromal cells during fetal development. Although not expressed in the majority of healthy adult tissues, it is transiently re-expressed during wound healing and persistently re-expressed by both tumor and tumor-associated stromal cells in common human cancers. MSF is also systemically re-expressed at distant, uninvolved sites in patients with cancer. MSF exhibits a number of potent bioactivities of direct relevance to both fetal development and disease progression, including the stimulation of hyaluronan (HA) synthesis, cell motility, and angiogenesis. In this chapter we shall review MSF in terms of (i) its molecular characterization and spectrum of bioactivities, including the role of HA in mediating the motogenic response of certain target cells; (ii) the oncofetal pattern of its local and systemic expression; (iii) the tissue-level control of target cell response to MSF; and (iv) the postulated epigenetic control of MSF expression within the context of an ''extended'' oncofetal model of cancer inception and progression. We conclude with a brief discussion of the potential clinical implications of these concepts for improving the management of patients with cancer.

MSF: ITS MOLECULAR CHARACTERIZATION AND SPECTRUM OF BIOACTIVITIES

MSF was first identified in studies comparing the behavior of different cell types in a then recently developed collagen gel migration assay (Schor, 1980; Schor et al., 1985a; 1988a). As part of this work, we reported that (i) fetal fibroblasts migrated into a 3-dimensional collagen matrix to a significantly greater extent than did their normal adult counterparts; (ii) this behavioral difference resulted from the production of a soluble ''migration stimulating factor'' (MSF) by the fetal, but not adult cells; and (iii) tumor-derived and skin fibroblasts obtained from patients with various types of cancer resembled fetal cells with respect to their elevated migration and production of MSF (Durning et al., 1984; Schor et al., 1985b; 1988b; Haggie et al., 1987). Normal adult fibroblasts did not produce MSF or an inhibitor of MSF; the migration of these cells was, however, stimulated by conditioned media from fetal and cancer patient fibroblasts, thereby providing a sensitive bioassay for the subsequent purification of MSF. MSF was eventually cloned and, most unexpectedly, shown to be a genetically truncated isoform of fibronectin (Schor and Schor, 2001; Schor et al., 2003).

Fibronectin is a ubiquitously distributed macromolecule, present as an insoluble constituent of the extracellular matrix (ECM) and as a soluble component of serum (Hynes, 1990). Both forms consist of two similar, but not necessarily identical, protein chains (with individual molecular masses in the region of 260 kDa) covalently linked by disulfide bonds at their respective C-termini. Each protein chain consists of a tandem array of ''functional domains'' defined on the basis of their binding proclivities for other matrix molecules and integrin receptors on the cell surface (Fig. 15.1). Starting at the N-terminus, these include: the *Fib1/Hep1-binding domain* (exhibiting affinity for fibrin and heparin), the *Gel-binding domain* (affinity for collagen/gelatin), the *Cell-binding domain* (affinity for certain cell surface integrins, such as $\alpha 5\beta 1$), the *Hep2-binding domain* (affinity for heparin), and the C-terminal *Fib2-binding domain* (affinity for fibrin). Each of these functional domains consists of different combinations of three ''structural homology modules'' designated types I, II, and III. This modular structure is reflected by a corresponding modularity in the fibronectin gene, in which types I and II modules are each coded by a single exon, while the majority of type III modules are coded by two exons (designated ''a'' and ''b''). There are approximately 20 previously described ''full-length'' fibronectin isoforms, all having molecular masses in the region of 260 kDa. These are generated by alternative splicing of the primary fibronectin gene transcript involving the retention or deletion of two particular type III exons (EDA and EDB), as well as a more complex splicing repertoire within the downstream IIICS region (Hynes, 1990).

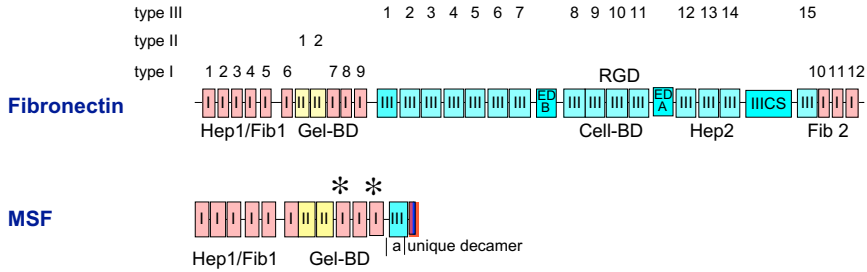

FIGURE 15.1 MSF is a truncated isoform of fibronectin. Fibronectin is a modular glycoprotein consisting of the following functional domains: Hep-1/Fib-1 (N-terminal low affinity binding to heparin and fibrin), Gel-BD (binding to gelatin/collagen), Cell-BD (RGD-mediated binding to cell surface integrins), Hep-2 (high affinity heparin binding), and Fib-2 (C-terminal fibrin binding site). Each functional domain is composed of three possible structural modules: types I, II, and III. MSF is a 70 kDa truncated isoform of fibronectin, identical to its N-terminus, up to and including the amino acid sequence coded by exon III-1a. MSF terminates in a unique intron-derived 10 amino acid sequence. Asterisks mark the location of the two IGD motifs that mediate the bioactivity of MSF on fibroblasts.

MSF message is transcribed from the fibronectin gene by an unusual "failure" of normal alternative splicing involving read-through of the intron separating exons III-1a and III-1b, and subsequent premature transcript cleavage (Schor et al., 2003). The retained intronic sequence contains a 30 bp in-frame coding sequence (immediately contiguous with exon III-1a), followed by several in-frame stop codons and a cleavage/poly-adenylation signal. The resultant MSF protein is consequently a truncated isoform of fibronectin, identical to its 70 kDa N-terminus, up to and including the amino acid sequence coded by exon III-1a, and ends in a unique, intron-derived, 10 amino acid sequence not present in any previously identified "full-length" fibronectin isoform (Fig. 15.1). As is the case with many cytokines and stress response molecules, MSF message exhibits an extremely short half-life as a consequence of an AU rich instability element in its 3′-UTR (Schor et al., 2003; Bakheet et al., 2001; Chen et al., 1994).

Zhao et al. (2001) have identified a similarly foreshortened fibronectin message in Zebrafish embryos and tissues of the adult fish which is identical to the 5′-end of Zebrafish fibronectin, up to and including exon III-3, and, like MSF, terminates in an intron-derived sequence coding for a unique peptide not present in any full-length Zebrafish fibronectin. Similar truncated isoforms of fibronectin have been detected in cDNA libraries from (i) fetal and adult human liver, prostate, ovary, and spleen; (ii) mouse liver, spleen, intestine, brain, heart, thymus, kidney, testis, stomach, lung and muscle; as well as (iii) goldfish and rainbow trout (Liu et al., 2003). Taken together, these various observations make it apparent that genetically truncated isoforms of fibronectin are widely expressed

across the phylogenetic tree. The functionality of these truncated fibronectins remains to be determined. Initial results indicate that, unlike MSF (terminating at exon III-1), the larger truncated isoforms (terminating at exon III-3) promote adhesion and appear to inhibit basal and MSF-stimulated migration (Zhao 2001; our unpublished observations).

Recombinant MSF exhibits a broad spectrum of bioactivities, including the (i) stimulation of fibroblast, epithelial, and endothelial cell migration (Fig. 15.2); (ii) up-regulation of HA synthesis by target fibroblasts (Fig. 15.2); (iii) induction of endothelial cell activation (“sprouting”) *in vitro* (Fig. 15.3); (iv) induction of angiogenesis *in vivo* (Fig. 15.4) and (v) proteolysis (Schor and Schor; 2001; Schor et al., 1989; 2003; Houard, 2005). These activities are unusually potent, commonly being manifest *in vitro* and *in vivo* at femtomolar concentrations. *In vitro* mutagenesis studies (Schor et al., 2003; Millard et al., 2007) indicate that MSF stimulation of fibroblast migration is mediated by two of its constituent IGD tripeptide motifs located in structural modules I-7 and I-9 (Fig. 15.1). The IGD motif is a highly conserved feature of fibronectin type I modules (Hynes, 1990), although no biological functionality had previously been ascribed to it. Significantly, synthetic tri- and tetra-peptides containing the IGD motif mimic all MSF bioactivities (Schor et al., 1999; Schor et al., 2003; unpublished data). Initial data indicate that cellular response to MSF/IGD requires maintenance of integrin αvβ3 functionality and is mediated, at least in part, by the PI-3 kinase signal transduction pathway (Schor et al., 1999, unpublished observations).

Full-length fibronectin isoforms do not express any of MSF’s potent bioactivities, presumably as a consequence of steric hindrance of their constitutive bioactive motifs. The functional “unmasking” of these in MSF is accordingly postulated to result from appropriate alterations in its tertiary structure (higher order folding) resulting from truncation. Analogously,

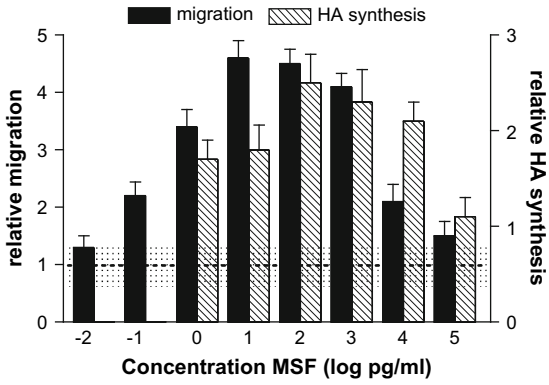

FIGURE 15.2 MSF stimulation of cell migration and HA synthesis by adult skin fibroblasts. The effect of MSF on migration and HA synthesis by confluent adult skin fibroblasts growing on a 3D native type I collagen matrix were determined as previously described (Ellis et al., 1992). Data are expressed relative to control cultures incubated in the absence of MSF.

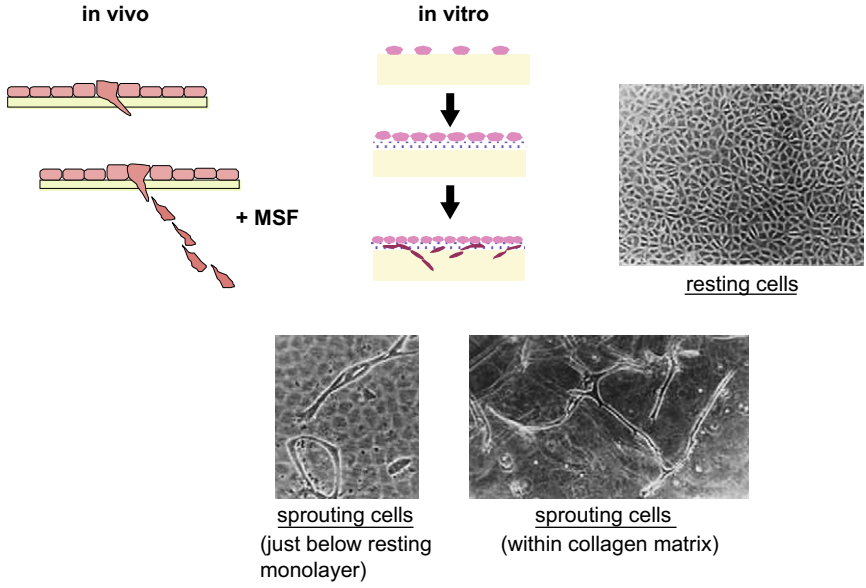

FIGURE 15.3 Induction of endothelial cell sprouting *in vitro* by MSF. The induction of new blood vessel formation by an angiogenic factor *in vivo* commences with the migration of "activated" endothelial cells from the vessel lumen into the surrounding tissue stroma where they adopt an elongated "sprouting cell" morphology. Endothelial cells *in vitro* may be induced to mimic this process. In this example, confluent endothelial cells growing on the surface of 3D collagen gel form a monolayer of resting "cobblestone" cells reminiscent of cells lining the vessel lumen. This resting monolayer remains stable in culture in the absence of exogenous angiogenic factor. Sprouting cells are induced by the addition of MSF (as well as any other angiogenic factor). In analogy with angiogenesis *in vivo*, these sprouting cells migrate down into the 3D collagen matrix where they form an interconnected network of cells, both just below the cobblestone monolayer and deeper within the collagen matrix.

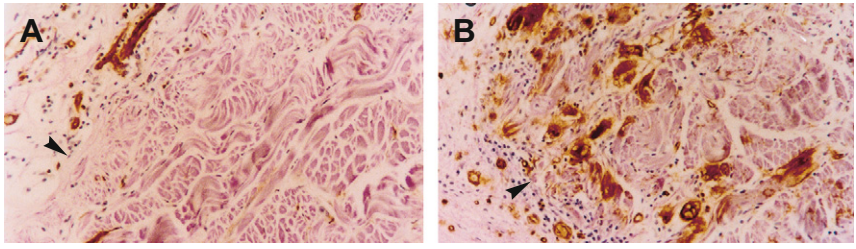

FIGURE 15.4 Induction of angiogenesis *in vivo* by MSF. Collagenous matrices were implanted subcutaneously in the rat. Tissues were excised, fixed, and paraffin-embedded 28 days later. Arrowheads mark the edge of the implants. **A**, Control implant. Blood vessels (stained brown) and fibroblasts are principally confined to the host tissue surrounding the implant. **B**, MSF impregnated implant (10 ng/ml). Significantly more blood vessels and fibroblasts have infiltrated the matrix. Indistinguishable results were obtained with IGDS-impregnated implants. (See Page 9 in Color Section at the back of the book).

proteolytically generated fragments of fibronectin display a host of ''neo-activities'' not expressed by the parental molecule (Fukai et al., 1995), including the stimulation of monocyte migration (Clark et al., 1998), the inhibition of cell proliferation (Muir and Manthorpe, 1992), the induction of protease gene expression by adherent synovial fibroblasts (Werb et al., 1989), adipocyte differentiation (Fukai et al., 1993) and an RGDS-independent mediation of cell migration (Fukai et al., 1991). The two active IGD motifs in MSF (in I-7 and I-9) reside in the gelatin-binding domain (Gel-BD), and as predicted, Gel-BD generated by proteolytic cleavage of fibronectin mimics all MSF bioactivities (Schor et al., 1996; 2003). Interestingly, the proteolytically generated cell-binding fragment of fibronectin (containing the RGD motif) inhibits the motogenic activity of MSF, Gel-BD and IGD synthetic peptide (Schor et al., 1999; unpublished observations).

Another bioactive motif has been identified in MSF. The putative zinc-binding motif (HEEGH), located in module I-8, is required for fibronectin–proteinase activity (Houard 2005). These workers further demonstrated that this motif is required to promote the migration of a breast tumor cell line (MCF-7). Mutagenesis of the two histidine residues to phenylalanine (FEEGF) abolished both the proteinase and motogenic activities of MSF. We have recently observed that the HEEGH motif functions in addition to the bioactive IGD motifs in stimulating the migration of target endothelial cells: i.e. both the HEEGH and IGD motifs in MSF must be mutated in order to abolish the motogenic response of these cells, whereas only the IGD motifs appear to be required to stimulate fibroblast migration.

Available evidence suggests that the motogenic response of target fibroblasts to MSF is mediated by its stimulation of HA synthesis: i.e. (i) exogenously provided HA stimulates the migration of adult-derived fibroblasts; and (ii) the motogenic response of adult fibroblasts to MSF is abrogated by co-incubation with *Streptococcal* hyaluronidase (Schor et al., 1989). MSF is not unique in this regard, as other, but not all, motogenic cytokines also up-regulate HA synthesis which similarly appears to mediate cytokine-stimulated migration (Schor, 1994; Ellis et al., 1992; 1997; 2007). As predicted, fetal fibroblasts produce significantly more HA and migrate to a greater extent compared to adult cells; hyaluronidase and MSF function-neutralizing antibody reduces the elevated migration of fetal cells down to that of their adult counterparts. In contrast, endothelial cells *in vitro* do not synthesize HA, either in the presence or absence of MSF (Winterbourne et al., 1983; Amanuma and Mitsui, 1991; unpublished observations); the stimulation of endothelial cell migration and adoption of a sprouting cell phenotype by MSF *in vitro* must accordingly be mediated by an HA-independent mechanism. The situation *in vivo* is clearly more complex. In view of the well-documented effect of HA and HA fragments on angiogenesis, it is likely that the pro-angiogenic activity of MSF in animal model systems may also be indirectly mediated by its effect on HA

synthesis by other cell populations (e.g. fibroblasts, carcinoma cells) within a multi-component tissue environment.

Section Summary and Significance

- MSF is a 70 kDa truncated isoform of fibronectin.
- MSF exhibits a number of potent bioactivities, including the stimulation of cell migration, angiogenesis, and the synthesis of HA. These bioactivities are, at least in part, mediated by two IGD tri-peptide motifs respectively located within the I-7 and I-9 type I structural modules.
- Another bioactive motif, HEEGH, appears to complement IGD in stimulating the migration of endothelial cells.
- MSF bioactivities are cryptic within all full-length fibronectin isoforms, probably as a consequence of steric hindrance. Fragments of fibronectin generated by proteolytic degradation also exhibit bioactivities which are held cryptic within the full-length parental molecule.
- The transcriptional control of fibronectin truncation provides a tightly controlled mechanism for regulating the spatial and temporal expression of potent bioactivities without the concomitant generation of potentially competing/confounding activities expressed by the full range of fibronectin proteolytic fragments.

THE ONCOFETAL PATTERN OF MSF EXPRESSION: CONTEXTUAL CONTROL BY ECM AND SOLUBLE FACTORS

MSF exhibits an "oncofetal" pattern of expression as defined by its (i) constitutive production by keratinocytes, fibroblasts, and endothelial cells in fetal skin; (ii) reduced or undetectable expression in skin and other tissues in healthy adults; and (iii) re-expression by both carcinoma and stromal cells in the majority of common human tumors, including those of the breast, lung, colon, prostate, and oral mucosa (Schor et al., 2003; unpublished observations). As is the case with other oncofetal regulatory molecules, including full-length fibronectin isoforms containing the EDA and/or EDB type III modules (ffrench-Constant et al., 1989), MSF is *transiently* re-expressed during acute wound healing (Fig. 15.5). This is first apparent approximately three days after wounding, when MSF is re-expressed by keratinocytes in the migrating epithelial tongue. In this experimental protocol, the epithelium was resealed by day 7, at which point MSF was exuberantly expressed by both the new epithelium and underlying granulation tissue (fibroblasts and endothelial cells). MSF expression declined to low or undetectable levels of expression by 21–28 days. MSF may play

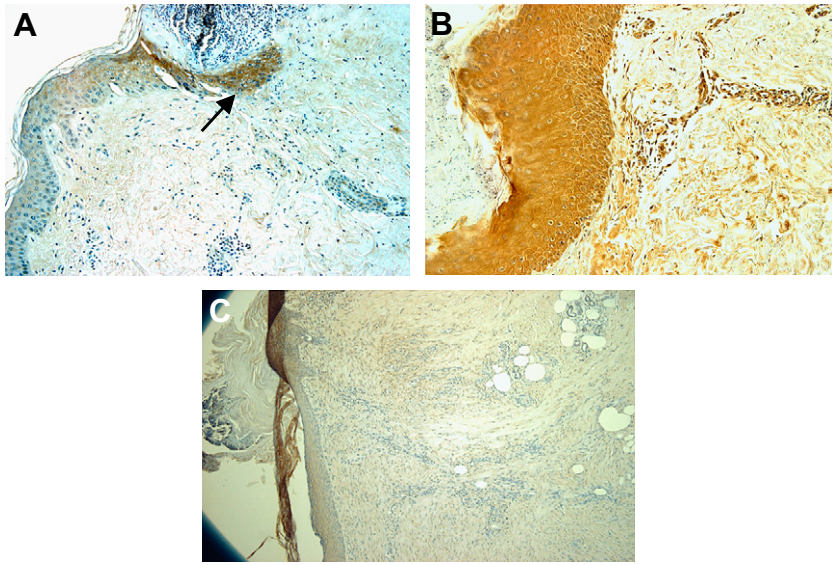

FIGURE 15.5 The transient re-expression of MSF during acute wound healing. **A**, Day 3: MSF expression is first apparent in the epithelial tongue migrating beneath the wound scab (arrow). **B**, Day 7: MSF is abundantly expressed by keratinocytes in the newly re-epithelized cell sheet covering the wound and by fibroblasts and endothelial cells in the subjacent granulation tissue. **C**, Day 21: Undetectable or low levels of MSF expression. (See Page 10 in Color Section at the back of the book).

a hitherto unrecognized role in orchestrating the spatial and temporal control of tissue repair (Schor et al., 2005), as topically applied MSF and IGD synthetic peptides to full-thickness wounds in diabetic mice significantly increase both the kinetics of wound closure and neovascularization (Fig. 15.6). Pilot studies have additionally demonstrated that MSF is re-expressed in association with other common pathologies, including scleroderma and rheumatoid arthritis (unpublished observations).

Expression of MSF protein by adult skin fibroblasts is regulated by an unusual post-transcriptional mechanism involving the initial generation of a 5.9 kb MSF pre-message by read-through of the majority of the intron separating exons III-1a and III-1b of the fibronectin gene (Kay et al., 2005). This precursor remains sequestered within the nucleus where it exhibits a short half-life. In response to an interdependent array of regulatory signals (as discussed below), the intron-derived 3′-UTR of the precursor mRNA is cleaved to produce the shorter 2.1 kb mature MSF message, which is then exported to the cytoplasm for translation (Kay et al., 2005). This post-transcriptional control mechanism, coupled with the aforementioned message instability (see section on p. 287), allows the cell to respond rapidly to microenvironmental cues, thereby controlling the temporal and spatial

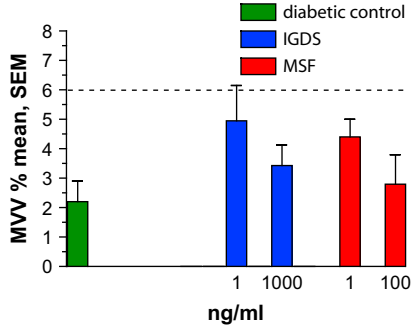

FIGURE 15.6 Stimulation of angiogenesis by MSF and IGD-peptide during wound healing in diabetic mice. A standard full-thickness wound (7.5 × 7.5 mm) was made in diabetic and non-diabetic mice and 20 microliters of PBS were applied to both control groups at days 0, 2, and 4; 20 microliters of the indicated concentrations of recombinant MSF or IGDS peptide were applied to the wounds in diabetic mice at the same time intervals. Animals were sacrificed on day 10 and microvascular volume (MVV) was assessed in histological sections as a surrogate measure of angiogenesis as described in Schor et al. (1998). MMV in non-diabetic controls is indicated by the dashed line. The lower concentrations of MSF and IGDS induced an increase in MMV (compared to the diabetic mouse control) to levels approaching that of the non-diabetic control. The study utilized eight mice per experimental group (unpublished data).

expression of MSF bioactivities. In contrast to this post-transcriptional mechanism, epithelial and endothelial cells appear to constitutively express MSF protein and regulate the manifestation of its potent bioactivities by the variable co-expression of selective inhibitors (as discussed below).

In addition to its *local* expression by tumor cells and tumor-associated stromal cells, patients with cancer may also exhibit an aberrant *systemic* expression of MSF. For example, MSF is inappropriately expressed by skin fibroblasts obtained from distant uninvolved sites in patients with cancer; these aberrant "fetal-like" cells further differ from their normal adult counterparts in terms of their migratory phenotype and elevated expression of HA (Fig. 15.7). We have also reported that the tumor-free, histologically normal, tissue margin adjacent to resected breast carcinomas commonly contain MSF-expressing intra-lobular fibroblasts (Schor et al., 1994; unpublished observations). The presence of such a functionally perturbed peritumor field may carry as yet unrecognized prognostic significance, as well as provide useful insight into the factors (systemic and local) which contribute to cancer pathogenesis (to be discussed below).

MSF bioactivity has also been detected systemically in the serum of approximately 90% of patients with breast cancer, as compared to only 10% of age- and sex-matched healthy controls (Fig. 15.8) (Picardo et al., 1991; additional unpublished data). This systemic expression of MSF does not appear to be a measure of tumor burden (as is the case with other oncofetal tumor markers), as it may persist for decades after resection of

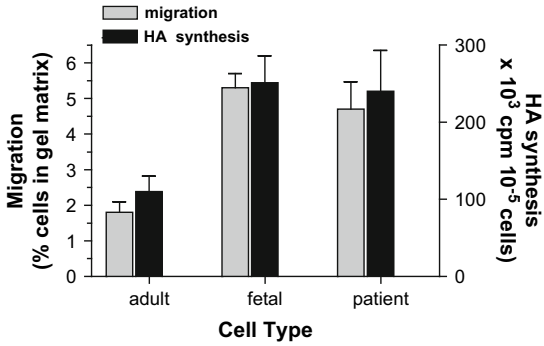

FIGURE 15.7 Fetal-like pattern of migration and HA synthesis by skin fibroblasts from patients with breast cancer. Cell migration and HA synthesis by confluent skin fibroblasts growing on 3D type I collagen gels were ascertained as described in Ellis et al. (1997) and unpublished data. Comparative data (mean ± SD) are presented for four lines each of fetal-derived, healthy adult and breast cancer patients.

the primary tumor in patients with no evidence of recurrent disease. The presence of bioactive MSF in the serum of cancer patients stands in marked contrast to results obtained in a corresponding study in patients recovering from post surgical trauma; in these individuals, MSF bioactivity was not present in serum, in spite of being clearly detected locally in wound fluid (Picardo et al., 1992).

A number of lines of evidence indicate that the post transcriptional control of MSF expression by adult skin fibroblasts may be manipulated by the concerted action of cytokine and matrix signals. For example, a brief (6–8 hour) exposure of adult skin fibroblasts growing on matrices characteristic of ''wounded'' tissue to certain cytokines, such as TGFβ1 (Kay et al., 2005), results in the switch-on of MSF expression. Significantly, these ''activated'' cells *continue to express MSF for the entire duration of their* in vitro *lifespan* when subsequently cultured under standard conditions *in the absence of inducing matrix molecule or cytokine* (Fig. 15.9). Remarkably, a second transient exposure of these activated fibroblasts growing on a matrix characteristic of healthy tissue *to the same cytokine* results in the equally persistent ''switch-off'' of MSF expression (Fig. 15.9). This inducible and persistent switching on and off of MSF expression may be repeated numerous times.

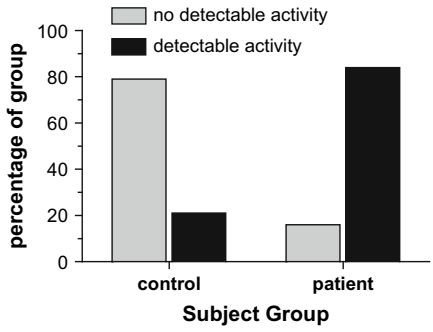

FIGURE 15.8 MSF bioactivity present in the serum of a majority of patients with breast cancer. The presence of detectable levels of MSF bioactivity in the serum of breast cancer patients ($n = 30$) and age- and sex-matched healthy controls ($n = 30$) was ascertained as described in Picardo et al. (1991). Data are presented as the percentage of positive (detectable) and negative (not detectable) samples in the two subject groups.

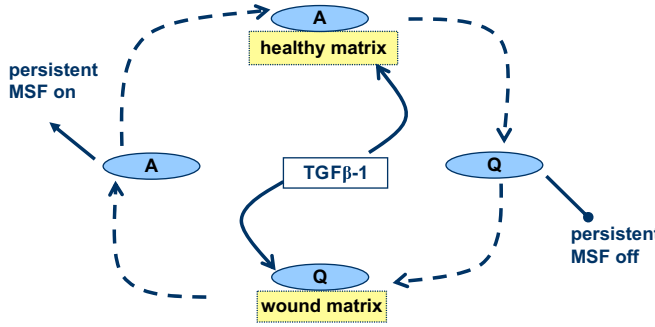

FIGURE 15.9 The persistent and reversible switch on and off of MSF expression by adult skin fibroblasts. Quiescent (Q) adult skin fibroblasts do not produce MSF. They may be induced to do so ("switched-on") by a single transient (8 h) exposure to TGF-β1 when cultured on a "wound" matrix, such as fibrin. These activated (**A**) cells continue to express MSF for the entire duration of their *in vitro* lifespan when cultured under standard tissue culture conditions. MSF expression may, however, be persistently switched-off by a subsequent transient exposure of such activated cells, this time when growing on a "healthy tissue" matrix (such as native type I collagen), to TGF-β1. This switch on and off remains completely reversible and may be repeated several times.

Our data further indicate that exposure to either matrix or cytokine alone is not sufficient to switch MSF expression either on or off, the concerted signaling of both is required. The inducible, heritable, and reversible nature of this on-off switch is most consistent with the underlying involvement of epigenetic control mechanisms (see section on p. 300).

Section Summary and Significance

- MSF is constitutively expressed during fetal development and is re-expressed in association with cancer and other common pathologies in the adult.
- Significantly, MSF may be expressed by both epithelial and stromal cells (such as fibroblasts and endothelium) within a given tissue.
- In addition to such localized expression by cells in fetal and diseased adult tissues, MSF may also be systemically re-expressed in cancer patients by epithelial and stromal cells in distant uninvolved sites, as well as a circulating component in serum.
- MSF expression by fibroblasts is regulated by a post transcriptional regulatory mechanism involving the interdependent signaling of cytokines and matrix macromolecules.
- MSF expression may be repeatedly switched on and off by the concerted action of microenvironmental cues. The inducible, persistent (heritable), and reversible nature of this on/off switch suggests that epigenetic control mechanisms may be involved.

THE CONTEXTUAL CONTROL OF TARGET CELL RESPONSE TO MSF BY ECM AND SOLUBLE FACTORS

The migratory response of target cells to putative motogenic factors is most commonly studied *in vitro* with the transmembrane (Boyden chamber) assay. This experimental protocol may be used to distinguish between *chemokinesis* (i.e. stimulation of non-directionally biased cell motility in response to an isotropic field of soluble factor) and *chemotaxis* (the stimulation of directional motility in response to a concentration gradient of soluble factor) (Zigmond and Hirsch, 1973). The polycarbonate membrane used in the transmembrane assay does not support cell attachment and must consequently be coated with an adhesive compound, gelatin (*denatured* type I collagen) being a popular choice. Such adhesion-promoting coatings have generally been assumed, either explicitly or tacitly, to provide a "neutral" substratum which does not influence target cell response to the evaluated motogenic factor.

In order to study cell behavior in a more "tissue-like" environment *in vitro*, we developed means for obtaining quantitative data regarding cell migration and proliferation on and within 3-dimensional gels of *native* type I collagen fibers (Schor, 1980). The production of MSF by fetal and cancer patient fibroblasts was first demonstrated using this assay. The critical role played by the substratum in determining cell migratory response to MSF became evident in later studies utilizing the transmembrane assay. In this assay, adult fibroblasts adherent to membranes coated with *native* type I collagen (as used in the collagen gel assay) responded to MSF, whereas the same target cells attached to *denatured* collagen (as typically used in the transmembrane assay) were completely unresponsive (Fig. 15.10). Matrices may accordingly be classified as either "permissive" (as is the case with native type I collagen) or "non-permissive" (as with denatured collagen) with respect to the manifestation of MSF motogenicity. This

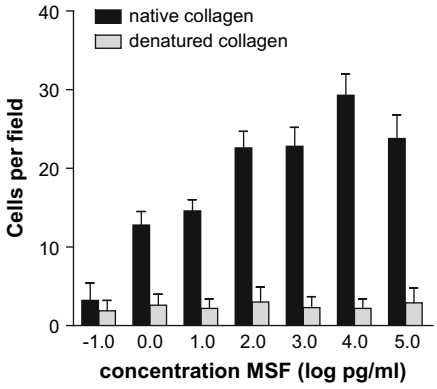

FIGURE 15.10 Matrix-dependence of MSF stimulation of fibroblast migration. Fibroblast migration was assessed in the transmembrane or Boyden chamber assay using membranes coated with either native type I collagen or denatured type I collagen (i.e. gelatin) as described in Schor et al. (1999). Fibroblasts adherent to a native collagen substratum exhibited a motogenic response to MSF (as well as IGD peptide), whereas those adherent to denatured collagen were unresponsive.

matrix-dependency was also apparent in studies of the induction of an early signal transduction response, as generically indicated by the enhancement of plasma membrane proton pump activity (Schor et al., 1999). IGD synthetic peptides exhibit an identical matrix-dependence with respect to both early signaling events and stimulation of cell migration (Schor et al., 1999). Insight into the molecular mechanisms responsible for such matrix-modulation has been provided by observations that (i) the ligation of RGD peptide by integrin $\alpha5\beta1$ inhibits the motogenic response of adult fibroblasts to MSF/IGD; and (ii) the α-chains of type I collagen contain cryptic RGD motifs which are rendered accessible by denaturation (Schor et al., 1999).

The permissive or non-permissive nature of the substratum is not invariant, but may vary with respect to the particular target cell population. For example, although denatured type I collagen is non-permissive for fibroblast response to MSF, it is permissive for endothelial cells (unpublished observations). Similarly, small amounts of added HA or thrombospondin-1 render a native type I collagen matrix non-permissive for endothelial cells, but do not alter the permissive nature of this substratum for target fibroblasts.

Cellular response to other motogenic factors is also modulated by the precise nature of the ECM (Schor, 1994). In this regard, we have recently reported that although the migration of target fibroblasts into a 3-dimensional native collagen matrix is equally stimulated by both EGF and TGF-α in serum-containing medium, this substratum is only permissive for the expression of EGF motogenic activity under serum-free conditions (Ellis et al., 2007). This finding is particularly unexpected, as the cellular response to these two structurally homologous cytokines is contingent upon their respective binding to the same cell surface EGF receptor (EGFR1). Interestingly, the addition of vitronectin (a principal ligand of integrin $\alpha v\beta3$) to serum-free medium is sufficient to restore cell motogenic response to TGF-α. The differential requirement for $\alpha v\beta3$ in the manifestation of TGF-α motogenicity was further suggested by the observed ability of function-neutralizing $\alpha v\beta3$ antibody to abrogate of cellular response to TGF-α, but not EGF. In a similar manner, HA appears to mediate the motogenic activity of EGF, by not TGF-α.

Cellular response to MSF is also modulated by soluble factors. For example, resting endothelial cells and keratinocytes constitutively express MSF which is rendered functionally inactive by the co-expression of an MSF inhibitor. In the case of human keratinocytes (or HaCaT cells, a keratinocyte cell line), this inhibitor has been identified as *neutrophil gelatinase-associated lipocalin*, or NGAL (Jones et al., 2007). The neoplastic transformation of HaCaT cells, either spontaneously or as a consequence of oncogene transfection, results in the unmasking of MSF activity, apparently as a result of a shift in the relative activities of MSF and

NGAL. MSF expressed by resting (“cobblestone”) endothelial cells is similarly rendered functionally inactive by co-expression of an as yet unidentified inhibitor. Activation of these cells by angiogenic factors (i.e. as manifest *in vitro* by the adoption of a “sprouting” cell phenotype) is similarly accompanied by the unmasking of MSF functionality.

The complex contextual control of MSF expression and target cell response to it by the concerted action of matrix and soluble factors is summarized in Fig. 15.11.

Section Summary and Significance

- Cellular response to MSF is matrix-dependent: certain matrices are permissive for manifestation of a motogenic response, whereas other matrices are not.
- Cellular response to MSF is also modulated by the presence of soluble factors.
- As a consequence of these various modulatory influences we conclude that the particular bioactivities manifest by MSF *in vivo* are not invariant and are likely to change during the temporal course of disease progression, especially in pathologies characterized by extensive matrix remodeling. A corollary of this dynamic control of MSF functionality is that the presence of MSF in tissues

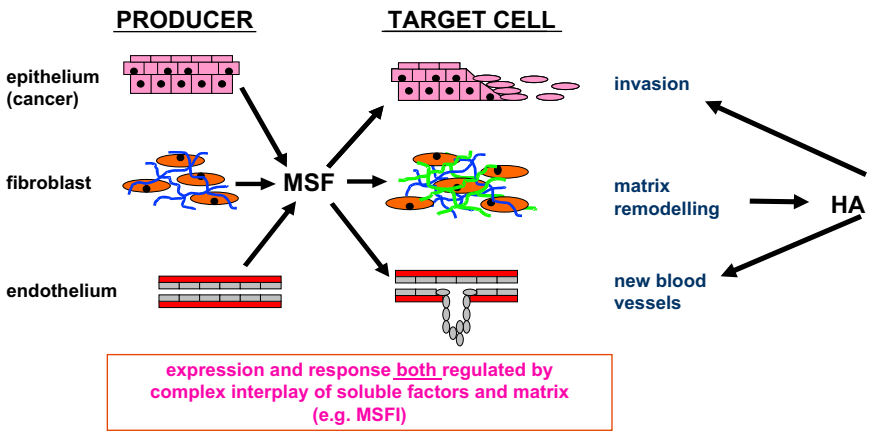

FIGURE 15.11 Contextual modulation of MSF expression and bioactivity. MSF expression by epithelial cells, fibroblasts, and endothelial cells is modulated by a complex and interdependent network of regulatory cues, including soluble factors and matrix constituents. The response of target cells to MSF is similarly controlled by soluble factors and matrix macromolecules. MSF-induced up-regulation of HA synthesis by target fibroblasts may influence the behavior of target epithelial and endothelial cells. This hierarchical regulatory circuitry may contribute to the temporal and spatial control of developmental and pathological processes, such as wound healing and cancer progression.

(as demonstrated by immunohistochemical localization) does not in itself indicate which, if any, of its multiple bioactivities are being expressed nor which of its potential target cells are responding to it.

AN EXTENDED ONCOFETAL PARADIGM OF CANCER PATHOGENESIS: THE INTER DEPENDENT CONTRIBUTION OF GENETIC AND EPIGENETIC MECHANISMS

The term ''oncofetal'' is used to denote those protein isoforms which are constitutively expressed during fetal development, are generally not expressed in healthy adult tissues, but are re-expressed in association with cancer and other pathological processes, such as wound healing and chronic inflammation. Available data suggest that oncofetal isoforms contribute to the control of relevant tissue-level events, including cell migration and angiogenesis, which are shared features of both fetal development and disease progression. Interestingly, disease-associated stromal cells also display a number of characteristic morphological features variously referred to by pathologists as ''fetal-like,'' ''activated,'' or ''plump'' when explicitly distinguishing them from their ''resting'' or ''quiescent'' healthy counterparts (McNeal, 1984).

Morphological and biochemical similarities between fetal and cancerous tissues have lent credence to successive incarnations of a ''perturbed development'' model of cancer pathogenesis, according to which, disease inception and progression result from a reversal of normal differentiation pathways. In analogy to fetal development, epigenetic control mechanisms have been suggested to drive this de-differentiation process. Such hypotheses have, however, been effectively overshadowed by an alternative genetic model postulating that cancer results from the activation of oncogenes and/or the inactivation of tumor suppressor genes by point mutations, as well as larger scale alterations to DNA sequence (Ilyas et al., 1999). Recognition that cancer pathogenesis is a multi step process occurred contemporaneously with the ascendance of the genetic model of carcinogenesis. Merging of these two precepts resulted in the now widely held view that carcinogenesis commences with the inception of a genetic lesion affecting the expression of a cancer-critical gene. This ''initiating'' event is further posited to confer a relative growth advantage to the progeny of the affected cell. Subsequent disease ''progression'' results from the random accumulation of complementary genetic lesions within the initiated clonal population and the selection of derivative sub-clones displaying ever increasing growth advantage. Successive iterations of this clonal selection process ultimately results in the emergence of

progressively dysfunctional populations of pre-neoplastic and overtly neoplastic cells, culminating in the appearance of a clinically identifiable malignancy. A corollary of this genetic model is that cancer inception and progression are ''deterministic'' processes inexorably driven by essentially irreversible genomic lesions.

Cancer-relevant genetic lesions have also been detected in epithelial cells within histologically normal peritumor tissue. According to the ''field cancerization'' hypothesis, such lesions are postulated to have arisen from prior exposure to environmental genotoxins, such as tobacco smoke in the case of oral cancer (Nees et al., 1993). The formation of subsequent independent tumors from these initiated cells is speculated to account for the elevated propensity of affected individuals to develop multiple, non-synchronous primary cancers following the successful (''free margins'') resection of the presenting lesion.

Related studies have definitively demonstrated the local and systemic presence of host stromal cells displaying both biochemical and behavioral features (such as anchorage-independent growth and formation of multi-layered regions) routinely used to define neoplastic transformation *in vitro* (reviewed in Schor and Schor, 1997; Schor et al., 1987; see also Azzarone et al., 1984; Kopelovich, 1988). These aberrant cells were non-tumorigenic *in vivo* and therefore have commonly been referred to as ''partially-transformed.'' Several genetic mechanisms have been invoked to account for the systemic disposition of these cells, including whole body exposure to environmental genotoxins (in analogy to local field cancerization) and the inheritance of germ-line mutations (as in patients with familial cancers). Seen within the confines of the then prevailing genetic model of cancer pathogenesis, these aberrant host cells were generally not considered to make a direct contribution to cancer development. Instead, their presence was interpreted as telltale indicators of an inherited or incurred genetic lesion which *only* leads to tumor formation when also present within a suitable (usually epithelial) progenitor cell population.

It is important to note, however, that the same phenotypic characteristics used to define ''partially-transformed'' host cells are also well recognized attributes of fetal cells. We have consequently elected to employ the descriptor ''fetal-like'' when referring to such aberrant host cells in patients with cancer (Schor and Schor, 1987, 2001; Schor et al., 1987). This is not a mere semantic distinction, as explicit use of the term ''fetal'' invites speculation that an inappropriate deployment of epigenetic mechanisms may also contribute to the genesis of these cells. This possibility is strongly supported by recent reports documenting the presence of aberrantly methylated CpG islands in the promoter regions of cancer-critical genes in histologically unremarkable peritumor tissue (Eads et al., 2000; Shen et al., 2005). Observations regarding the contextual control of MSF expression are also consistent with the involvement of epigenetic

mechanisms. For example, our data indicate that a transient exposure of normal adult fibroblasts to an appropriate combination of cytokine and matrix results in a switch-on of MSF expression. Although this change is heritable (persisting for the entire subsequent *in vitro* lifespan of the treated cells), it may be reversed at any time by a second transient exposure to a different combination of cytokine and matrix. Recent pilot studies further indicate that constitutive MSF expression by both *bona fide* fetal fibroblasts and the fetal-like fibroblasts of cancer patients may similarly be persistently switched-off by a transient exposure to an appropriate combination of cytokine and matrix (unpublished data). Finally, related observations indicate that a transient exposure of normal adult fibroblasts to either fluorodeoxyuridine (FUDR) or 5-azacytidine, two agents which induce changes in gene expression by epigenetic mechanisms, also result in a persistent switch-on of MSF expression which, again, may be switched off by a subsequent exposure to combinations of cytokine and matrix. Taken together, these observations suggest that aberrantly behaving host cells in cancer patients may have arisen by a prior exposure of the individual to agents which, like 5-azacytidine and FUDR, induce persistent (although reversible) epigenetic alterations in gene expression. The key question is whether these ''fetal-like'' host cells, which may precede the initiation and subsequent progression of an overt malignant lesion, actually contribute to the disease process. In this context, initial observations indicate a statistically significant inverse association between MSF expression by carcinoma cells and the survival of patients with breast cancer (unpublished observations).

Explicit use of the term ''fetal-like'' also engenders a broader ''contextual'' framework in which to consider the disease process. In this regard, it has long been recognized that cancer progression can be an extremely indolent process, with many decades elapsing between documented or inferred exposure to an environmental genotoxin and the emergence of a clinically overt malignancy. Indeed, a significant (age-related) proportion of apparently healthy individuals have been shown to harbor pre-malignant and/or early stage malignant lesions prior to their death by non-cancer related causes (Nielsen et al., 1987). We have previously speculated that the inappropriate production of MSF, as well as other oncofetal regulatory molecules, by fetal-like stromal cells may significantly *accelerate* the kinetics of cancer progression by creating a more nurturing (permissive) microenvironment for the clonal expansion of genetically initiated tumor progenitor cells (Schor and Schor, 1997; 2001; Schor et al., 1987). The effects of MSF on cell migration, angiogenesis, and HA synthesis would all be expected to function in this capacity.

In the light of these considerations, we propose an ''extended'' oncofetal paradigm of cancer pathogenesis according to which (i) both genetic and epigenetic mechanisms contribute to the creation of a perturbed tissue field

containing tumor progenitor and/or aberrant host stromal cells; and (ii) the progression of initiated tumor progenitor cells is driven by both genetic (deterministic) and epigenetic (contextual) mechanisms. In agreement with a previously published observation: "*there is nothing new under the sun*" (Ecclesiastes 1:9), this "extended" oncofetal model is essentially a re-statement of the traditional "seed-soil" hypothesis in the contemporary context of clonal selection and epigenetic/genetic mechanisms. Nonetheless, we hope that explicit recognition of the multi factorial (local and systemic, genetic and epigenetic) factors contributing to cancer progression may encourage a more holistic view of patient management strategies. For example, if future studies confirm that MSF and other oncofetal regulatory molecules produced by "fetal-like" host cells do indeed accelerate tumor progression, the potentially reversible nature of their expression may make it possible to develop novel adjuvant therapies designed to switch them off. Such therapies may make it possible to (i) retard the kinetics of disease progression in patients with cancer; (ii) reduce the likelihood of a local recurrence in patients with a resected primary tumor embedded within a histologically normal, but functionally perturbed, field containing fetal-like host cells; and (iii) reduce the risk of cancer development in apparently healthy individuals (approximately 10% of the adult population) who exhibit an aberrant systemic expression of MSF.

Section Summary and Significance

- According to the prevailing clonal selection model, cancer progression is a multi step process driven by the successive emergence of increasingly aberrant clonal populations harboring accumulated genetic lesions. Aberrantly behaving "partially transformed" stromal cells present in patients with cancer have not generally been regarded to make a direct contribution to the disease process.
- We have proposed an "extended oncofetal" model which explicitly acknowledges the additional contribution of epigenetic and contextual control mechanisms to cancer pathogenesis.

ACKNOWLEDGMENTS

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CHAPTER

16

Hyaluronan Synthesis and Turnover in Prostate Cancer

Melanie A. Simpson

OUTLINE

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OVERVIEW OF PROSTATE CANCER PROGRESSION

Despite the prevalence of prostate cancer, prostate carcinogenesis is not fully understood, either from the standpoint of physical causes or initial molecular progression. Several genetic susceptibility loci have been identified that may account for higher risk through reduced ability to cope with environmental insults. Insults may be in the form of chronic or acute inflammation, bacterial infection, or exposure to carcinogens. Inflammation leads to tissue damage, matrix remodeling, fibrosis, hypoxia, selection for cell survival in extreme conditions, and triggering of normal angiogenic mechanisms. The infiltration of immune cells produces reactive oxygen species (ROS) that damage DNA while cells are being demanded to

repopulate the damaged areas of tissue, promoting mutagenesis and ultimately neoplasia.

In the prostate, the luminal epithelium is the site of tumorigenesis and normal proliferation is androgen dependent. Tumors detected early are often managed effectively by surgical resection and/or hormone ablation therapy. However, a significant percentage of tumors will resume growth in the absence of androgens (Pienta and Smith, 2005). The transformation from androgen dependent to androgen independent prostate cancer is incompletely understood, and such tumors are typically highly aggressive. Invasive spread, in which tumor cells are no longer confined to the prostate, may include surrounding smooth muscle tissue, seminal vesicles, or rectum. Metastasis may occur in either androgen dependent or androgen independent prostate cancer and most frequently involves lymph nodes and/or bone. Complications of bone metastatic cancer are the cause of $\approx$80% of prostate cancer mortality (Jemal et al., 2006).

HA SYNTHASES AND HYALURONIDASES

In humans, HA synthases HAS-1 (Shyjan et al., 1996), HAS-2 (Watanabe and Yamaguchi, 1996) and HAS-3 (Spicer and McDonald, 1998) catalyze the synthesis of HA polymers from $\approx$100–5000 kDa (Weigel et al., 1997). High levels of HA production are maintained by availability of UDP-esterified glucuronate, which is provided by UDP-glucose dehydrogenase (UGDH). The level of UGDH is normally high in the adult prostate relative to other tissues (with the exception of liver), though HAS expression is extremely low, and its main role may be in solubilization of androgens for excretion (Lapointe and Labrie, 1999; Spicer et al., 1998).

HA turnover depends on the activity of the hyaluronidase enzymes HYAL-1-HYAL-4 and PH-20, each of which catalyzes endolytic cleavage of HA polymers (Csoka et al., 2001; Stern, 2005). Sizes of the processed fragments range from tetrasaccharides to $\approx$20 kDa polymers, depending upon isozymes and microenvironment. HYAL-1 is found in serum and in extracellular spaces, while HYAL-2 and PH-20 are GPI anchored at the extracellular surface. Both HYAL-1 and HYAL-2 also occur in lysosomes, where they catalyze complete hydrolysis of endocytosed HA. Removal of HA from the cell surface is additionally promoted by surface receptors such as CD44, LYVE-1, and RHAMM. These receptors determine the responses of the cell to extracellular HA in a complex fashion that depends in part on their individual regulatory status and partly on the size of the HA fragment.

HA polymers may be fragmented in the extracellular space by inflammation-induced ROS (Al-Assaf et al., 2006; Jiang et al., 2007), such as hydroxyl radicals, through locally acidified microenvironment activation

of extracellular matrix (ECM) hyaluronidase, through release from lysosomes during necrotic cell death, or through mechanical disruption of ECM integrity (reviewed in Stern et al., 2006). Fragments may induce cell proliferation, motility, or apoptosis, while polymers are either inert or suppress these effects. In general, HA is a critical stimulus regulating cellular behavior during wound healing, inflammation, and development (Camenisch et al., 2000; Delpech et al., 1997; Evanko et al., 1999; Fujimoto et al., 1989; Gakunga et al., 1997; Jiang et al., 2007; Laurent et al., 1996; Toole, 1997). The following sections discuss the clinical correlation and functional roles of HA synthases, hyaluronidases, and HA receptors in specific aspects of prostate cancer.

GENETIC SUSCEPTIBILITY TO PROSTATE CANCER

Relatively few genetic determinants of prostate cancer have been identified. Recently, chromosome locus 8q24 was found to be overrepresented in human prostate cancer and prostate tumor cell lines, as a result of its duplication and translocation to another chromosome (Tsuchiya et al., 2002). This locus contains, among others, the gene encoding the essential isozyme of the HA biosynthetic enzyme family, HAS-2 (Camenisch et al., 2000). Extra gene copies of the biosynthetic enzyme may account for elevation of HA with the progression of prostate cancer in patients with this genetic lesion. Interestingly, single nucleotide polymorphisms in untranslated DNA at chromosomal locus 8q24 were also newly confirmed by three independent groups simultaneously as a genetic correlate of up to 40% of prostate cancers, in particular accounting for early appearance of the disease in certain European lineages and African-American men (Gudmundsson et al., 2007; Haiman et al., 2007; Yeager et al., 2007).

INFLAMMATION RESPONSE AND HYPOXIA

Several recent reviews address the well documented role of inflammation in prostate carcinogenesis (De Marzo et al., 2007; Goldstraw et al., 2007; Wagenlehner et al., 2007). The prostate may become inflamed as a result of physical insults such as urine reflux, chemical components of the diet such as heterocyclic amines, or bacterial infections (Fig. 16.1). The net result is pro-inflammatory cytokine production, which differentially elevates HAS-2 and/or HAS-3 mediated HA synthesis (Mohamadzadeh et al., 1998). HA recruits macrophages, which infiltrate the damaged site and release copious amounts of ROS to induce epithelial cell death and degrade the HA polymers to oligomers (Jiang et al., 2007). HA oligomers

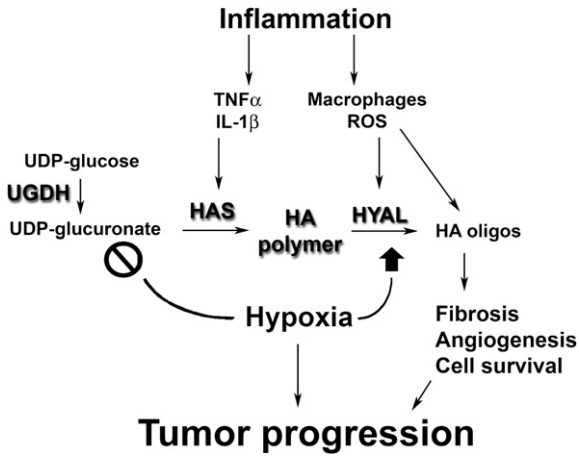

FIGURE 16.1 Flow diagram of inflammation-related prostate carcinogenesis and progression.

promote collagen synthesis, angiogenesis, and epithelial cell proliferation to repair the injured tissue (Mast et al., 1992; Toole, 2004). In a glandular tissue such as the prostate, experiencing chronic inflammation, the new matrix is disorganized and fibrotic (Wernicke et al., 2003). Fibrosis contributes to extreme hypoxia, a hallmark of several cancers including prostate (Movsas et al., 1999), which suppresses proliferation and activates autophagic processes to promote cellular survival (Degenhardt et al., 2006; Jin and White, 2007). These lesions continue to be infiltrated by inflammatory cells and adjacent epithelium may become hyperproliferative as the tissue attempts to regenerate. These lesions are termed Proliferative Inflammatory Atrophy (PIA), and are thought to precede high-grade prostatic intraepithelial neoplasia (HGPIN) and prostate adenocarcinoma.

Areas of PIA are damaged tissues high in mutagenic ROS. The elevated HA polymers in response to the initial damage have a wound healing function, in that HA deposition is observed to preserve cell-free space for organized replacement of basement membrane and polarized epithelium. In the presence of chronically elevated ROS, however, HA degradation to oligomers pathologically causes excess stromal collagen synthesis, reducing oxygen and nutrient permeability, despite concurrently increased cell proliferation. The resulting hypoxia may potentiate rapid aggressive progression of the cancer (Harris, 2002). Hypoxia-induced genes such as HIF-1α and TGF-β1 are then expressed (Liu and Simon, 2004), activating further collagen deposition and initiating angiogenesis cascades to augment blood supply. Hypoxia creates an anti-proliferative microenvironment for the epithelial tumor cells, which then cease efficient aerobic metabolism in favor of lactate production and elevated glycolytic flux (Harris, 2002; Liu and Simon, 2004). Chronically increased glycolysis

provides a metabolic advantage by facilitating survival in hypoxic conditions.

Hypoxic regions have been identified with microelectrodes and molecular markers in prostate tumors (Movsas et al., 1999; Wouters et al., 2002). In development and normal wound healing, hypoxia induces angiogenesis, in part because ROS-generated HA fragmentation occurs and because secretion of angiogenic factors such as VEGF is signaled. However, HA polymers are anti-angiogenic and accumulation of HA in hypoxic tumor regions may thereby exacerbate hypoxia and prolong tumor cell survival by potentiating slow proliferation. Tumor cells in a hypoxic microenvironment are highly resistant to chemo- and radiotherapy treatment efforts (Harris, 2002; Wouters et al., 2002) because of their reduced proliferation and autophagic nutrient conservation. Presence of elevated ROS nonetheless leads to mutations in tumor cell DNA of cells residing in these extreme conditions. Ultimately, as the matrix is slowly turned over by activation of proteases and hyaluronidase, the former of which is activated by HIF-1α and the latter by acidification of the local microenvironment, an aggressive prostate tumor may be the outcome.

TUMORIGENESIS

Synthesis and deposition of HA is normally associated with cell division, motility, transformation, and vascular development in embryogenesis (reviewed in Fraser et al., 1997; Toole, 2004). These normal functions continue into adulthood for maintenance of cell and tissue turnover. Additional utility of HA as an adhesion substrate is important for recruitment of circulating cells during wound healing and immune system function. HA-mediated functions are exploited by transformed cells, so the enzymes and receptors which synthesize, process, and signal cellular responses to HA are valuable predictive tools for cancer aggressiveness, and their complex interplay is an active area of research.

Many studies have demonstrated the strong correlation between HA accumulation and malignancy in solid tumors. Prostate histopathology first indicated increased HA content of the stroma in benign prostatic hyperplasia (BPH) relative to normal tissue (De Klerk, 1983), and found the magnitude of the increase directly mirrored level of dedifferentiation within the tumor (De Klerk et al., 1984). Subsequent studies investigated the diagnostic and prognostic potential of HA detection in tumors. In general, HA is detected in tumor stroma if Gleason score is $\geq$5 and does not indicate severity. Tumor cell-associated HA is detected at high Gleason scores and in metastases, each of which correlates with poor prognosis (Aaltomaa et al., 2002; Lipponen et al., 2001; Lokeshwar et al., 2001). In retrospective studies with minimum 5-year follow-up, high HA levels in

radical prostatectomy specimens did not independently predict biochemical recurrence (PSA elevation after tumor resection indicating possible local or distant metastasis) (Ekici et al., 2004; Posey et al., 2003). However, combined staining of HA and HYAL-1 is 87% accurate for predicting disease progression (Ekici et al., 2004). It is noteworthy that in early stage prostate cancer specimens, HYAL-1 is exclusively expressed by tumor cells, and HA is primarily in the tumor-associated stroma (Posey et al., 2003), but its cellular origin becomes uncertain as compartmental boundaries erode in progression.

HA production in cultured tumor cells is significantly stimulated by growth factors and proinflammatory cytokines known to be elevated in human prostate cancer specimens. Notably, IGF-1, bFGF (Kuroda et al., 2001), PDGF (Pullen et al., 2001), IL-1β and TNFα (Mohamadzadeh et al., 1998), have all been shown to trigger HA synthesis. PDGF in particular was shown to stimulate HAS-2 expression and HA production in prostate stromal fibroblasts during the transition from normal to BPH. These studies suggest an important link between clinically relevant tumor proliferation signals and inappropriate HA biosynthesis. Targeted disruption of HAS-2 in mice illustrated that HA can signal epithelial to mesenchymal transition during development (Camenisch et al., 2000). This function of HA as a transformation signal has been demonstrated in cancer progression as well, in which case neoplastic transformations may be promoted by HA-mediated activation of receptor tyrosine kinases such as ErbB2 (Ghatak et al., 2005; Misra et al., 2006; Toole et al., 2005).

Despite its documented activation of growth factor receptors, large HA quantities of high molecular mass (i.e., from ≈100 kDa to ≈2 MDa) are antiproliferative (Rooney et al., 1995; West and Kumar, 1989), capable of suppressing growth of both tumor cells (Enegd et al., 2002; Simpson, 2006) and vascular endothelial cells (West and Kumar, 1989). Constitutive engagement of HA receptors by small amounts of newly synthesized HA polymers was shown to stimulate cell cycling through assembly of protein complexes including PI3-kinase, cdc37, and the cytoskeletal adaptor ezrin (Ghatak et al., 2002; Ghatak et al., 2005). HA responses are also context dependent with respect to two-dimensional versus three-dimensional culture conditions (Toole, 2002). Anchorage-independent growth of prostate tumor cells requires HA polymer ligation of cell surface receptors to stabilize cytoskeletal architecture by intracellular association with cytoskeletal proteins and adaptors (Ghatak et al., 2002; Zhu and Bourguignon, 1998). Disruption of these interactions *in vitro* and *in vivo* by administration of HA oligomers induces tumor apoptosis (Ghatak et al., 2002; Zeng et al., 1998).

Among cultured human prostate tumor cell lines, elevated HA production was found specifically in aggressive, metastatic cells, in which HAS-2 and HAS-3 isozymes were upregulated ≈3-fold and ≈30-fold, respectively (Simpson et al., 2001). Suppression of HAS-2 and/or HAS-3

expression by stable antisense RNA reduced the synthesis and cell surface retention of HA (Simpson et al., 2002a), and inhibited primary subcutaneous (Simpson et al., 2002b) or intraprostatic growth (McCarthy et al., 2005). Reduced primary tumor growth was associated with comparable apoptotic and proliferative fractions in culture and in tumors, but virtually no vascularization of tumors. These results implicate HA, and specifically HAS-2 and HAS-3, in tumor angiogenesis, as well as intrinsic growth rate modulation (Simpson et al., 2002b). Interestingly, HA exogenous addition to knock-down cells upon injection restored subcutaneous tumor growth and angiogenesis, implying the existence of a tumor or stromal factor (i.e. a hyaluronidase) that could modulate effects of HA in trans, with the same malignant outcome. HAS-2 overexpression in prostate cells impacted tumor initiation but did not significantly affect size of tumors that formed subcutaneously (Simpson, 2006). In contrast, overexpression of HAS-3 suppressed growth (Bharadwaj et al., 2007). Two alternatives explain this dichotomy: HA synthesis is dose responsive, with enhanced tumorigenesis at low synthesis amounts and inhibited tumorigenesis at high amounts (Itano et al., 2004); and hyaluronidase co-expression with HA synthases is required for maximum tumorigenesis.

Overexpression of HYAL-1 in prostate tumor cells enhanced rate of proliferation (Simpson, 2006) and promoted cell cycle progression (Lin and Stern, 2001). However, tumorigenic potential either subcutaneously (Simpson, 2006) or in the prostate (Kovar et al., 2006) was not altered. Inhibition of HYAL-1 expression showed significant impairment of subcutaneous tumorigenesis and reduced proliferation through cell cycle arrest (Lokeshwar et al., 2005), consistent with a role for HYAL-1 as a tumor promoter. HA production by the tumor stroma correlates with HYAL-1 levels in tumor cells, suggesting crosstalk between the tumor and the tumor-associated stroma (Lokeshwar et al., 2005). Relative to cells expressing either HAS-2 or HYAL-1 alone, which showed modest but not significant increases in tumorigenesis, cells co-expressing HAS-2 and HYAL-1 gave rise to tumors of several-fold increased size (Simpson, 2006). Synergy between HA and its processing enzymes supports an active role for crosstalk between the HA metabolic pathways in tumor progression (Fig. 16.2).

In addition to its effects on transformation and proliferation of prostate cells, HA polymers diminish ECM adhesion and enhance motility of tumor cells. Excess production and retention of HA at the surface of prostate tumor cells by overexpression of HAS-3 was found to reduce adhesion to ECM proteins by downregulation of specific cell surface integrin receptors (Bharadwaj et al., 2007), thereby increasing migration potential. Time lapse videomicroscopy revealed that treatment of tumor cells with prostate fibroblast conditioned media, which caused formation of HA matrices on the tumor cells, also increased their motility (Ricciardelli et al., 2007).

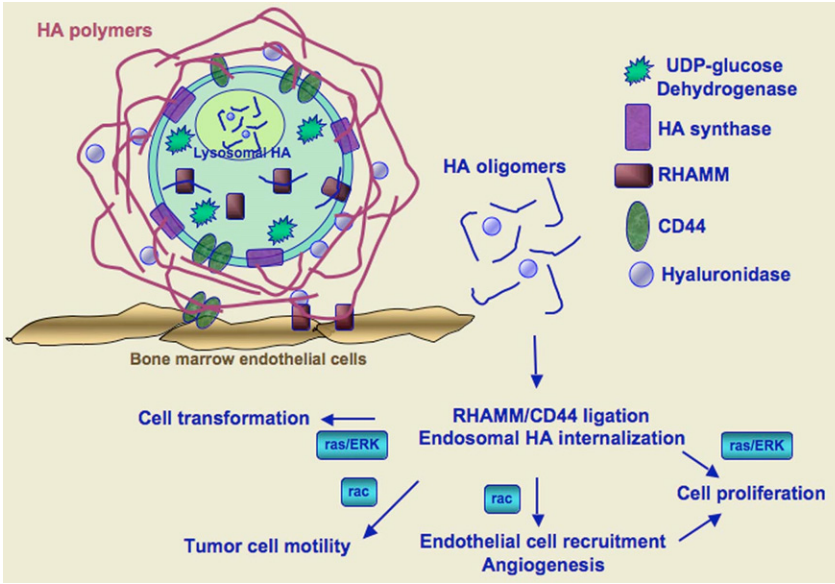

FIGURE 16.2 Model for HA involvement in tumor cell biology. The prostate stromal and tumor-associated HA matrices found in aggressively progressing tumors are maintained by a complex interplay of enzymes and receptors. Production of hairlike HA polymers occurs at the cell surface from membrane embedded HA synthases. High level HA synthesis places demands on intracellular UDP-glucose dehydrogenase to provide HA precursors in excess of the cell's energy needs. HA polymers are retained at the cell surface by transmembrane and extracellular HA binding proteins. Degradation of HA by ROS or hyaluronidases generates a decrement of lower molecular weight fragments of HA, which may be internalized through the action of specific cell surface receptors such as CD44 and RHAMM, and act in autocrine or paracrine fashion on both tumor cells and associated stromal cells. Increased cellular motility among endothelial cells within the prostate stroma in response to HA fragments may directly regulate angiogenesis. Response of prostate epithelial cells to HA fragments versus HA polymers determines cellular transformation, proliferation, motility, and ultimately may be required for sustained tumor growth and metastasis.

HA was most densely deposited at the trailing edge of the polarized motile cells, consistent with a potential role in de-adhesion. A related observation is that levels of the extracellular HA-binding proteoglycan, versican, rise as a function of prostate cancer severity (Ricciardelli et al., 1998) and are independently prognostic. Versican/HA complexes have been implicated in the mediation of prostate stromal cell motility. In fact, TGFβ1 secreted by tumor cells induces stromal fibroblast secretion of versican (Sakko et al., 2001). Thus, the combined accumulation of versican and HA matrix is required for prostate tumor cell remodeling of the ECM and acquisition of intrinsic motility (Ricciardelli et al., 2007).

Complexity of response to HA is increased by the involvement of HA receptors and binding proteins. In addition to versican and RHAMM, the

cell surface HA receptor CD44 has been directly linked to prostate cancer progression. CD44-mediated responses are very complex, as it has been shown to function as a tumor suppressor, but activated signaling through CD44 promotes proliferation, motility, and metastasis. It has been proposed (Herrlich et al., 2000), that the activity of CD44 as a tumor suppressor is mediated by the local equilibrium of HA polymers and oligomers, whereas its signaling activities are the result of CD44 having the alternative HA independent function of growth factor presentation to relevant surface receptors. Interestingly, prostate cancer stem cells, recently implicated in prostate cancer progression (Maitland and Collins, 2005), were isolated and characterized by their capacity for self renewal, expressing among other markers, CD44 (Collins et al., 2005). Several splice variants of CD44 have been reported in prostate carcinomas (Liu, 1994), but there are conflicting reports on its relation to carcinoma stage. For example, CD44 standard isoform is reported to decrease with increasing cancer grade (De Marzo et al., 1998; Noordzij et al., 1997) due to hypermethylation of the promoter (Kito et al., 2001), and loss of this form is an independent predictor of clinical recurrence (Aaltomaa et al., 2002; Vis et al., 2000). However, levels of CD44 variant isoforms, CD44v3 and CD44v6, have been shown to increase with Gleason sum and T-stage (Aaltomaa et al., 2002). Expression of CD44v3, but not CD44v6, independently predicts PSA biochemical recurrence. Among cultured prostate tumor cell lines, poorly aggressive androgen sensitive LNCaP cells do not express CD44, whereas metastatic androgen independent cell lines express standard and variant isoforms of CD44 (Liu, 1994; Welsh et al., 1995).

Certainly one important function of CD44 is to mediate the effects of HA polymers and oligosaccharides on cell signaling and response. In particular, CD44 is required for uptake and turnover of HA polymers (Culty et al., 1992; Harada and Takahashi, 2007). Opposite responses to polymers and oligomers are thought to result from multivalent clustering of receptors bound to polymers that transduces intracellular signals differently from the monovalent unclustered CD44 monomer. CD44 is localized to lipid rafts in which HYAL-2 is associated, possibly through simultaneous binding of both to the same HA strand, and activated by CD44-mediated acidification through a Na+/H+ exchange pump (Bourguignon et al., 2004). CD44-mediated HA polymer adhesion is at least partially responsible for stabilization of cell-ECM contacts through interaction of its C-terminal tail with cytoskeletal proteins (Legg et al., 2002; Welsh et al., 1995; Zhu and Bourguignon, 1998), disruption of which can occur by HA-oligomeric activation of merlin, an inhibitor of cytoskeletal complex formation (Morrison et al., 2001). CD44 ligation with HA polymers can induce multidrug resistance (Misra et al., 2003; 2005) and disruption of signaling by oligomeric HA induces apoptosis (Ghatak et al., 2002), inhibits anchorage-independent growth and reduces *in vivo* tumorigenesis (Zeng et al., 1998).

A final point to emphasize is that HA has been specifically implicated in the progression of prostate tumors from hormone dependence to independence following androgen ablation therapy. Rats treated long term with androgens developed neoplastic prostate tissue with elevated stromal HA (Li et al., 2001). Castration, on the other hand, reduced levels of HA in the rat prostate, and these were stimulated subsequently up to 10-fold by androgen administration (Terry and Clark, 1996). In fact, HA may directly influence the transition from androgen dependence to independence. In human tissue samples, elevation of the HA receptor, RHAMM, correlated with aggressive, hormone resistant cancer and metastasis (Lin et al., 2008). Stimulation of androgen dependent prostate tumor cells concurrently with androgen and HA increased proliferation *in vitro* and tumorigenesis *in vivo*, in an androgen receptor and RHAMM dependent fashion. In addition, siRNA knock-down of Rho kinase (ROK) could reverse HA-RHAMM malignant properties of androgen-insensitive prostate tumor cell lines (Lin et al., 2007), so antagonism of this signaling pathway may have potential therapeutic value for hormone resistant cancer patients.

ANGIOGENESIS AND LYMPHANGIOGENESIS

HA oligomers, specifically of 4–25 disaccharides, have been shown to stimulate angiogenesis (West et al., 1985), despite the antiproliferative effect of larger HA polymers on endothelial cells that suppresses angiogenesis (Rooney et al., 1995; West and Kumar, 1989). Furthermore, the antiangiogenic effect of HA polymers on endothelial cells is irreversible once engaged except in the sustained presence of antagonistic HA oligosaccharides (Deed et al., 1997). Thus, this normal function of HA has the potential to allow tumor cells to directly signal their own vascular development. Several reports have implicated HA in prostate tumor angiogenesis. For example, as mentioned above, inhibition of HA polymer synthesis suppressed growth of prostate tumors (McCarthy et al., 2005; Simpson et al., 2002b) but vascular density within the resultant tumors was also $\approx$80% diminished. Seemingly contrary to this finding, excess deposition of HA has been shown to suppress angiogenesis of prostate tumors. This suggests HA is required for angiogenesis but modification or further metabolism of the polymeric form is critical for the angiogenic response. In support of this hypothesis, angiogenic HA fragments (10–15 disaccharide units) have been detected in high-grade prostate cancer tissues (Lokeshwar et al., 1997; 2001) and the knock-down of HYAL-1 also dramatically impairs angiogenesis (Lokeshwar et al., 2005). Interestingly, HA production in relatively low quantities has a potently stimulatory effect on angiogenesis in prostate tumors (Simpson, 2006), which is consistent with

motility experiments described above that show low HA concentrations stimulate, while high levels inhibit, migration.

Tumor lymphangiogenesis, growth of new lymphatic vessels within a developing tumor, has also been implicated in progression of prostate cancer. LYVE-1, a member of the link module domain containing family of HA receptors, like CD44, is primarily expressed on lymphatic endothelial cells and responsible for HA adhesion of lymphocytes, as well as mediation of HA clearance through the lymphatic system during inflammation (Johnson et al., 2007; Prevo et al., 2001). Expression of LYVE-1 is used as a marker for lymphangiogenesis and lymphatic content of human tumors, and has been used specifically to show that lymph vessels are destroyed in the transition from benign to malignant prostate cancer, rather than created (Trojan et al., 2004). Further studies showed a correlation between VEGF-C and lymph node metastasis, suggesting lymph vessel permeability, though not lymph vessel density, may be the key factor underlying lymph node metastasis (Trojan et al., 2006). Surprisingly, LYVE-1 null mice also showed no phenotype with respect to normal lymphatic development, and formed tumors comparably to wild-type mice, suggesting potential redundancy in lymphatic endothelial receptors (Gale et al., 2007). Mouse subcutaneous and orthotopic tumorigenesis and metastasis studies have given rise to conflicting results. In one study, VEGF-C stimulated lymphangiogenesis was strongly correlated with lymph node metastasis following surgical excision of subcutaneously implanted prostate tumors (Brakenhielm et al., 2007), while another group found lymphangiogenesis was not required for lymph node dissemination of orthotopically implanted tumor cells and that lymphatic vessel counts actually decreased (Wong et al., 2005). In either case, it appears that HA binding and turnover by LYVE-1 in lymphatic vessels is not required for metastasis.

METASTASIS AND BONE TURNOVER

HA deposition is observed in the stroma of cancer patients in early stages, but its levels appear to be highest in advanced cancer and remote metastases (Lokeshwar et al., 1996; 2001). In addition, high levels of HA (Lipponen et al., 2001) and/or HYAL-1 correlated with more invasive cancer (Madan et al., 1999), and metastatic prostate cancer lesions were found to have higher HYAL-1 levels than the high-grade primary tumor (Lokeshwar et al., 1996; 2001).

Prostate tumor cells that produce endogenous large quantities of HA polymer are more metastatic to lymph nodes when injected intraprostatically in mice, and this can be increased by overexpressing HYAL-1 (Patel et al., 2002). In low HA-producing prostate tumor cells, normally not metastatic in a six-week time course, HYAL-1 overexpression induced

rapid metastatic dissemination to lymph nodes following orthotopic implantation (Kovar et al., 2006). Stable antisense inhibition of HA synthesis abrogated ≈90% of spontaneous lymph node metastasis from orthotopic injections of prostate tumor cells (McCarthy et al., 2005). HA on the tumor cell surface may be functioning in metastasis at the level of facilitated arrest/adhesion to endothelial surfaces in remote tissues and also signaling of cell survival.

Predilection of metastatic prostate tumor cells for bone has been postulated to result from a combination of anatomic and molecular factors (Cher, 2001). Bone endothelium is the first constrained microvasculature encountered by cells that intravasate from the prostate, so physical arrest may be a significant component. Receptor-ligand interactions may then be engaged, signaling transendothelial migration. Colonization requires growth factors, new blood vessels, and bone tissue remodeling. Bone metastasis in prostate cancer patients is both osteoblastic, characterized by bone growth, and osteolytic, associated with bone breakdown. Prostate tumor cells have been termed osteomimetic because in coculture with bone derived cells, they acquire an osteoblast-like gene expression profile, and this ability to alter in response to bone microenvironment may underlie their tendency to colonize bone tissue (Chung et al., 2005; Koeneman et al., 1999).

Recent studies have shown that specific factors such as RANK, a surface receptor on osteoclasts, and RANKL, its cognate ligand expressed on osteoblasts, can be upregulated in the presence of tumor cells (Corey et al., 2002). Subsequent increased presentation of RANKL by tumor cells and osteoblasts triggers differentiation of osteoclasts, the mediators of bone breakdown and resorption, thereby creating physical space to be occupied by the tumor cells. Importantly, HA signaling through CD44 has been shown to increase the expression of RANKL on the surface of bone-derived stromal cells (Cao et al., 2005), and low molecular weight HA polymers specifically stimulated osteoclast differentiation (Ariyoshi et al., 2005), suggesting tumor-borne HA may be a significant contributing factor in osteomimicry and bone remodeling (Prince, 2004).

In addition to its effects on cell differentiation, there is a strong rationale for HA-mediated adhesion in metastatic potential of tumor cells. HA is a well-recognized substrate for circulating cell adhesion, as originally determined by its ability to arrest lymphocytes in high endothelial venules, and confirmed by *in vitro* adhesion assays (Aruffo et al., 1990; Miyake et al., 1990). Retention of high molecular weight pericellular HA by prostate tumor cell lines correlates with their metastatic potential in mice and promotes adhesion specifically to bone marrow endothelial cells *in vitro* (Simpson et al., 2001). This was proposed to be one mechanism underlying the skeletal metastatic frequency of clinical prostate cancer, and other bone-homing tumors have been shown to use CD44–HA interactions to

orchestrate transmigration of the bone endothelium (Okada et al., 1999). Increased HA synthesis and pericellular retention by non-adherent prostate tumor cells rendered them adherent to bone marrow-derived endothelial cells and inhibition of HA synthesis in adherent cells by stable antisense RNA expression diminished their adhesion potential (Simpson et al., 2002a).

CD44 is the proposed receptor mediating HA-facilitated arrest on microvascular endothelial cells, since anti-CD44 antibody treatment blocks adhesion, while RHAMM is essential for migration *in vitro* and angiogenesis *in vivo* (Savani et al., 2001). The involvement of CD44 in HA-mediated adhesion of prostate and breast tumor cells to bone marrow-derived endothelial cells has specifically been demonstrated (Draffin et al., 2004), which highlights the potential capacity of CD44 to function as a bone "metastasis receptor" for HA-bearing tumor cells. However, as mentioned above, clinical studies do not consistently demonstrate CD44 association with metastasis to the bone or any other location.

In summary, HA and its metabolic enzymes and receptors clearly play a significant role in multiple aspects of the progression of prostate cancer. While HA is not intrinsically carcinogenic, its broad functions in cellular transformation, proliferation, adhesion, migration, inflammation, and angiogenesis are coordinately timed and executed in response to other genetic and epigenetic alterations that precede full-blown cancer. Thus, the sequence of responses initiated by elevated HA production are ultimately suited to potentiate tumor growth and metastatic spread. Since its signaling functions may additionally give rise to multi drug resistance, it is critical to understand the interplay of HA metabolism and cellular recognition from a therapeutic standpoint. Furthermore, the identification of aggressive prostate cancer stem cells that express CD44, in conjunction with the production of HA and its oligomers in the hostile environments that promote stem cell activation, highlight the immediate importance of defining these complex relationships.

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CHAPTER

17

Role of Hyaluronan and CD44 in Melanoma Progression

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HYALURONAN AND MELANOMA

Hyaluronan (hyaluronic acid, HA) is a polysaccharide found ubiquitously in the cutaneous extracellular matrix. It is synthesized as an unbranched polymer of repeating disaccharides of glucuronic acid and *N*-acetylglucosamin: [-β(1,4)-GlcUA-β(1,3)-GlcNAc-]$_n$ (for review see Toole, 2004)]. There is growing evidence that HA is involved in the course of melanoma: for example Dietrich and co-workers show that high expression levels of CD44 (the principal cell surface receptor for HA) correlate with poor prognosis of melanoma patients (Dietrich et al., 1997). Another study demonstrates a correlation between serum HA levels of

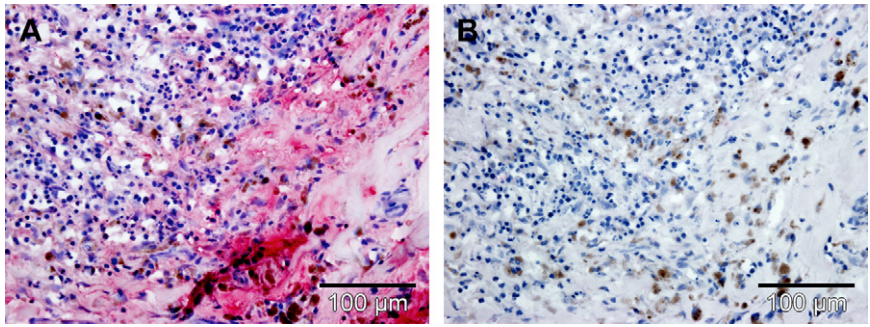

FIGURE 17.1 HA-deposition in the peritumoral stroma of melanoma metastasis. HA can be detected in high amounts in the stroma surrounding a melanoma metastasis (primary tumor: nodular malignant melanoma, Breslow thickness 4 mm) (**A**). HA was stained with the HA binding protein and visualized with amplex red, sections were counterstained with hematoxylin. As demonstrated by the strong red staining, high amounts of HA are synthesized by the melanoma cells (arrows) and the surrounding stroma tissue (arrowhead). Specificity was ensured by staining after incubation with hyaluronidase (**B**). These sections show no reactivity. (See Page 10 in Color Section at the back of the book).

melanoma patients and disease stage (Burchardt et al., 2003), showing high serum HA levels in patients with advanced stage disease. Furthermore, in histological sections of advanced melanoma, high levels of HA surrounding the tumor can be observed (Fig. 17.1). The contradiction to a report in which poor prognosis of melanoma patients was associated with reduced CD44 and HA expression in melanoma stage I patients (Karjalainen et al., 2000) is most likely due to several factors: (1) the different disease stages investigated in these studies. (2) Differences between *in vitro* and *in vivo* settings. For example, cutaneous melanomas evolve in basal epidermis, surrounded by keratinocytes with high levels of CD44 and an environment rich in HA. Thus, results on CD44 and HA metabolism in melanoma cell lines may not be comparable to the clinical behavior of human melanoma. (3) The same group demonstrate in a recent publication, that C8161 melanoma cells express different levels of CD44, depending on their depth of invasion into collagen lattices (Edward et al., 2005). This could mean that either a sub-population of CD44-positive cells in the original cell population has the ability to invade the lattices, or the process of invasion itself induces CD44 expression on the cells.

But importantly, direct CD44/HA interaction, i.e. the binding of HA to CD44, directly regulates tumor development (Bartolazzi et al., 1994), implicating a functional connection between the observed high expression levels of HA and CD44.

In addition, accumulating evidence shows the concomitant increase of HA in the course of melanoma. In mice, HA on the cell surface correlates

with the metastatic potential of MM cells (Zhang et al., 1995) and in highly metastatic melanoma cell lines, HA synthesis is increased (Goebeler et al., 1996). Furthermore, inhibition of HA synthesis (Kudo et al., 2004; Yoshihara et al., 2005) and blocking HA by different means (Mummert et al., 2003; Xu et al., 2003) reduce tumorigenicity of melanoma cells.

INTERACTION OF HYALURONAN AND MELANOMA CELLS

The Hyaluronan Receptors

The interaction between HA and cells is mediated by different cell surface receptors, among which CD44 is the most prominent one on melanoma cells (reviewed in Ponta et al., 2003), although other HA receptors, such as RHAMM (receptor for hyaluronic acid mediated motility; reviewed in Cheung et al., 1999), HARE (hyaluronan receptor for endocytosis; Zhou et al., 2000) and LYVE1 (lymphatic vessel endothelial hyaluronan 1; Banerji et al., 1999; Prevo et al., 2001) have been identified. For example RHAMM is found to be expressed on melanoma cells and its expression increases during melanoma progression (Ahrens et al., 2001a). On the other hand we find RHAMM to be expressed exclusively within the cytoplasm of melanomas, thus rendering it unlikely that RHAMM mediates interaction of melanomas with extracellular HA (Ahrens et al., 2001a). Furthermore, CD44 interacts with a wide variety of different proteins, including osteopontin, collagen, fibronectin, growth factors, cytokines, and chemokines, as well as metalloproteinases (reviewed in Ponta et al., 2003).

CD44 Influences MM Proliferation

CD44 is a transcript of a single gene containing 20 exons. Since 10 of these exons are regulated by alternative splicing, multiple isoforms of CD44 exist. In addition to alternative splicing, CD44 is subject to different degrees of posttranslational modifications, such as glycosylation, sulfation, and phosphorylation, resulting in CD44 isoforms ranging from 85 to 200 kDa (reviewed in Ponta et al., 2003). The expression of standard CD44 and variant isoforms of CD44 has been discussed as prognostic marker in the course of MM (Dietrich et al., 1997; Manten-Horst et al., 1995). Taking together the expression studies and the findings that CD44 is the principal mediator of HA induced melanoma cell proliferation, one can conclude that the CD44–HA interaction promotes melanoma development (Ahrens et al., 2001a). Consequently, Ahrens and co-workers report, that (1) the expression of CD44 is increased during melanoma progression; (2) CD44 is the principal HA surface receptor on melanoma cells; and (3) HA induced increase of melanoma proliferation is mainly dependent on CD44–HA

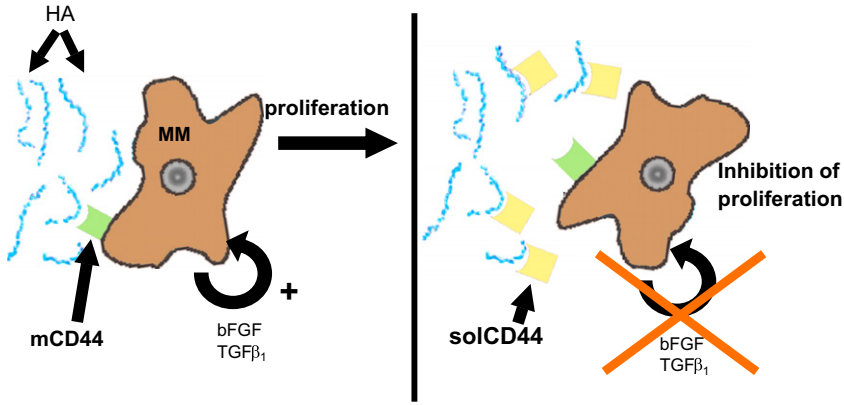

FIGURE 17.2 HA induces MM proliferation via membrane bound CD44; solCD44 is able to block this effect. HA binds to membrane CD44 (mCD44) and induces MM proliferation. This is mediated partly by autocrine secreted bFGF and TGFβ$_1$. Shedded/soluble CD44 (solCD44) blocks this autocrine proliferative loop and inhibits MM cell proliferation. (See Page 11 in Color Section at the back of the book).

interactions. The observed increase of the proliferative capacity of melanoma cells after HA binding is partly due to a stimulation of autocrine, secreted TGF-β1 and bFGF, thus showing for the first time a direct impact of HA on growth factor release in MM (Fig. 17.2).

In addition to the already mentioned post-translational modifications and the alternative splicing, the extracellular portion of CD44 can be cleaved by proteases from the cell surface thus generating a soluble form of CD44 (solCD44) (Ahrens et al., 2001b; Friedl et al., 1997; Okamoto et al., 2001). More than one protease seems to be involved in the shedding of CD44 from the cell surface, among which MT1-MMP (MMP14) is the best studied occurring in pancreatic tumor cell lines. Which protease is involved in CD44 cleavage in melanomas is currently under investigation (Kajita et al., 2001; Nakamura et al., 2004). The released CD44 is able to affect cellular behavior in several ways. (1) solCD44 is able to block the HA induced increase in proliferation of MM (Fig. 17.2) and importantly the HA binding ability of solCD44 is crucial for this effect, since solCD44 with mutations in the HA binding domain is not able to block the HA induced proliferation of MM (Ahrens et al., 2001b) and mammary carcinoma cells (Peterson et al., 2000). (2) CD44 cleavage regulates cell migration (Goebeler et al., 1996; Kajita et al., 2001), since enhanced shedding of CD44 by MT1-MMP induces cell migration in pancreatic tumor cell lines (Kajita et al., 2001) and highly aggressive MM shed significant amounts of CD44 compared to MM with lower tumorigenicity (Goebeler et al., 1996).

Interestingly, cytokines like oncostatin M and TGFβ are able to attenuate the shedding process itself and the subsequent capacity of HA

binding by the soluble CD44 molecule (Cichy et al., 2005). These findings obtained with human lung squamous carcinoma cell line HTB58 might indicate that the local cytokine environment may also determine the effect exerted by the shed solCD44.

Hyaluronan Affects MM Migration via CD44

Since HA is the major component of the extracellular matrix of the skin, the question arises if HA influences migration and motility of MM, with possible consequences for local tumor progression and metastasis. Indeed several publications show an impact of HA on MM migration. It was demonstrated that migration of MM on HA coated surfaces is increased (Fig. 17.3) in cell lines expressing high amounts of CD44 (Thomas et al., 1993) and that MM cells with high metastatic potential synthesize high amounts of HA and CD44 (Goebeler et al., 1996). Blocking of CD44 by different means abolishes the observed effects of HA in both studies. Moreover, Goebeler and colleagues demonstrate that the HA binding epitope of CD44 is responsible for the observed changes in MM migration and metastasis. Furthermore, in mice the HA cell surface levels correlate with the metastatic potential of cells (Zhang et al., 1995) and transfection of MM cells with cDNAs encoding the HA synthesizing enzymes HAS-1 and HAS-2 induces melanoma cell motility as well (Ichikawa et al., 1999).

Similarly Sugahara et al. report that the small HA degradation products (HA-oligomers, sHA) are able to induce tumor cell migration (Sugahara et al., 2003). Again, the CD44 molecule seems to be involved in this process: the sHA fragments are able to induce CD44 shedding and increase cell motility that coincides with rearrangement of F-Actin and CD44 in the cells (Murai et al., 2004; Sugahara et al., 2003). Recently this group could demonstrate hyaluronidase activity in a pancreatic carcinoma cell line generating active sHA fragments. Obviously, the tumor cells can stimulate

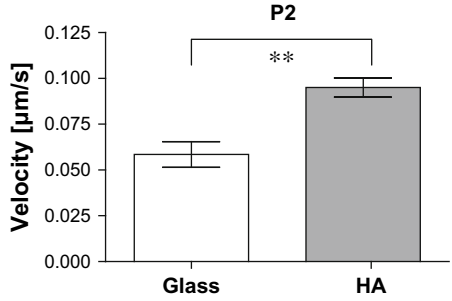

FIGURE 17.3 HA increases melanoma cell motility. Random motility of P2 melanoma cells is displayed on uncoated glass coverslips (clear bars) and HA coated coverslips (grey bars). Values are displayed as means ± SEM. The significance of differences was determined by *t*-test analysis (*$P < 0.05$; **$P < 0.01$).

their own CD44 shedding and migration in a vicious cycle driven by sHA fragments (Sugahara et al., 2006). These mechanistic findings underline the facts of increased hyaluronidase activities found in many aggressive tumors (Bertrand et al., 1997; Lokeshwar et al., 1997; Pham et al., 1997). This suggests that tumor spread and metastasis is enabled by hyaluronidase-generated sHA. These fragments decrease the CD44 anchoring of the cells by induced shedding and facilitate detachment and cell motility (Stern et al., 2006).

HYALURONAN METABOLISM, MELANOMA, AND UV-B

The HA metabolism and its role in cancer is complex and as of yet, not fully understood (Stern, 2005). There are numerous enzymes involved in HA metabolism (three HA synthases termed HAS-1-3 and three validated hyaluronidases in the skin, HYAL-1-3) which are susceptible to different steps of regulation (Stern, 2003; 2004; 2005). Several recent publications suggest a prominent role of HA in the progression of melanoma (Ichikawa et al., 1999; Mummert et al., 2003; Yoshihara et al., 2005), but less is known about the expression of different enzymes regulating HA anabolism and catabolism in MM. Recently, a link between UV-B and HA metabolism was observed (Sudel et al., 2004). This finding, together with the known importance of HA in MM progression and the fact that UV-B is critically involved in the development of MM (Berking et al., 2004; De Fabo et al., 2004; Jhappan et al., 2003) prompted us to question if all enzymes of HA metabolism are expressed in MM and if they are affected by UV-B. Indeed, the three HA synthases and the three hyaluronidases are expressed by melanoma cell lines and are, at least in the case of HAS-1, HAS-2, HYAL-1, and HYAL-3, subject to regulation by UV-B irradiation (Fig. 17.4). However, the subsequent analysis of HA content in the supernatants of irradiated cells showed no difference in HA levels indicating that UV-B irradiation does not affect significantly the release of HA into extracellular spaces *in vitro* (Fig. 17.5).

Nevertheless, the influence of UV-B on HA, with consequences for tumor progression in skin should not be underestimated. In recent years, the importance of tumor–stroma interaction has been emphasized. Although most of the research has focused on proteases (Labrousse et al., 2004; Pasco et al., 2004), recently the influence of MM on the HA synthesizing capacity of fibroblasts was shown (Edward et al., 2005). The authors report an up-regulation of HA synthesis of fibroblasts by conditioned supernatants of MM. Moreover, we could demonstrate, that UV-B irradiation influences the enzymes of the HA metabolism in HaCat keratinocytes and fibroblasts *in vitro* with an up-regulation of HAS-2 and HAS-3 in HaCats and to a lower extend in fibroblasts 24 hours after irradiation with 30 mJ/cm^2 (Averbeck

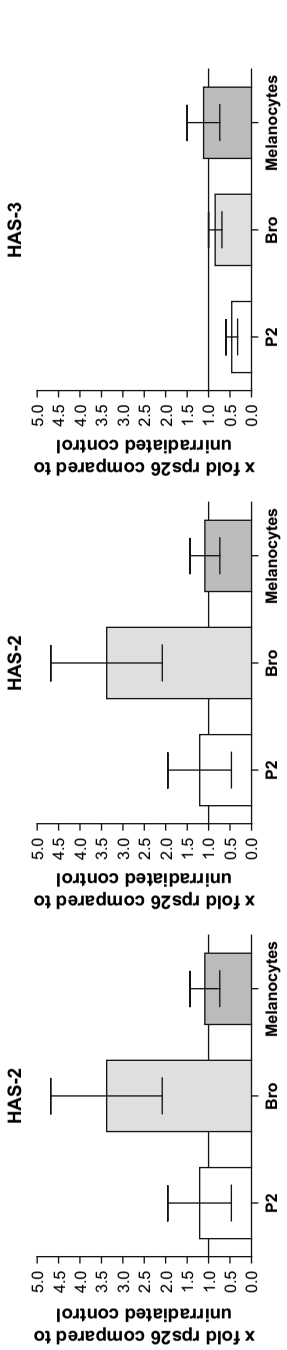

FIGURE 17.4 UVB influences the expression of HA synthases and hyaluronidases. Quantitative rt-PCR the three HA synthesizing enzymes (HAS-1-3) and the three HA degrading enzymes (HYAL-1-3). Values were normalized to the housekeeping gene (rps26) of un-irradiated controls. RNA of irradiated cells was collected 24 h after UVB treatment with 30 mJ/cm^2. Values are displayed as means of three independent experiments ± SEM.

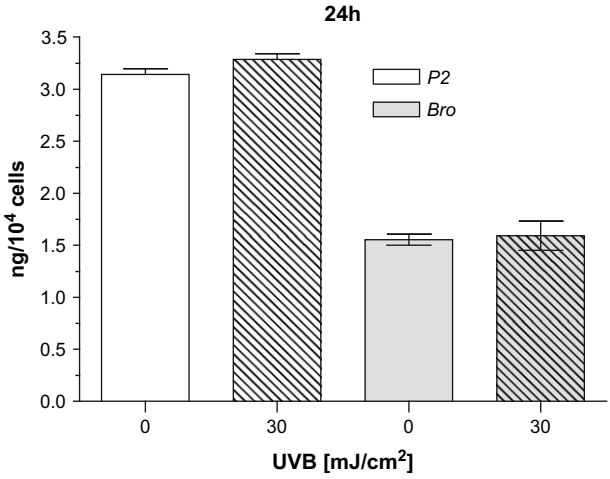

FIGURE 17.5 Total amount of HA in the supernatants of irradiated and un-irradiated cells. The total amount of HA in the supernatants of P2 (white bars) and Bro (grey bars) melanoma cells without (clear bars) and with (hatched bars) UVB treatment is shown 24 h after irradiation with 30 mJ/cm^2. HA was measured by ELISA. Values are displayed as means of duplicate measurements ± SD. One out of two independent experiments is shown. Values are displayed as means of duplicate measurements ± SD.

et al., 2007). The HA degrading enzymes showed only a slight regulation by UV-B. Interestingly, HA in culture supernatants of irradiated HaCats increased dramatically after UV-B treatment, while it was decreased in fibroblasts. Additionally, *in vivo* studies with punch biopsies of healthy volunteers also showed an increase of HA synthesizing and degrading enzymes after UV-B irradiation, resulting in an increase of epidermal HA and a decrease of dermal HA levels. These investigations emphasize the importance of the stromal tissue surrounding malignant melanomas.

CONCLUSION AND PERSPECTIVES

HA, which was regarded a simple biological "grease" just some decades ago, has had an exceptional career. It has become evident that besides it's remarkable biophysical properties (reviewed in Toole, 2004), HA is a co-regulator in the progression of malignant melanoma in several ways. The induction of proliferation in melanomas by HA via CD44 (Ahrens et al., 2001a) provides a new theory for the observed correlation between HA serum levels and disease stage (Burchardt et al., 2003). CD44 which is shed from the cell surface, also influences malignant melanoma, but the observed effects of cytokines on the affinity of shedded CD44 for HA and the shedding process itself (Cichy et al., 2005) add new complexity to this component of the picture.

Regarding the influence of HA on melanoma cell motility, a recent publication showed that the pro-migratory effects of HA are also performed by HA fragments, and that tumor cells are able by themselves to generate these fragments (Sugahara et al., 2003; Sugahara et al., 2006), resulting in autocrine motility induction, detachment, and spread (Stern et al., 2006). It appears that even if a part of the puzzle is unraveled, new investigations bring further insight into known effects, but also add new complexity to the total picture.

Regarding the influence of UV-B on the HA metabolism of MM, our investigations show no increase in the net amount of HA released into the extracellular space, but we demonstrate a regulation of HA metabolizing enzymes at the mRNA level. For skin, further studies are needed to investigate the quality of the HA following UV-B exposure. If UV-B leads to an increase in low molecular weight HA, a tumor modulating effect can be expected without a change in the total amount of HA, since the assays used to detect hyaluronan bind also short chains of HA. Another possibility is that cytokines secreted by MM stimulate HA production of tumor surrounding stroma cells, or modulate the enzymes of HA metabolism (Edward et al., 2005).

The more complex the picture becomes, the more difficult are the questions to be answered. Future experiments will focus more on the interplay of MM, its surrounding stroma and HA, including HA metabolism and the quality of the accumulated HA. Not only is HA metabolism not fully understood (Stern, 2003; 2005), but also the regulation of the HA receptors. Especially CD44, with its complex regulation (alternative splicing, post-translational modifications, and shedding) remains an important topic in the struggle to fully understand the role of HA in MM and tumor progression. Further insight into this network will hopefully enable us to find possible new targets for therapeutic strategies that will inhibit melanoma growth and metastasis.

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CHAPTER

18

Role of Hyaluronan Metabolism in the Initiation, Invasion, and Metastasis of Breast Cancer

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THE TUMOR MICROENVIRONMENT OF BREAST CANCER

The evolutionary process of breast cancer involves progression through defined pathological and clinical stages, initiating with ductal hyperproliferation, subsequent development into *in situ* and invasive carcinomas, and finally metastatic disease (Allred and Fuqua, 2001; Burstei

et al., 2004). In carcinogenesis, the importance of multiple genetic changes in epithelial cell oncogenes and tumor suppressor genes is well recognized (Vogelstein, 2004) but more recent focus on the role of host stroma has emphasized that efficient tumorigenesis requires an interaction between epithelial cells and the microenvironment. In pre-cancerous tissue, epigenetic changes can alter and activate the stroma, thereby providing the aberrant microenvironment necessary for tumor cell proliferation (Bhowmick and Moses, 2005). The activated stroma consists of altered extracellular matrix (ECM), various non-transformed cells (e.g. fibroblasts, myofibroblasts, leukocytes, and myoepithelial and endothelial cells) and growth factors that play a role in the initiation and progression of breast cancer (Bhowmick and Moses, 2005; Hu and Polyak, 2008). Reciprocal communication between epithelial cells and the tumor microenvironment enables transmittance of signals to the host stroma resulting in ECM remodeling adjacent to cancer cells (Howlett and Bissell, 1993; Elenbaas et al., 2001). The tumor-stroma pathology is similar to the embryonic state which is rich in glycosaminoglycans (Burstein et al., 2004). This pathology promotes an environment suited to cellular proliferation, angiogenesis, and stimulation of tissue-degrading proteases (Bissell et al., 2002), all cellular processes essential to carcinogenesis. This chapter will focus on the intratumoral metabolism of the ECM glycosaminoglycan, hyaluronan (HA) and more specifically the role that its anabolic and catabolic products play in the initiation, progression, and invasion of breast cancer.

INTRATUMORAL HYALURONAN LOCALIZATION DURING BREAST CANCER TUMORIGENESIS AND PROLIFERATION

Hyaluronan is a major constituent of the ECM where its linear structure, comprising of repeating disaccharide units of (β1-3) D-glucuronate-(β1-4) *N*-acetyl-D-glucosamine, and its large molecular weight (reaching up to 10 mega Daltons) provides unique physiochemical properties enabling it to bind up to 1000 times its weight in water (Fraser et al., 1997). Even at very low concentrations, high molecular weight (MW) HA forms inter- and intra-molecular associations resulting in a viscous milieu, making it an ideal biological scaffold to provide structural integrity in tissue, maintenance of hydration homeostasis (Toole, 2004), and the sequestration of growth factors, cytokines, and nutrients essential for cellular proliferation (Day and de la Motte, 2005). The multi-factorial functions of HA within the tumor microenvironment are regulated by its interaction with a variety of HA binding proteins such as versican and TSG-6, which promote the formation of both an HA-rich pericellular and extracellular matrix (Evanko et al., 2007). Through interaction with receptors, CD44, RHAMM, and

LYVE-1, the HA-containing ECM is able to be retained by cells, consequently reinforcing the structural integrity of the tumor while ensuring the maintenance of biologically relevant concentrations. The interface of HA and its receptors play a crucial role in cancer where the interaction has been implicated in the intracellular signaling cascades associated with tumor growth (Entwhistle et al., 1996), tumor cell adhesion (Toole and Hascall, 2002), neovascularization (Rooney et al., 1995), and metastasis (Toole, 2002). Refer to chapter 15 for an in-depth review of the localization of HA within tumors.

Relevant information on the clinical significance of HA in breast cancer is still emerging. In studies investigating clinical breast carcinoma samples, immunohistochemistry demonstrated that the expression of intra-tumoral HA was up-regulated in the surrounding stroma enabling it to be used as an independent prognostic factor for patient survival (Auvinen et al., 2000). The presence of high levels of HA in stromal myxoid changes in breast cancer was strongly associated with high tumor grade, tumor emboli with lymph node involvement, and increased mortality (Wernicke et al., 2003). Sonographic examination of invasive breast cancer showed that the tumor shape correlated with that of the HA ECM (Vignal et al., 2002). Furthermore, serum HA levels were significantly elevated in women with metastatic breast cancer when compared to non-metastatic carcinoma and benign breast diseases (Delpech et al., 1990). Contrary to the increase in extracellular HA, cytosolic HA has been studied in breast cancer where a strong correlation exists between cancer progression and reduced HA levels (Ruibal et al., 2000; 2001a; 2001b) suggesting that in progressive breast cancer the primary HA requirements are within the tumor microenvironment, where it can act as an integral component of the ECM and associated metabolic pathways.

METABOLISM OF HYALURONAN IN BREAST CANCER – DYSREGULATION OF A FINELY TUNED PROCESS

In healthy tissue the catabolic and anabolic rates of HA are tightly regulated resulting in precise concentrations which are indicative of the location and the specific function of the HA (Fraser et al., 1997). In general, medium and high MW HA (>400 kDa) is representative of the non-diseased state, but when processes such as inflammation occur this prompts the generation and accumulation of depolymerized or degraded HA, a scenario evident in cancer progression and dissemination (Stern, 2005). Due to the multi-factorial role of HA within the tumor microenvironment, changes in its concentration and molecular weight can exert significant epigenetic effects on tumor cells and the associated stroma. In breast cancer,

the invasive phenotype can be correlated with the differential expression of specific isoforms of hyaluronan's catabolic and anabolic enzymes, thereby highlighting the functional importance of the metabolic end-products.

Comparative Expression and Isoform Functionality of the Hyaluronan Synthases

The hyaluronan synthases (HAS) are integral plasma membrane glycosyltransferases that are predicted to be topologically and structurally homologous, with clusters of transmembrane domains and a large intracellular region that constitutes approximately half of the overall protein. Three isoenzymes (HAS-1, HAS-2, and HAS-3) share 55–71% amino acid sequence identity, where the majority of HAS sequence consensus is in the large cytoplasmic loop which is predicted to contain the glycosyltransferase activity and substrate binding sites (Itano and Kimata, 2002). Hyaluronan biosynthesis is thought to occur by alternate addition of UDP-glucuronic acid and UDP-N-acetyl glucosamine substrates to the reducing end of the elongating chain, allowing unconstrained polymerization and extrusion into the extracellular matrix (Prehm, 1983; 2006). The mechanism of export of HA into the ECM is yet to be fully elucidated but it is thought that HAS may interact with activity-related phospholipids to form a pore which associates and dissociates from the growing chain as it is synthesized (Weigel et al., 2006). Additionally, it has been proposed that HA may be exported via an ABC transport system (Ouskova et al., 2004; Prehm and Schumacher, 2004).

The HA synthases appear to be separately and differentially regulated and expressed (Jacobson et al., 2000) where responses to stimuli such as TGF-β and IL-1β (Oguchi and Ishiguro, 2004) or mechanical stretching (Mascarenhas et al., 2004) can result in transcriptional up-regulation of the genes. As changes in HA production do not always coincide with alterations in prevalence of HAS transcripts (Recklies et al., 2001) modulation of the synthetic rate could also occur through regulation of post-translational modifications such as activation by phosphorylation (Goentzel et al., 2006), altered availability of synthetic substrates (Spicer et al., 1998), or cytokine-mediated changes in protein turnover or synthetic rate. Characterization of enzymatic properties of the HAS isoforms has revealed notable differences in enzyme kinetics and molecular size of their end product (Itano et al., 1999a). The HA elongation rate is inherently faster in HAS-1 and -2 when compared to HAS-3; these isoforms are also associated with the synthetic products of high MW HA ($2 \times 10^5 - 2 \times 10^6$) while HAS-3 is associated with production of lower molecular weight HA ($1 \times 10^5 - 1 \times 10^6$) (Itano et al., 1999a). The synthetic products of the HAS isoforms have generally been determined in transformed cells or under experimental conditions where simultaneous degradation had not been inhibited. One study investigated

the MW produced by breast cancer cell lines after the inhibition of concurrent degradation (Udabage et al., 2005a). It was demonstrated that all HAS isoforms produced a 10 mega Dalton product and that the simultaneous degradation generated the variety of MWs detected and reported by other researchers. Functionally, the different roles of HAS isoforms are reiterated by their varied catalytic rates, substrate affinities and size distributions of the synthetic products.

HAS-2 is a Prognostic Indicator of Breast Cancer Tumorigenesis and Metastasis

To date, all synthase isoforms have been demonstrated to elicit functions within breast cancer initiation and progression, but the bulk of the experimental data highly implicates HAS-2 as the strongest prognostic factor in the evolution of breast cancer.

Breast cancer initiation appears to require HAS-1 and/or HAS-2 expression, as oncogenic malignant transformation of fibroblasts up-regulated both HAS-1 and -2, while tumorigenicity was reduced after HAS-2 inhibition in v-Ha-ras transformed cell lines (Itano et al., 2004). However, rigid control of expression appears to be required to facilitate tumor initiation, as HAS overexpression can reduce tumorigenic potential (Itano et al., 2004; Bharadwaj et al., 2007; Enegd et al., 2002), thereby indicating a narrow functional range in the optimal HAS expression and HA concentrations required to promote growth. The role of HAS-2 in spontaneous breast cancer was investigated using a transgenic murine model that purposely overexpressed HAS-2 with the objective of simulating the hyperproduction of HA found in breast cancer (Koyama et al., 2007). Forced expression of HAS-2 within spontaneous mammary tumors contributed to the formation of an HA-rich ECM, which promoted microvessel infiltration and an intratumoural stroma (Koyama et al., 2007). Malignant transformation in breast cancer is evident through the matrix remodeling conducted by stromal myofibroblasts and stiffening of the stroma (Kass et al., 2007) which is mediated by the production of matrix components and the release of angiogenic factors such as SDF-1/CXCL12 (Orimo et al., 2005). HAS-2 and HA production induce increased expression of pro-angiogenic factors in breast cancer stromal cells (Koyama et al., 2007) readying the microenvironment for progression and survival of the developing tumor mass.

During *adenocarcinoma progression*, the breast cancer epithelial cells lose their stable, polarized, non-malignant properties and transdifferentiate into fibroblastic, migratory cells acquiring mesenchymal characteristics, a process known as the epithelial–mesenchymal transition (EMT). EMT is characterized by epithelial cell loss of cell junctional proteins and cytoskeletal elements, increased expression of mesenchymal cadherins and vimentin rich intermediate filaments, and acquisition of motility observed

in actin cytoskeletal rearrangement (Thiery and Sleeman, 2006). Transition can manifest in resistance to anoikis (Frisch, 2001) and enhanced survival (Thiery and Sleeman, 2006) where it can occur in up to 18% of breast carcinomas *in vivo* (Dandachi et al., 2001; Jones et al., 2001; Sørlie et al., 2001). The crucial role that HA synthesis plays in EMT and subsequently cancer progression was demonstrated when breast cancer cells infected with an HAS-2 adenovirus acquired several mesenchymal characteristics, including upregulation of vimentin, dispersion of cytokeratin, and loss of organized adhesion proteins at intercellular boundaries (Zoltan-Jones et al., 2003). In addition, increased HA production induced several other tumor cell survival mechanisms such as the capacity for anchorage-independent growth, resistance to apoptosis, stimulated invasiveness and gelatinase production, increased phosphoinositol 3-kinase (PI3 kinase)/Akt pathway activity, and recruitment of stromal cells (Koyama et al., 2007; Zoltan-Jones et al., 2003). Interestingly, EMT has recently been identified to induce expression of stem cell markers (Rodriguez-Pinilla et al., 2007) suggesting that HA may indirectly promote formation of a sub-population of tumorigenic stem cells. This notion is supported by the role of HA in the hemopoietic stem cell niche (Haylock and Nilsson, 2006) and the identification of a $CD44^{+}/CD24^{-/low}$ tumorigenic stem cell subset within a heterogeneous breast cancer population (Al-Hajj et al., 2003) which indicated that HA–CD44 interactions may be of intrinsic importance in cancer stem cell biology. The role of HA in regulation of ErbB2 activation and downstream activation of the anti-apoptotic phosphoinositide 3-kinase/Akt pathway and its implications in tumour growth, progression, and chemoresistance are reviewed elsewhere in this series.

The correlation between HAS-2 and breast cancer progression was further established when Udabage et al. characterized HAS expression and HA production in 10 breast cancer cell lines and demonstrated a correlation between HAS-2 expression and invasive potential (Udabage et al., 2005a). Low levels of HAS-3 were detected in all cell lines, however, a glycocalyx could only be visualized in exponentially growing cells which predominantly expressed HAS-2 (Udabage et al., 2005a). On reaching a plateau in proliferation, all cell lines produced detectable amounts of cell-associated HA (Udabage et al., 2005a) demonstrating that after development of the critical tumor mass, retention of a cancer cell glycocalyx was not HAS isoform-specific. Characterization of the size distribution of the synthesized HA highlighted that both HAS-2 and HAS-3 extruded high MW HA and that invasive cell lines rapidly depolymerized this into fragments ranging from 10–40 kDa (Udabage et al., 2005a), which would feasibly be capable of initiating tumor neovascularization *in vivo* (Rooney et al., 1993; Sattar et al., 1994; West et al., 1985).

To date, the localization of HAS isoform expression within cancerous tissues has not been established due to the lack of widely available and

reliable antibodies, therefore limiting the immunodetection of the native proteins (Weigel and DeAngelis, 2007). The essential requirement for high MW HA during invasion suggests that HAS-2 would be significantly up-regulated at the invasion front of the tumor which would account in part, for the increased HA concentration found at the stromal-tumor interface (Bertrand et al., 1992). The functional importance of HAS-2-mediated enhancement of HA production in breast cancer was identified by anti-sense inhibition of HAS-2 in a highly invasive breast cancer cell line (Udabage et al., 2005b). Inhibition of HAS-2 reduced proliferative potential and induced transient cell cycle arrest in 79% of the cell population *in vitro*, and completely abrogated tumor initiation and progression following subcutaneous mammary fat pad inoculation into nude mice (Fig. 18.1A) (Udabage et al., 2005b). These observations support a recent study which indicated that RNA interference mediated silencing of HAS-2 expression in an invasive breast cancer cell line reduced HA production leading to a less aggressive phenotype of reduced proliferative and migratory potential (Li et al., 2007).

HAS-2 has also been implicated *in breast cancer metastasis*. Injection of antisense-inhibited HAS-2 breast cancer cells in an intracardiac metastasis and localized intradermal mammary pad model demonstrated that HAS-2 expression was essential for breast cancer metastasis because the inhibition of HAS-2 significantly reduced organ metastasis (Fig. 18.1B & C) and conveyed longer survival times (Fig. 18.1D) (Udabage et al., 2005b). It has been reported that breast cancer cell lines secrete soluble factors which mediate up-regulation of HAS-1 and HAS-2 in CD44/RHAMM expressing osteoblasts (Bose and Masellis, 2005). Breast cancer cells interact with osteoblasts and release products which activate osteoclast formation and bone resorption (Bendre et al., 2002; Dickson and Lippman, 1995; Guise et al., 1996; Pederson et al., 1999), inhibit differentiation of mature osteoblasts, or induce apoptosis in differentiated (Mastro et al., 2004; Mercer et al., 2004; Thomas et al., 1999). It is conceivable that osteoblast HA-receptors may anchor HA producing breast cancer cells to the malignant site, resulting in paracrine stimulation of HA production and chemotactic migration of additional breast cancer cells. While breast cancer cells exhibit a predilection for metastasis to bone, the absence of osteolytic lesions in the previously described HAS-2 inhibition study (Udabage et al., 2005b) suggests that the absence of HA production may inhibit anchoring of breast cancer cells, limiting stimulation of osteoblast HA synthesis and subsequent bone resorption. The extracellular phosphoglycoprotein, osteopontin, may also influence regulation of HAS-2 expression in breast cancer as osteopontin overexpression dramatically increased HAS-2 transcripts and HA production (Cook et al., 2006). Osteopontin is associated with the progression and metastasis of several cancers, and can bind cell surface receptors including HA-receptor CD44, to instigate signaling

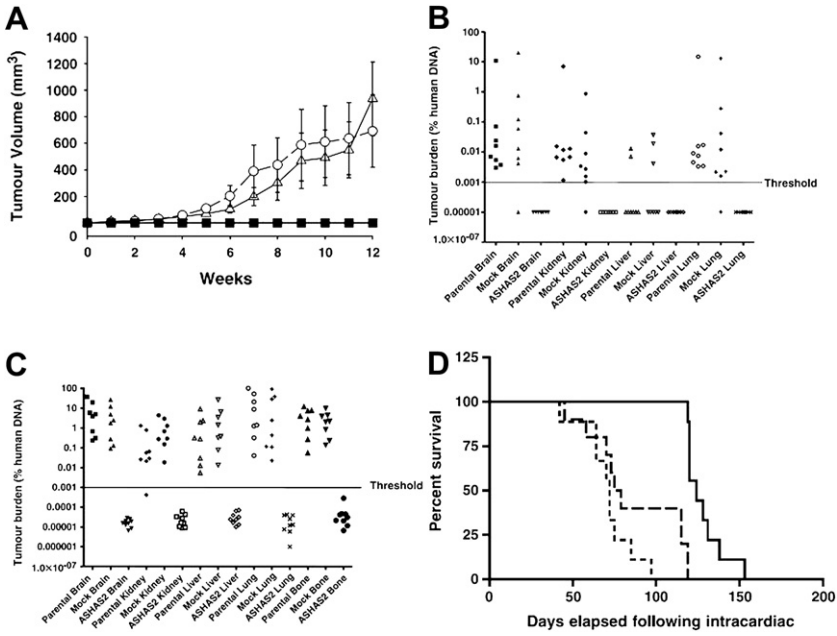

FIGURE 18.1 HAS-2 expression is an essential component of breast cancer tumorigenicity and metastasis. The MDA-MB 231 invasive breast cancer cell line was stably transfected with an anti-sense HAS-2. Parental, mock, and ASHAS-2 transfectants were inoculated into the mammary fat pad of nude mice.

A, Primary tumor growth was followed over a 12-week period with tumor progression recorded twice weekly. The results graphed represent the average tumor volume (mm^3) $\pm$ SE, where $n = 9$ to 13.

B, Alu PCR was used to detect the metastasis of human cancer cells to the brain, kidney, liver, and lung of the mouse. Results are expressed as the percentage of human tumor DNA in mouse soft organs ($n = 8$ per group).

C, Alu PCR was used to determine the extent of soft organ metastasis after intracardiac inoculation of the parental, mock, and ASHAS-2 cells lines. Results are expressed as the percentage of human tumor DNA in mouse soft organs ($n = 9$ per group). No metastasis to these organs could be detected where animals had been inoculated with MDA-MB-231 ASHAS-2-transfected cells.

D, The survival rate and number of days elapsed after intracardiac inoculations. There were no differences in the animal survival rate ($P = 0.0840$) between the parental and mock-transfected mice. Survival curve for ASHAS-2 was significantly different ($P < 0.0001$) from the both control groups. MDA-MB-231 ASHAS-2 transfectants (solid); parental cell line (short dash); mock transfectants (long dash). (This figure was reproduced from Udabage, L., et al. (2005). *Cancer Res* **65**, 6139–6150.)

cascades promoting migration, invasion, cell survival, and angiogenesis (Heldermon et al., 2001; Senger et al., 1996; Tuck et al., 2000).

Whilst there is a significant body of work in breast cancer indicating that HAS-2 overproduces HA promoting tumorigenesis and invasion, HAS-1

transfection can restore the metastatic potential of highly metastatic mammary carcinoma cells which have been rendered non-invasive by treatment with a chemical mutagen that abrogates HA production (Itano et al., 1999b). Therefore, it appears that although HAS-2 is preferentially expressed in breast cancer, the HA produced by HAS-1 is capable of eliciting similar functional effects. Moreover, the dynamic balance of HA production and HA degradation and the profoundly different signalling capacity of native and degraded HA ensures that anabolism and catabolism of HA are of significant importance in carcinogenesis.

Hyaluronan Degradation – An Essential Component of Breast Cancer Progression

The degradation of high MW HA within tissue occurs through the concerted effort of non-enzymic reactive oxygen species (ROS) (Knudson et al., 2002; Karihtala et al., 2007) and a group of multi-isoform enzymes known as the hyaluronidases (HAase) (Stern, 2003). The extensive volumetric domain of endogenous HA necessitates that it must be processed into more manageable fragments by ROS or hyaluronidase cleavage before cellular catabolism can occur (Knudson et al., 2002). Following the preliminary depolymerization, the HA follows two fates: (i) passive diffusion into the lymphatics and subsequently bloodstream where it binds to the lymph node LYVE-1 receptor (Prevo et al., 2001) or the liver endothelial cell receptor which rapidly (plasma half life of 2–5 min) clears and metabolizes the HA to monosaccharides (Fraser et al., 1984); or (ii) in the tissues, HA is internalized via CD 44 receptor-mediated endocytosis and degraded.

The degradation of HA occurs in cells by a series of sequential enzymatic reactions which involve several HAase isoforms, namely, HYAL-1 and -2 and PH-20 (refer to chapter 13 for a comprehensive review of hyaluronidases in cancer biology). In brief, the hyaluronidases associated with HA cellular metabolism are HYAL-1, known as serum hyaluronidase, which is thought to be an acid-active (pH 4.0–4.2) lysosomal enzyme (Afify et al., 1993; Frost et al., 1997) that is able to utilize HA of any size as a substrate, and which predominantly generates tetrasaccharides. HYAL-2 (Lepperdinger et al., 1998; 2001) is also proposed to be an acid-active enzyme and is linked to plasma membranes by a glycosylphosphatidylinositol anchor (Rai et al., 2001). This hyaluronidase has unique substrate specificity, cleaving high molecular mass HA polymers to intermediate size fragments of ~20 kDa, or about 50 disaccharide units. A third HAase, PH-20 is considered a neutral HAase as it does have activity at pH 7, but it can demonstrate activity at pH 4.0 to 8.0 (Cherr et al., 1999). PH-20 is thought to be the testicular HAase, but there are a number of reports of PH-20 in cancer cells (Liu et al., 1996; Madan et al., 1999), and particularly in breast cancer where it is thought to promote tumorigenesis

by accelerating the release of FGF-2 from tumor cells, decomposing HA into small fragments, and promoting angiogenesis (Gao and Underhill, 2002; Wang et al., 2004).

In tissues that are not involved in the plasma or tissue clearance of HA, the primary metabolic receptor for HA is CD44 (Culty and Underhill, 1992) more particularly in breast cancer, where CD44 has been definitively identified as the receptor responsible for the binding and internalization of HA (Culty et al., 1994). Initiation of HA depolymerization and catabolism is thought to occur within a caveolin-rich lipid raft which contains CD44, HA synthase, and HYAL-2 (Stern, 2005). The HA is anchored to the cell surface via the cooperation of the GPI-anchored HYAL-2 and CD44 (Rai et al., 2001). Before metabolism occurs the high MW HA is initially cleaved by hyaluronidases (Stern, 2003) or ROS which generates intermediate molecules suitable for internalization within unique acid endocytic vesicles (Tammi et al., 2001) and further degradation by HYAL-2, resulting in 20-kDa HA fragments. The fragments are internalized, transported to endosomes and then to lysosomes where they are further digested by HYAL-1 to generate tetrasaccharides oligosaccharides (Rai et al., 2001) and the final catabolic end-products are monosaccharides generated through the concerted activity of β-exoglycosidases, β-glucuronidase, and β-N-acetyl glucosaminidase. As previously highlighted, oligosaccharide by-products of HA degradation are pivotal in cancer progression and invasion. For brevity, refer to Table 18.1 which correlates HA chain length with functionality within the malignant phenotype implicating the diversity of function of HA catabolic products.

Due to the lack of verification of the exact nature of HA turnover and the accumulation of HA degradation products within tumors *in vivo*, Brown et al. (manuscript in preparation) intravenously injected 825 kDa [^{3}H] HA into mice bearing CD44-positive, human breast cancer xenografts. The tumor cells and ECM were separated via trypsin digestion and the MW of the intra- and intercellular HA was determined using size exclusion chromatography. These data demonstrated that within a 72 h time-frame, the extracellular half-life of the 2 ± 0.4 μg of HA (amount distributed per gram of tumor) was 11 h. Within the initial 4 h, the high MW HA persisted in the tumor ECM primarily as 825 kDa after which 5% of the radioactivity presented as a heterogeneous HA species ranging from 1.2 kDa to 125 kDa, with the most prevalent molecular weight being 20 kDa; while the remaining metabolic products were [^{3}H]acetate and [^{3}H]water. Only minor concentrations (82–220 fg/cell) of tetrasaccharides were identified within the cells; no 20 kDa HA was observed, indicating that the intracellular metabolism of HA is a rapid event. These data suggest that a broad range pH-active such as PH-20 is active within the ECM and HYAL-2 is the predominant ECM hyaluronidase. In addition there was no prolonged accumulation of high concentrations of low MW HA fragments.

TABLE 18.1 The Molecular Weight of Hyaluronan Catabolic Products Promote Specific Functionality Within the Initiation, Progression, and Invasion of Breast Cancer

Oligosaccharide length	Function in cancer	Reference
Tumorigenic potential		
8	Abrogation of hyaluronan-rich stroma	Hosono et al., 2007
Progression		
1000	Induction of inflammatory chemokines required as a stimulus for HA synthesis	Noble, 2002
10–40	Promotion of tumor cell migration	Sugahara et al., 2003
	Induction of CD44 cleavage	Sugahara et al., 2003; 2006
8–32	Stimulation of angiogenesis	Sattar et al., 1994; West et al., 1985; Slevin et al., 1998; 2002; 2004; Lokeshwar, 2000
	Stimulation of tumor neovascularization	Rooney et al., 1995
12	Endothelial cell differentiation	Takahashi et al., 2005
12	Inhibition of anchorage-independent growth	Ghatak et al., 2002
Invasion		
~170	Activation of MAP kinase facilitating invasion	Kobayashi et al., 2002
4–6	Transcription of metalloproteinases	Fieber et al., 2004

The functional diversity of high MW HA and its lower MW degradation products in breast cancer is well established (see Stern et al., 2005 for review) but there is a lack of experimental evidence correlating HA catabolism with breast cancer phenotype. Quantitative identification of HAase genes and the functional characterization of the gene products in breast cancer cell lines found that invasive tumor cells primarily expressed HYAL-2 where the co-expressed HYAL-1 had a 5- to 10-fold lower transcript number. The turnover of HA was significantly higher in aggressive cell lines, but the differentiating factor was the ability of growth arrested cells to continue metabolizing HA, contrary to the less invasive phenotype which ceased turnover after reaching plateau growth (Udabage et al., 1999a). These findings were consistent with the proposed function of the

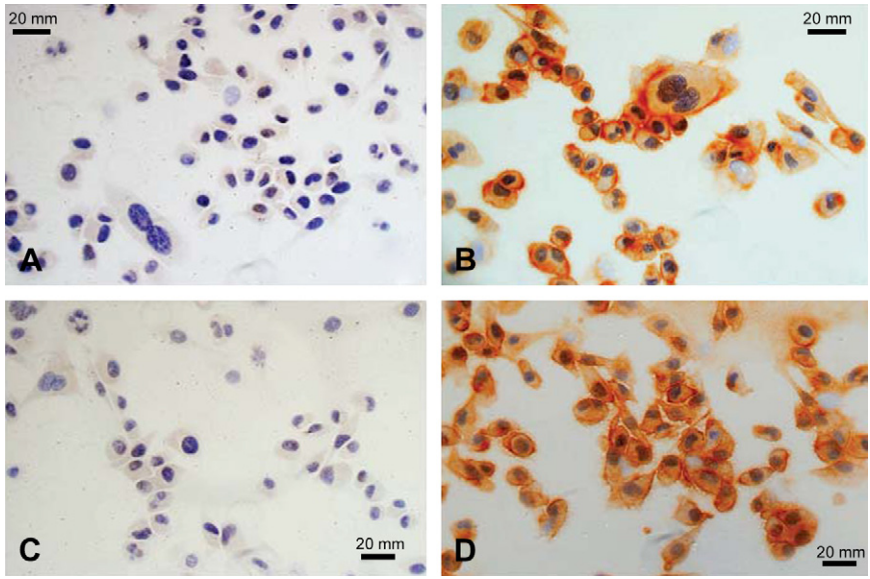

FIGURE 18.2 In breast cancer the expression and function of HAS-2, HYAL-2 and CD44 are intra-dependently regulated. The MDA-MB 231 invasive breast cancer cell line was stably transfected with an anti-sense HAS-2-pCl-Neo construct and mock control (pCl-neo vector without insert) using LipofectAMINE. One month prior to commencing the experiments, transfected cells were selected using G418 antibiotic and stable cell lines were established by harvesting and pooling of antibiotic-resistant colonies. The effect of HAS-2 inhibition on the expression of CD44 was determined using immunohistochemical detection of HAS-2 and CD44. Subconfluent stable transfectants of MDA-MD-231 expressing antisense mRNA to HAS-2 (**A**) and parental MDA-MB-231 (**B**) were reacted with an HAS-2-specific antibody. Cell surface reactivity to CD44H was also tested in subconfluent stable transfectants containing ASHAS-2 (**C**) and parental MDA-MB-231 (**D**) using an antihuman CD44 monoclonal antibody. (This figure was reproduced from Udabage, L., et al. (2005). *Cancer Res* **65**, 6139–6150.) (See Page 11 in Color Section at the back of the book).

catabolic products of HA within tumors (Stern, 2005). The co-dependency of CD44 and HYAL-2 in HA turnover in breast cancer was confirmed when the inhibition of HAS-2 concomitantly decreased CD44 and HYAL-2 expression (Fig. 18.2) which resulted in elevated concentrations of 10 mega Dalton HA (Fülop et al., 1997). The inability of the cancer cells to degrade the HA totally ablated tumor initiation, an observation potentially explained by the proposed anti-angiogenic effects of high MW HA (West and Kumar, 1989) emphasizing that when a tumor reaches a critical volume, it is necessary to establish neovascularization via the degradation of HA into small angiogenic fragments (<10 kDa). See Chapters 12 and 13 which further explore the role of HAases in other cancers and in the regulation of CD44 expression.

THE CO-ORDINATED FUNCTION AND IMPORTANCE OF HA METABOLISM IN BREAST CANCER

A comprehensive understanding of the role of HA metabolism and the functional diversity of the resultant polymers is yet to be fully elucidated. The genetic manipulation of the expression of HAS and HAases, and the use of specific inhibitors for these enzymes has made it possible to form a collective understanding of the role of HA metabolism in tumorigenesis, progression, and invasion (Fig. 18.3). It is posed that, during malignant transformation the acquisition of multiple genetic mutations in the epithelial cells (Vogelstein, 2004) result in altered rates of apoptosis and proliferation which is evidenced by an accumulation of pericellular and extracellular high MW HA, primarily synthesized by breast cancer HAS-2. The increase in HA concentration contributes to (i) the formation of a hydrated and volumetrically expanded versican/HA matrix which supports cancer–host interactions via the unhindered penetration of stromal cells and diffusion of nutrients (Folkman, 2002; Koyama et al., 2007); and (ii) the inactivation of tumor macrophages ensuring the immunological suppression of a potential attack on the tumor cells (Kuang et al., 2007). The next step in breast cancer progression eventuates when the increased synthesis of HA represses E-cadherin transcription which induces epithelial–mesenchymal transition (Haylock and Nilsson, 2006). In concert with ECM degrading enzymes, the tumor cells detach and migrate into the underlying stroma where they contribute to the formation of intratumoral stroma (Zoltan-Jones et al., 2003). The specialized stroma and tumor cells simultaneously produce, and then rapidly degrade the newly synthesized HA and the degradation products in turn promote angiogenesis (West et al., 1985; West and Kumar, 1989; Liu et al., 1996), increased inflammatory cell and fibroblast recruitment to the specialized stroma (Ronnov-Jessen et al., 1996; Tlsty and Hein, 2001), and enhanced expression of matrix metalloproteinases, MMP-9 and MMP-13 (Fieber et al., 2004). The inflammatory cells appear to have dual roles: (i) secretion of cytokines, growth factors, and chemokines necessary for stimulation of HA synthesis; and (ii) production of ROS required for the initiation of extracellular HA degradation and the generation of HA fragments which drive the endothelial cell proliferation and neovascularization essential for tumor progression. In addition, it is thought that circulating bone marrow-derived endothelial cells are recruited to the area of neovascularization where they form new blood vessels (Gottfried et al., 2008). Finally, in preparation for cancer cell dissemination to distant sites, the development of the peritumoral lymphatic system is initiated by the secretion of lymphangiogenic activators (VEGF-C and -D) by stromal and tumor cells (Von Marschall et al., 2005).

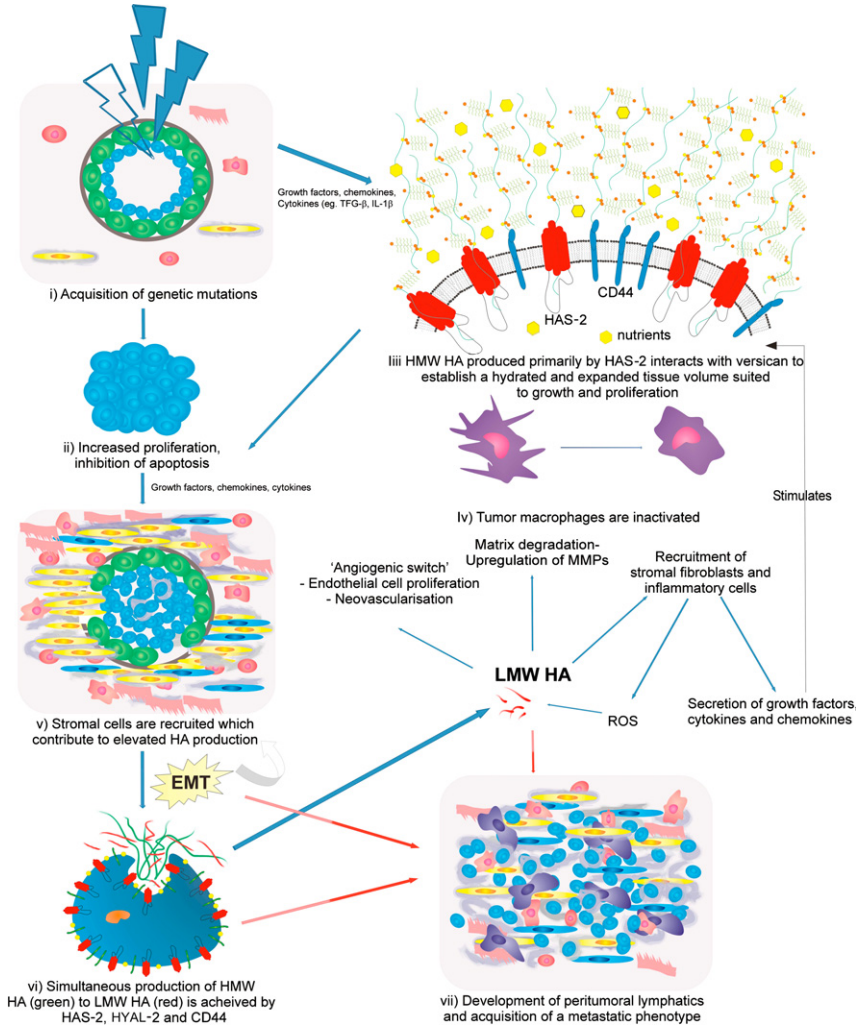

FIGURE 18.3 Dynamic hyaluronan metabolism contributes to the pathogenesis of breast cancer. Multiple genetic mutations (i) can lead to epithelial cells being at a proliferative advantage where apoptosis is inhibited (ii); this is manifested in an accumulation of pericellular and extracellular high MW HA primarily synthesized by HAS-2 (iii). HA within the pericellular and extracellular matrix can bind to the HA synthase (red), cell surface receptors such as CD44 (blue), and interact with other matrix components including versican, creating a hydrated and expanded tissue volume which facilitates the diffusion of nutrients and promotes growth (iii). Hyaluronan also inactivates tumor macrophages ensuring the immunological suppression of potential attack on the tumor cells (iv). Increased HA synthesis can stimulate recruitment of stromal cells which contribute to microenvironmental changes (v), and induction of epithelial–mesenchymal transition (EMT) which facilitates acquisition of a more invasive, metastatic phenotype. In concert with ECM degrading enzymes, EMT can induce tumor cell can detachment and migration into the underlying stroma where they contribute to the formation of intratumoral stroma. The synergistic

◀ actions of HAS-2 (red), CD44 (green), and HYAL-2 (yellow) ensure simultaneous production and degradation of HA (vi). Low molecular weight HA (red) can promote angiogenesis, increased inflammatory cell and fibroblast recruitment and enhanced expression of matrix metalloproteinases. Inflammatory cells can in turn secrete cytokines, growth factors, chemokines necessary for ongoing stimulation of HA synthases, and produce reactive oxygen species (ROS) which degrade HA. These events contribute to acquisition of a metastatic phenotype, which is accompanied by development of the peritumoral lymphatic system (vii). (See Page 12 in Color Section at the back of the book).

CONCLUSIONS

The dynamic balance between HA synthesis and degradation within the tumor microenvironment plays an integral role in the complex, multistep process of carcinogenesis. Substantial preclinical work has elegantly elucidated the potential participation of both the synthetic and degradative enzymes in the metabolic processes of breast cancer. Tumor initiation, progression and maintenance appear to be highly dependent on the accumulation of high MW HA within the breast cancer stroma, where it provides a hydrated growth matrix for tumor cells, promotes tumor survival by prevention of apoptosis, camouflages cancer cells from cytotoxic attack by host immunocompetent cells and ultimately stimulates invasion. In addition, after the epithelial–mesenchymal transition of cancer epithelial cells, the high MW HA acts as the substrate for further degradation into biologically active, low MW fragments that have the primary function of initiating cell signaling cascades during ECM remodeling. The specific signaling events induce neovascularization, lymphangiogenesis, and ECM degradation via the enhanced expression of matrix metalloproteinases. By no means is HA and its receptors the only instigators of these processes, to date numerous cellular ligands and molecules have been shown to participate in the process of HA metabolism and carcinogenesis of the breast, but a complete understanding of the significance with translation into clinical benefit requires substantially more work and elucidation.

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CHAPTER

19

Clinical Use of Hyaluronidase in Combination Cancer Chemotherapy: A Historic Perspective

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INTRODUCTION

Testicular hyaluronidase was first described in 1928 as "spreading factor" by Duran-Reynals and further characterized in 1940 by Chain and Duthie as mycolytic enzyme that mediated increased uptake of substances

into tissues (Duran-Reynals, 1929; Chain et al., 1939; 1940). Clinical application of this enzyme started in 1952, when Breu demonstrated that hyaluronidase helps to distribute dyes and other substances in tissues and increases the permeability of dermis and connective tissue by degradation of hyaluronan (Breu, 1952). Atkinson pioneered the use of hyaluronidase in ophthalmology using the enzyme in combination with epinephrine and procaine in 1949 (Atkinson, 1949). The effects of hyaluronidase enables this enzyme to be used therapeutically to increase the speed of absorption and to diminish discomfort due to subcutaneous or intramuscular injection of fluid, to promote resorption of excess fluids and extravasated blood in tissues, and to increase the effectiveness of local anesthesia. Hyaluronidase has been used to reduce the extent of tissue damage following extravasation of parental nutrition solution, electrolyte infusions, antibiotics, aminophyline, and 'mannitol' (Farr et al., 1997; Frost, 2007). Hyaluronidase was widely used in many fields, i.e. in orthopedics, surgery, ophthalmology, internal medicine, oncology, dermatology, gynecology, etc (Kluza and Moritz, 1985). The application of this enzyme in tumor therapy in combination with chemotherapeutic agents has been studied for more than two decades. However, many aspects of hyaluronidase usage in anticancer regimens continues to be experimental (Baumgartner et al., 1998).

The term hyaluronidase was introduced to denote enzymes which degrade hyaluronan, although these enzymes are able to cleave other glycosaminoglycans (Stern, 2006). There are three main groups of enzymes with the same specificity, however, with different reaction mechanisms. The one available for clinical pilot experiments belongs to the class of testicular-type hyaluronidases (hyaluronate 4-glycanohydrolase, EC 3.2.1.35), like Permease®, which randomly cleaves β-N-acetyl-hexosamine $(1 \rightarrow 4)$ glycosidic bonds in hyaluronan, chondroitin, and chondroitin sulfates. The reaction generates even-numbered oligosaccharides, with mainly tetrasaccharides as the smallest fragments. In the human body, hyaluronidase is found in many organs (testes, spleen, skin, eye, liver, kidney, uterus, and placenta), body liquids (tears, blood, urine, sperm and others), and in tumors (Farr et al., 1997). Generally, hyaluronidases are extracted from bovine and sheep testes or from bacteria, respectively. Highly purified preparations, e.g. hyaluronidase from bovine testes, achieve an activity of 40,000–50,000 IU/mg. The most frequent contaminating enzymes in less pure preparations are proteases and glucuronidases. Hyaluronidase is rapidly eliminated from plasma: upon intravenous (i.v.) injection of 5000 IU hyaluronidase per kg body weight, no activity can be demonstrated after 45 min (serum half-time 2.1 ± 0.2 min; Oettl et al., 2003). The short half-life of the enzyme in plasma cannot be accounted for by excretion in urine and bile. Tissue distribution measurements reveal that the major site of uptake is the liver (Muckenschnabel et al., 1998). All hyaluronidases, including tumor hyaluronidases, are most active at an acid

pH 3.7–4.0. Testicular hyaluronidase has a broad pH-optimum and at pH 5.0 has approximately 70% of the activity at pH 4.0. When measured by viscosity reduction or by turbidity methods, the enzyme is still active at neutral pH (Oettl et al., 2003).

CLINICAL APPLICATION OF HYALURONIDASE IN PERIPHERAL CIRCULATORY DISORDER

A review of the various applications of hyaluronidase in benign diseases was published by Farr and Menzel (Farr et al., 1997). One peculiar clinical application, which may be of relevance for the use of the enzyme as an adjunct in chemotherapy, is described by Baumgartner et al., namely for the experimental therapy of peripheral circulatory disorders (Baumgartner et al., 1998). In a patient with gangrene of the toes, the problem was successfully treated with 7500 IU hyaluronidase intravenously, in close agreement with published observations (Elder et al., 1980). Following this clinical observation, 16 patients were treated with 200,000 IU hyaluronidase intra-arterially (12/16 patients with diabetes) resulting in complete recovery of three patients, improvement in eight patients and progression of disease in three patients, leading to amputation. To confirm these results, a randomized trial comparing 200,000 IU hyaluronidase intra-arterially (nine patients) with placebo (nine patients) was performed. In the hyaluronidase group a statistically significant reduction of ulceration was achieved compared to the placebo group. Since parameters of local circulation were not improved in large vessels, the enzyme seems to improve transport of oxygen and nutrients in the small capillaries, a property which could similarly assist delivering chemotherapeutic drugs to solid tumors.

CLINICAL APPLICATIONS OF HYALURONIDASE IN MALIGNANT DISEASE

Expression of hyaluronidase was detected in various tumors and at the time was regarded as an important factor supporting tumor growth and dissemination. This led to an interest in inhibitors of this enzyme (Kiriluk et al., 1950; Rosenthal, 1952; Cameron et al., 1979). Paradoxically, both hyaluronan and hyaluronidases can correlate with cancer progression. Increased levels of hyaluronan on the surface of tumor cells are an indicator of poor outcome (Stern, 2005). Such information would indicate that cancer progression is inhibited by hyaluronidase. Yet progression of certain cancers also correlates with levels of hyaluronidase activity (Lokeshwar et al., 1996). Although hyaluronidase became an established preparation

for the management of the extravasation of cytotoxic drugs, its use in treatment of tumors was considered contra-productive in regard to promotion of metastasis (Herp et al., 1968, Bertelli, 1995).

One observation of tumor response accidentally made during treatment of extravasation of a chemotherapeutic infusion in 1982 stimulated interest here in Vienna in the possibility that the enzyme hyaluronidase may improve the transport of cytotoxic drugs to and within tumor tissue by degradation of the extracellular matrix (Baumgartner and Baumgartner, 1985; Baumgartner, 1987). A myeloma patient treated with a polypeptide complex of L-phenylalanine mustard (Peptichemio®, Peptichemio AG, Switzerland; drug now discontinued) who had received several prior administrations without response, suffered from an extensive extravasation. A large dose (approximately 54,000 IU) of hyaluronidase was applied in order to resolve the extravasation. The combination of the cytotoxic drug with the enzyme however led to rapid reduction in bone pain from the bone manifestations of myeloma. Since this surprising observation pointed to hyaluronidase as crucial additive, a more systematic investigation of the enzyme as an adjunct to chemotherapy was initiated and reported for the first series of patients by Baumgartner and Baumgartner in 1985. Forty-six patients (17 myelomas, 11 malignant lymphomas, eight mammary carcinomas, seven head and neck carcinomas, two gastrointestinal carcinomas, and one ovarian carcinoma) that became resistant to their specific type of chemotherapy were treated again with the same regimen combined with bovine testicular hyaluronidase (7500 IU Permease®, Sanabo, Vienna, Austria), either intravenously, intramuscularly one hour prior to or intraperitoneally in combination with cytotoxic chemotherapy. Two cases of local irritation at the site of injection and one case of reversible anaphylactoid reaction were described. Responses reported in patients were as follows (complete response – CR; partial response – PR): myeloma – CR 2/9, subjective improvement – 7/9; non-Hodgkin-lymphoma (NHL) – CR 2/5, PR 2/5, and breast cancer – PR 2/4. Complete regression of ascites was achieved in most cases of intraperitoneal therapy in later studies (Baumgartner et al., 1998). The effectiveness of Permease® was ascribed to a reversal of the resistance of tumor cells or an alteration of pharmacokinetic parameters of cytotoxic agents, respectively.

HYALURONIDASE PREPARATIONS IN ADDITIONAL CLINICAL STUDIES IN VIENNA

Since the pilot study described above had yielded promising results, further attempts were made to improve and expand clinical applications of hyaluronidase by using more enriched preparations with higher specific activity. Bovine hyaluronidase had been available in various formulations

for therapeutic use for over 40 years. These preparations were registered during different times and varied widely with regard to purification standards and dosage per vial. The two preparations of lyophilized bovine testicular hyaluronidase (Stettbacher, 1953; Aderhold, 1954) that were used for clinical application by our group at the III Department of Internal Medicine, Hanusch Hospital Vienna, V Department of Internal Medicine, Hietzing Hospital Vienna, and the Ludwig Boltzmann Institute for Clinical Oncology and Photodynamic Therapy were Permease® 750 IU/vial (available from 1982, Biochemie, Vienna, no longer available) and the further purified Neopermease® 200,000 IU/vial (available from 1984; discontinued 1998, Sanobo/Sandoz, Austria). The third available hyaluronidase preparation for clinical use at that time was Hylase® Dessau 1500 IU/vial (Impfstoffe, Dessau, Germany; now Riemser, Germany). Results of Oettl et al. demonstrated that Neopermease® and Hylase® Dessau are hyaluronidase preparations with nearly the same enzymatic properties (Oettl et al., 2003), though the molecular sizes and other properties of the enzymes appeared to differ. Although the existence of isoenzymes could not be definitely ruled out, the pattern of proteins appeared to depend primarily on different proteolytic cleavage reactions during the isolation procedures. Within the limits of experimental error, both pharmaceutical bovine testicular hyaluronidase preparations are characterized by identical pH dependencies. Enzyme activity was observed over a broad range of up to pH 8, with a distinct maximum at pH 3.5. For intramuscular application, 7500 IU Permease® (10 vials containing 750 IU each, dissolved in 5 ml isotonic NaCl) were given 1 h prior to chemotherapy, for intravenous application, 7500–22,500 IU Permease® (infusion time 15 min) or 200,000 IU Neopermease® (infusion time 15 min–24 h) in 100 and 500 ml isotonic NaCl, respectively. For intravesical therapy, 200,000 IU Neopermease® in 20 ml isotonic NaCl were instilled in combination with 20 mg mitomycin C following transurethral resection of bladder cancer.

EXTENDED STUDIES OF THE USE OF HYALURONIDASE AS ADJUNCTS IN CANCER CHEMOTHERAPY

During the years that followed, a total of 260 patients were treated with combinations of hyaluronidase and chemotherapeutic drugs at our institutions and in cooperation with other departments and hospitals. These included cases of brain tumors, head and neck cancers, bladder cancer, myeloma, lymphoma, breast cancer, non-small cell and small cell lung cancer, colon cancer, renal cell cancer, glioma, and others (Baumgartner, 1987). Sixty-nine patients received low-dose hyaluronidase intramuscularly, 11 patients low-dose intravenously, and 180 patients high-dose (20,000 IU) intravenously or intravesically.

To obtain more definite information concerning the contribution of hyaluronidase to the therapeutic effect of chemotherapy, the unchanged treatment cycle was repeated with addition of hyaluronidase in 103 patients, who had been resistant to various types of chemotherapy. The other patients were treated with a combination therapy from the beginning. Cytotoxic drugs included alkylating compounds, doxorubicin, Vinca alkaloids, cisplatin, etoposide, 5-fluorouracil, and methotrexate. At that time no alternative chemotherapy regimens were available for those patients. In a significant number of these patients, new responses could be achieved by the addition of hyaluronidase. Regression or remission could be maintained for varying but limited periods: myeloma patients (5 CR + PR/23), Morbus Hodgkin patients (5 CR + PR/9), NHL high-grade patients (9 CR + PR/14), mammary carcinoma patients (7 CR + PR; 4 stable disease/14) and colorectal carcinoma patients (5 PR + 7 stable disease/16). In 17 out of the 23 myeloma patients bone pain was reduced and mobility improved for a prolonged period.

In 27 patients with squamous cell carcinomas of the head and neck region, hyaluronidase was added to cytostatic chemotherapy (bleomycin/cisplatin/methotrexate/5-fluorouracil), in some from the beginning of treatment, and in others, after the development of chemoresistance. We administered either vials containing 750 IU of Permease® in a dosage of 7500 IU or 22,500 IU or a preparation of Neopermease® containing 200,000 IU. Hyaluronidase was well tolerated and reversible allergic reactions were observed in only two patients. Overall CR 14/27, PR 5/27 and stable disease 3/27 was achieved. After giving hyaluronidase to chemoresistant patients, CR 8/16, PR 3/16 and stable disease 3/16 were achieved. The course of disease in chemoresistant cases makes it very likely that hyaluronidase improved the outcome in such patients. Disease-free survival times have been extremely long. For example, in resistant patients with squamous cell head and neck carcinomas, complete remissions (median of 37 months) were achieved by the addition of hyaluronidase (Baumgartner and Neumann, 1987).

Hyaluronidase in combination with chemotherapeutics from the beginning was also tested in patients bearing brain tumors, either primary cerebral tumors or secondary tumor metastases. Furthermore this pilot study included high-grade astrocytomas and NHL, including HIV-positive patients. Although hyaluronidase is not able to cross the blood–brain barrier under normal conditions, it may pass a leaky barrier that appears to be prevalent in brain tumor patients (Vick et al., 1997; Baumgartner et al., 1987).

In six patients with mammary carcinoma and cerebral metastases, chemotherapy according to either the ACO-scheme (adriamycin/cyclophosphamide/vincristine) or MTX/DDP (methotrexate/cisplatin) was combined with hyaluronidase without radiation therapy. There was one

CR (brain and lung metastases), two PR, two that remained stable, and one case in which there was progression of disease. These remissions were maintained for a median of nine months. Eight patients with high-grade cerebral lymphomas (seven primary and one secondary) were treated with MTX/DDP/hyaluronidase without radiation therapy. Three CR and three PR were achieved (Baumgartner et al., 1987b). CR could be maintained for 11, nine and one month, respectively. In three further HIV-positive patients with primary cerebral lymphoma, one CR was achieved with CHOP (cyclophosphamide/doxorubicin/vincristine/prednisone)/hyaluronidase lasting for 6 months without radiation therapy.

In 1986, a 75-year-old female patient with inoperable glioblastoma stage IV in poor general condition was treated. Cytotoxic chemotherapy consisted of 60 mg lomustine in a single dose, 150 mg methotrexate, and 2 mg vincristine at 3-week intervals and additionally 200,000 IU hyaluronidase were injected intravenously prior to cytostatic application. Without any additive radiotherapy, a complete remission lasting for 9 months was obtained, proven by using computer-assisted tomography. This patient died from the tumor after 12 months.

Based on this observation, 39 patients with high-grade astrocytomas, including primary tumors and relapses, were treated with polychemotherapy and hyaluronidase in a second pilot study. In six patients who suffered from primary tumors, after partial resection, three partial remissions could be achieved. The same was observed in two of 12 patients with recurrent astrocytoma. The longest disease-free interval achieved by chemotherapy with added hyaluronidase was nine months. In the next randomized study involving 43 patients with high-grade astrocytoma, in the randomized arm A without hyaluronidase the median survival was 8.7 months, while in arm B with hyaluronidase the median survival averaged 9.5 months, indicating no significant differences. The median survival time achieved in this study (9.2 months) correlated well with results by others in grade IV astrocytomas with chemotherapy plus radiation therapy, however, quality of life was improved in our own study, due to partial responses and a lower requirement for surgical interventions for reducing intracranial pressure (Baumgartner et al., 1998).

Intravesical instillation of mitomycin C can significantly reduce the rate of relapse after radical resection of superficial bladder cancer (Bolenz et al., 2006). In our group, a randomized study using mitomycin C with and without hyaluronidase applied by intravesical instillation after radical resection of superficial bladder cancer was performed. The aim of this trial was to investigate whether hyaluronidase can further reduce the rate of relapse. Of major concern was the possibility that this enzyme would lead to an enhanced release of mitomycin C into the blood stream, resulting in increased systemic toxicity. However, intravesical instillation of 20 mg mitomycin C with and without 200,000 IU hyaluronidase and

measurement of peak plasma concentrations ruled out any differences in resorption of the cytotoxic drug between these two patient groups (Maier and Baumgartner, 1988).

Following this pharmacokinetic study, 56 patients were randomized for mitomycin C with and without hyaluronidase treatment. The recurrence rate after a median observation time of 21.1 months was nine of the 28 patients (32%) who received 20 mg mitomycin C as monotherapy, in contrast to the two of 28 patients (7%) who received 20 mg mitomycin C together with 200,000 IU hyaluronidase intravesically. The local toxicity of mitomycin C was not enhanced by hyaluronidase (Maier and Baumgartner, 1988). After a median observation time of 50.4 months, the rate of relapse with mitomycin C single agent instillation was 59% compared to 27% after mitomycin C plus hyaluronidase (Maier and Baumgartner, 1989). It was concluded therefore that addition of hyaluronidase enhances the local effect of mitomycin C in the intravesical neoadjuvant treatment of bladder cancer.

Based on the results of this study, a group of 43 patients undergoing transurethral resection of Ta–T1 tumors and mitomycin C/hyaluronidase combination treatment was analyzed retrospectively after a mean observation period of 48.5 months. During two years of neoadjuvant therapy, tumor recurrence was seen in six patients (13.9%). Of the 37 patients who remained disease-free under treatment, five (13.5%) exhibited tumors later during a mean observation period of 24.5 months after treatment. These values were significantly lower than those obtained previously from a group of 63 patients treated with mitomycin C alone (mean observation period 50.4 months), with recurrence rates of 33.3% during and 26.2% after neoadjuvant chemotherapy (Hoebarth et al., 1989).

SIDE EFFECTS OBSERVED DURING CLINICAL AND EXPERIMENTAL USES OF HYALURONIDASE

Previously, Hylase® Dessau had been used clinically in large doses intravenously for the therapy of Bechterew's disease (Bellmann et al., 1972). The immunogenicity, formation of humoral antibodies, and frequency of anaphylactic reactions were studied in experimental animals in response to application of hyaluronidase (Storch et al., 1978). Intravenous and intramuscular administrations of 150 to 75,000 IU of Hylase® Dessau were followed by production of IgG antibodies against the enzyme. The formation of antibodies was reported to occur more extensively after intramuscular application. One third of the animals showed anaphylactic responses. 26% of patients developed antibodies after application of Hylase® Dessau. No anaphylactic reactions were observed in 17 patients with antibodies when intravenous application of hyaluronidase was continued.

Our first clinical studies were designed as phase I studies, mainly to obtain information concerning the tolerability of the hyaluronidases, the widely differing dosages (7500 or 200,000 IU) and the significance of different application routes. The large difference in the hyaluronidase content per vial in the two available preparations (Permease® and Neopermease®) was the main reason for the large variation in dosage schemes.

In general, hyaluronidase itself was very well tolerated when applied by the intramuscular or intravenous route with 20 out of 229 patients exhibiting reversible allergic reactions (Baumgartner, 1987). When given by the intravesical route, no side effects occurred. Local allergic reactions at the injection site were observed in three of 72 patients treated intramuscularly, with one patient showing systemic symptoms, such as nausea, vomiting and circulatory problems, which is typical of the allergic reactions when hyaluronidase is given intravenously. In all other systemically treated patients, the rate of allergic reactions was 10%, with the exception of astrocytomas where the rate was 20%, but always reversible and easily manageable by corticoids and calcium (4/10 patients received 22,500 IU and 13/149 patients 200,000 IU). This higher rate was observed in astrocytomas even though all these patients received corticoids as symptomatic prophylaxis against cerebral edema.

In 38 patients, measurements of the antibody level against hyaluronidase were performed. In 12 of these patients, antibody levels were already elevated before therapy. In further 18 patients, the increase in antibody levels was manifest until 6 weeks after the initiation of therapy (nine patients: 10–100 fold; one allergic reaction; Baumgartner, 1987). Five out of 16 pediatric patients with CNS tumors treated with hyaluronidase (Neopermease®) in addition to chemotherapy developed symptoms of immediate type allergic reactions (Szépfalusi et al., 1997). In sera from these five patients binding of specific IgE antibodies to proteins of the hyaluronidase preparation were found. No specific IgE binding was detected either in the sera of atopic patients, or in the control group. Further application of hyaluronidase is not indicated in cases of an allergic reaction that may be increased by impurities in these preparations.

APPLICATION OF HYALURONIDASE IN EXPERIMENTAL ANIMAL MODELS OF CANCER

Following studies concerning the use of hyaluronidase as an adjunct to chemotherapy for different tumor entities with promising results, investigations in regard to the pharmacokinetics and toxicity of the bovine enzyme were initiated in experimental animal models. Pharmacokinetic studies for hyaluronidase were performed by the group of Professor Schoenenberger at the Institute of Pharmacy, University of Regensburg

(Germany) in 1990 and have been continued by his successor, Professor Buschauer, systematically characterizing the distribution and effects of this enzyme *in vitro* and *in vivo*.

A first trial using a murine mammary carcinoma animal model showed that the efficacy of doxorubicin was enhanced by application of hyaluronidase in the vicinity of the tumor (Beckenlehner et al., 1992). The antitumor activity of doxorubicin was enhanced *in vitro* and *in vivo* by high doses of bovine hyaluronidase that had no cytotoxic activities themselves in different cell lines and in a mammary cancer tumor model (100,000 IU/kg).

A second trial tested the subcutaneous application of vinblastine close to a transplanted melanoma with and without hyaluronidase. Vinblastine as single agent therapy as well as with hyaluronidase alone were not effective in this resistant xenotransplant model, while the combination of hyaluronidase and vinblastine achieved curative results using one melanoma cell line and delayed tumor growth in three other melanoma lines (Spruss et al., 1995). A further trial in a malignant melanoma xenotransplant model investigated the impact of intraperitoneal hyaluronidase on the accumulation of melphalan in various organs and the tumor. It could be shown that accumulation of melphalan was increased by a factor of 5–32 after intraperitoneal application of hyaluronidase. This result indicates that hyaluronidase is able to cross the blood vessel barrier and reach the tumor, even when applied at some distance away from the tumor (Muckenschnabel et al., 1996). A further conclusion was that the extremely short time of decay of hyaluronidase in plasma, which amounts to a few minutes, is not due to the rapid inactivation but to a very efficient distribution to all tissues and also to a larger extent to tumor tissue. In summary, hyaluronidase alone even in very high concentrations revealed no antitumor activity, however, in combination with chemotherapeutic drugs significantly increased permeation and cytotoxicity (Muckenschnabel et al., 1998).

OVERVIEW AND ADDITIONAL OBSERVATIONS

Hyaluronidase has been in clinical use involving chemotherapy for the treatment of the extravasation, in order to prevent tissue necrosis. A key event in alerting us to the possible additional uses of hyaluronidase was the case of one myeloma patient who received a large dose of hyaluronidase locally to resolve extravasation of a highly toxic melphalan-containing drug. This local application of hyaluronidase not only resolved the marked extravasation, however, in addition, rapidly reduced pain from the bone manifestations of myeloma, a phenomenon not observed with the chemotherapeutic drug alone during previous administrations (Baumgartner and Baumgartner, 1985). Based on this observation, a series

of patients were treated with cytotoxic drug combinations including hyaluronidase, most frequently after progression of disease following state-of-the-art regimens, but with the last cycle of chemotherapy repeated with addition of hyaluronidase. For diverse tumor entities a significant number of complete or partial responses were observed or patient status was changed to stable disease.

Modified treatments were most successful in squamous cell head and neck carcinoma, breast cancer, and myeloma. Responses were documented in astrocytomas, resulting in improved quality of life due to lower frequency of required surgical care, although survival was not prolonged. Under normal conditions hyaluronidase is not able to cross the blood–brain barrier, however, this limitation can be overcome in patients with brain tumors, many of whom appeared to have compromised blood–brain barriers (Vick et al., 1997). This was additionally demonstrated by an improved accumulation of the label sodium borocaptate for boron neutron capture therapy in astrocytomas following intravenous injection of hyaluronidase (Haselsberger et al., 1996). Two trials using hyaluronidase as an adjunct to intravesical mitomycin C therapy for bladder cancer, one of the studies randomized and one using a historical patient control group, pointed to a significant reduction in the number of recurrences. The levels of mitomycin C in the systemic circulation were not increased by hyaluronidase, and in all patients treated with hyaluronidase signs of increased metastasis were not observed.

Several findings of other groups supported our own observation of a significant role of the added hyaluronidase in the improved outcome of patients undergoing combination chemotherapy (Possinger, 1988; Thiruvengadam and Moran, 1995). Hyaluronidase enhanced the therapeutic effect of vinblastine in intralesional treatment of Kaposi's sarcoma (Smith et al., 1997). A pilot study using hyaluronidase in combination with chemotherapy and radiation therapy was performed in inoperable head and neck tumors (Klocker et al., 1995).

Another pilot study tested the combination in cases of mesotheliomas, where in consideration of the excessive production of hyaluronic acid, the concept of hyaluronidase seemed particularly promising (Jones et al., 1995). Thirty-eight advanced cases with mesothelioma were treated with good results, namely a 44% response rate (Israel, 1992; Breau et al., 1993). Thus hyaluronidase was shown to be effective also as adjuvant to systemic palliative therapy, although most of these results have up to now, not been confirmed by randomized trials.

The hyaluronidase preparations available for clinical application more than 20 years ago were of limited purity, containing mixtures of hyaluronidase, fragmented proteins and other enzymatic activities and of limited potency (activities of 1500 or 7500 IU), except the 200,000 IU package size. These first pilot results gained with these widely differing

dosages did not indicate a clear-cut correlation of hyaluronidase dosage to therapeutic and side effects. Finally, those clinical studies had to be terminated due to the withdrawal of the hyaluronidase preparations because of safety concerns by the pharmaceutical companies. New preparations containing human recombinant hyaluronidase may solve the problems associated with the use of bovine protein and of enzymatic contaminations. Experiments using recombinant hyaluronidase will help to define the possible role of proteolytic and glycolytic contaminations in bovine testicular hyaluronidase preparations.

Important support for the concept of hyaluronidase as adjuvants for chemotherapy have come from multiple pharmacokinetic experiments in animal models. Application of high doses of hyaluronidase improved the effects of cytotoxic drugs in a breast cancer and a melanoma xenograft animal model significantly, not only when applied locally but most importantly, also when applied intraperitoneally, far from the tumor site (Muckenschnabel et al., 1996; 1998). Hyaluronidase alone had no effect *in vivo*, however, in combination with melphalan markedly increased the concentration of the drug in tumor tissue. Furthermore, it was reported that treatment with hyaluronidase blocked lymph node invasion by tumor cells in an animal model of T cell lymphoma (Zahalka et al., 1995).

CONCLUDING REMARKS AND SUMMARY

In conclusion, hyaluronidase as an adjunct to chemotherapy increases the response of tumors to chemotherapeutic drugs in experimental animal models and in the clinical trials described above. The mechanisms responsible for the synergism of hyaluronidase with cytostatics *in vivo* are not clear. The most simple explanation is a breakdown of the physical barrier shielding tumor cells against drugs (Gately et al., 1984; Kohno et al., 1994; Jones et al., 1995). Solid tumors are partially protected against cytotoxic drugs by a limited physical access due to irregular vascularization and blood supply and dense stromal areas that impede the diffusion of chemotherapeutics, in addition to mechanisms of resistance at the cellular level. Transient disintegration of the intercellular structures, such as the degradation of hyaluronan by hyaluronidase, would be expected to facilitate the transport of drugs to tumor cells and thereby increase their therapeutic efficacy, as demonstrated for liver metastases of colorectal cancer for cisplatin (Civalleri et al., 1996). Interestingly, many human solid tumors contain high concentrations of hyaluronan, which correlate with high hyaluronan serum levels of the patients (Toole, 2004).

The interstitial pressure, which is known to be elevated in tumor tissues, can be decreased by hyaluronidase as recently shown (Eikenes et al., 2005). Intratumoral injection of hyaluronidase (1500 IU) one hour prior to i.v.

injection of liposomal doxorubicin increased tumor uptake four-fold. Additonally, in human osteosarcoma xenografts grown in mice, the closure of vessels was prevented and perfusion improved in response to hyaluronidase or collagenase (Tufto et al., 2007).

A further resistance mechanism of solid tumors is the vascularization deficit resulting in hypoxia, which might also be modulated by hyaluronidase. Degradation of hyaluronan by hyaluronidase yields a heterogeneous mixture of oligosaccharides and hyaluronan fragments of different sizes, to which certain biological functions have been ascribed, e.g. induction of irreversible phenotypic and functional maturation of dendritic cells during inflammation, induction of angiogenesis by stimulation of proliferation, and migration of vascular endothelial cells via multiple signaling pathways (Toole, 2004). These fragments may be responsible for effects of hyaluronidase distant from a local injection site, since the enzyme may become rapidly inactivated in the peripheral circulation. Jain et al. has demonstrated that transient normalization of tumor vascularization creates a ''window of opportunity'' for chemotherapeutic intervention (Jain, 2005). At the tumor cell level, the group of Kerbel has shown an adhesion-dependent multicellular tissue chemoresistance which has been demonstrated to be sensitive to hyaluronidase (St. Croix et al., 1996).

The role of hyaluronan has undergone re-evaluation from a passive component of connective tissues controlling viscosity and permeability, to an active role in cell survival and modulation of the malignant state (Toole, 2004; Girish et al., 2007). This glycosaminoglycan can bind to specific cell surface receptors of tumor cells, such as CD44 and RHAMM (receptor of hyaluronic acid-mediated motility) providing a cell coat as well as for intracellular signaling. Hyaluronan strongly promotes anchorage-independent growth by supplying survival signals. Hyaluronan oligomers are both high and low affinity ligands, and together with soluble CD44 or RHAMM disrupt this positive regulatory mechanism via hyaluronan receptors. Oligosaccharides generated by hyaluronidase may act synergistically with increases in permeability of the cellular matrix to improve the cytotoxic effects of drugs. Various combinations of all of these mechanisms may be the basis for the clinical results described above.

In summary, hyaluronidase seems to constitute a valuable adjunct to chemotherapy. When administered locally for bladder cancer and mesothelioma, the protective layer of cells may be removed and the physical barriers of the tumors compromised. The shutdown of intratumoral vessels and blood supply, increased interstitial pressure, and impeded permeability may be reduced. In systemic therapy, indirect effects of the enzyme, for example release of oligosaccharides and hyaluronan fragments may play important roles in addition to the direct effects. Otherwise, the synergistic effects of systemic chemotherapy with small amounts of

locally administered hyaluronidase, for example in myeloma patients with bone manifestations, may be difficult to explain. Since expression of hyaluronan and hyaluronidase seems to have different effects in different tumors, hyaluronidase in combination with chemotherapy may be helpful only in specific tumor entities, however, in a wide dose range. The clinical studies performed more than 20 years ago need to be repeated and evaluated using human recombinant hyaluronidase, in order to exclude side activities of impurities of the bovine preparations. Additionally, investigations into the mechanisms by which hyaluronidase improves the efficacy of specific anti-cancer drugs should be conducted.

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CHAPTER

20

Exploiting the Hyaluronan–CD44 Interaction for Cancer Therapy

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EXPLOITING THE HYALURONAN–CD44 INTERACTION FOR CANCER THERAPY

Hyaluronan (HA) mediates the connection between a cell and its local environment by acting as a structural component of the extracellular matrix and by signaling through cell surface receptors. CD44, the main HA receptor, regulates cell motility, growth, and survival during tissue growth and maintenance (McKee et al., 1996). Malfunctions in tissue development caused by aberrant interactions between CD44 and HA (CD44–HA) contribute to cancer growth and progression (Naor et al., 2002). In many types of cancer, CD44 expression differs substantially from the expression normally seen in healthy tissue; CD44 expression is up-regulated or CD44 is alternately spliced to produce non-native variants (Naor et al., 2002). Signaling pathways downstream of CD44 activation, when deregulated, lead to tumor growth, progression, and metastasis (Bourguignon et al., 2000; 2003; 2007). The composition of the extracellular matrix is also directly modulated during progression of some cancers; HA levels within the tumor extracellular matrix change (Toole, 2004). Because of the intimate relationship between the CD44–HA system and cell survival and growth, it is an increasingly investigated area for applications to anti-cancer chemotherapeutics.

This chapter will review the potential ways in which the CD44–HA interaction can be exploited in cancer therapy. HA is a high-molecular weight saccharide signaling molecule; it can be used as a drug (Alaniz et al., 2006; Hosono et al., 2007; Gilg et al., 2008), a drug carrier (Luo and Prestwich, 1999; Luo et al., 2000; 2002), or a CD44-targeting ligand (Eliaz and Szoka, 2001; Eliaz et al., 2004a; 2004b; Peer and Margalit, 2004a; 2004b).

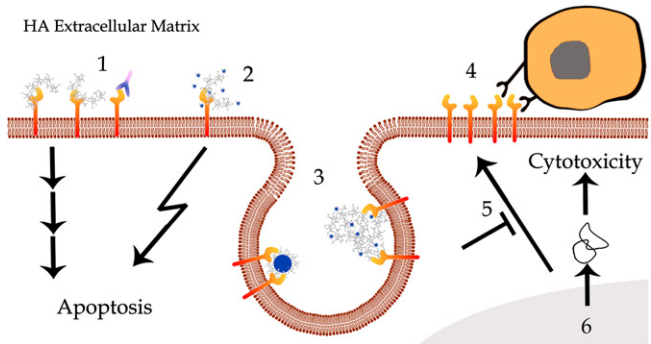

FIGURE 20.1 Schematic of CD44 Mediated Anti-Cancer Strategies (1) HA or anti-CD44 antibody induced matrix detachment which may lead to anti-growth signaling cascades, (2) HA induced cell sensitization to chemotherapeutic agents, (3) CD44 mediated HA drug carrier endocytosis, (4) CD44 targeted immune response (5) Genetic downregulation of CD44 production and (6) Production of cytotoxic proteins via CD44 splice-linked gene regulation within the nucleus.

This chapter will also discuss methods that reduce CD44 surface presentation (Subramaniam et al., 2007), interfere with the interaction of CD44 and the extracellular matrix (Peterson et al., 2000; Ahrens et al., 2001; Song et al., 2004), target CD44 (Tijink et al., 2006; Rupp et al., 2007), or activate CD44-mediated cell destruction (Harada et al., 2001; 2002; 2004; Dall et al., 2005).

The six distinct approaches to anti-cancer therapy which exploit the CD44–HA relationship are illustrated in Fig. 20.1: (1) Interference with CD44–HA interactions causing physical matrix disruption or modulation of the CD44 anti-growth signaling pathway; (2) HA-induced sensitization to chemotherapeutics; (3) Targeting of drugs to CD44; (4) Generation of an immune response to CD44; (5) Genetic down regulation of CD44 production; and (6) Expression of cytotoxic proteins by CD44 regulatory pathways.

INTERFERENCE WITH CD44-HA INTERACTIONS

Interference with CD44–HA Interactions by Soluble CD44

CD44, interacting with extracellular matrix HA, stabilizes and supports CD44 expressing cells. Disrupting association of tumor cells with the extracellular matrix can reduce the tumor burden by causing anti-growth signaling or by physically interfering with tumor expansion into surrounding tissue. Soluble HA binding proteins, anti-CD44 antibodies and exogenously added HA have the potential to reduce cancer progression by influencing CD44–matrix interactions through these mechanisms. A summary of studies which explore the effect of CD44–HA interactions on cancer progression is given in Table 20.1.

Soluble HA binding receptors, either expressed via stable transfection or added as immunoglobulin fusion constructs, efficiently decreased tumor growth (Sy et al., 1992; Bartolazzi et al., 1994; Peterson et al., 2000; Ahrens et al., 2001). The therapeutic effects of the soluble binding proteins were correlated with HA binding. Mutant proteins which could not bind HA had no anti-tumor benefits, therefore the disruption of CD44–HA interactions is implicated in the mechanism by which these treatments act to decrease tumor growth (Peterson et al., 2000; Ahrens et al., 2001). In murine models of lymphoma (Sy et al., 1992) and melanoma (Bartolazzi et al., 1994), CD44–immunoglobin fusion proteins inhibited tumor invasion and decreased cancer progression. Cancerous cells transfected to overexpress soluble CD44 exhibited significantly decreased HA binding *in vitro* and reduced tumor progression *in vivo* (Peterson et al., 2000; Ahrens et al., 2001). Mammary carcinoma cells that stably overexpressed soluble CD44 formed a much smaller primary tumor which failed to attach to the

TABLE 20.1A *In vitro* Interference with CD44–HA Interactions

Acting agent	Cell lines	Effect	Controls	Reference
Soluble CD44				
Soluble CD44	TA3/St (transfected murine mammary carcinoma)	Inhibited HA binding and internalization	Untransfected cells	Yu, 1997
Soluble CD44	TA3/St (transfected murine mammary carcinoma)	Inhibited anchorage-independent growth	Non-HA binding soluble CD44	Peterson, 2000
Soluble CD44	MV3 (human melanoma)	Inhibited HA binding, inhibited proliferation	Non-HA binding soluble CD44	Ahrens, 2001
Antibodies				
Mouse anti-CD44 monoclonal antibody (GKW.A3)	SMMU-2 (human melanoma)	Inhibited HA binding	HMW-HA	Guo, 1994
Mouse anti-CD44H monoclonal antibody (2C5)	IPNT-H (human pilocytic astrocytoma) IPSB-18 (human anaplastic astrocytoma) GO-G-CCM (human anaplastic astrocytoma) GO-G-UVW (human anaplastic astrocytoma)	Reduced matrigel invasion	Control antibodies	Merzak, 1994; Koochekpour, 1995
Mouse anti-CD44 monoclonal antibody (F10-44-2)	G-CCM (human anaplastic astrocytoma)	Reduced HA-matrigel invasion	HA-free matrigel	Radotra, 1997
Rat anti-CD44 monoclonal antibody	G-26 (mouse oligodendroglioma)	Reduced matrigel invasion	Untreated cells	Wiranowska, 1998

Mouse anti-CD44 monoclonal antibody	9L (rat gliosarcoma)	Inhibited ECM binding	Isotype immunoglobulins	Gunia, 1999
Mouse anti-CD44 antibodies (H90 and A3D8)	AML (human acute myeloid leukemia)	Induced differentiation	Control antibodies, HMW-HA, retinoic acid	Charrad, 1999
Mouse anti-CD44 monoclonal antibody	C6 (rat glioblastoma multiforme)	Inhibited ECM binding	Control antibodies	Breyer, 2000
Mouse anti-CD44 antibody (A3D8)	HL60 (human myeloblast leukemia) NB-4 (human promyelocytic leukemia)	Inhibited proliferation, inhibited drug-induced apoptosis	Control antibodies	Allouche, 2000
Mouse anti-CD44 antibodies (H90 and A3D8)	KG1a (human myeloblast leukemia) HL60 (human myeloblast leukemia) NB4 (human promyelocytic leukemia) THP-1 (human monocytic leukemia)	Inhibited proliferation, induced differentiation	Control antibodies, retinoic acid	Charrad, 2002
Anti-CD44 monoclonal antibody (HI44a)	AML (human acute myeloid leukemia)	Induced differentiation, induced apoptosis	Untreated cells	Song, 2004

(*continued*)

Table 20.1A (*continued*)

Acting agent	Cell lines	Effect	Controls	Reference
Free hyaluronan				
HA-O(3–12 disaccharides)	B16F10 (murine melanoma)	Inhibited proliferation	Chondroitin sulfate, HMW-HA	Zeng, 1998
HA-O(3–10 disaccharides)	TA3/St (murine mammary carcinoma) HCT-116 (human colon carcinoma) LX-1 (human lung carcinoma)	Inhibited anchorage-independent growth, induced apoptosis	Chitin, chondroitin sulfate, HMW-HA, anti-CD44 antibodies	Ghatak, 2002
HA-O(3–10 disaccharides)	C6 (rat glioblastoma multiforme) A172 (human glioblastoma) U87 (human glioblastoma)	Inhibited invasion, inhibited anchorage-independent growth	Chitin, glucuronic acid, *N*-acetyl glucosamine, HMW-HA, anti-CD44 antibodies	Ward, 2003
HA-O(2–7 disaccharides)	LBLa (invasive murine T-lymphoma) LBLc (murine T-lymphoma)	Increased apoptosis	HMW-HA, LMW-HA, anti-CD44 antibodies	Alaniz, 2006
HA-O(2–6 disaccharides)	MG-63 (human osteoblast osteosarcoma) LM-8 (murine osteosarcoma)	Inhibited growth, increased apoptosis, disrupted matrix attachment, inhibited matrigel invasion	HMW-HA, HA4, anti-CD44 antibodies	Hosono, 2007

TABLE 20.1B *In vivo* Interference with CD44–HA Interactions

Acting agent	Tumor model	Injection method	Effect	Reference
Soluble CD44				
Soluble CD44–Rg fusion protein	CD44N2.1 (transfected human Burkitt lymphoma) in BALB/c mice via intravenous injection	Co-injection with tumor implantation	Reduced tumor formation	Sy, 1992
Soluble CD44–Rg fusion protein	B16F10 (murine melanoma) in SCID mice via subcutaneous injection at the pump site	Subcutaneous pump	Reduced tumor formation	Bartolazzi, 1994
Soluble CD44	TA3/St (transfected murine mammary carcinoma) in A/jax mice via tail vein injection		Prevented tumor formation	Yu, 1997
Soluble CD44	TA3/St (transfected murine mammary carcinoma) in A/jax mice via intraperitoneal injection		Prevented peritoneal wall invasion	Peterson, 2000
Soluble CD44	1F6 (transfected human melanoma) in MF1 nude mice via subcutaneous flank injection		Prevented primary tumor growth	Ahrens, 2001

(*continued*)

Table 20.1B (*continued*)

Acting agent	Tumor model	Injection method	Effect	Reference
Antibodies				
Mouse anti-CD44 monoclonal antibody (1.1ASML)	AS-14 (CD44v transfected rat metastatic pancreatic adenocarcinoma) in BDX rats via subcutaneous foot-pad injection	Intravenous with tumor implantation	Prevented distant metastasis, increased survival time	Seiter, 1993
Mouse anti-CD44 monoclonal antibody (GKW.A3)	SMMU-2 (human melanoma) in SCID mice via subcutaneous injection	Co-injection with tumor implantation	Prevented primary tumor formation	Guo, 1994
		Intravenous	Prevented primary tumor formation if treated with or shortly after tumor cell injection	
		Intravenous	Prevented metastasis formation if given 7 days after tumor cell injection	
Rat anti-CD44 monoclonal antibody (IM 7.8.1)	LB (T-cell lymphoma) in BALB/c mice via subcutaneous flank injection	Subcutaneous	Prevented lymph node metastasis	Zahalka, 1995
Mouse anti-CD44 monoclonal antibody	9L (rat gliosarcoma) in athymic nude rats via intracerebral injection	Intracerebral	Reduce tumor size	Gunia, 1999
Mouse anti-CD44 monoclonal antibody	C6 (rat glioblastoma multiforme) in athymic nude rats via intracerebral injection	Intracerebral	Reduce tumor size	Breyer, 2000

Bispecific anti-CD44 × anti-Id antibody	38C-13 (murine B-cell lymphoma) in C3H/eB mice via subcutaneous injection.	Intraperitoneal	Reduced lymph node, bone marrow and spleen metastasis, increased survival time	Avin, 2004
Mouse anti-CD44 antibody (H90)	AML (human acute myeloid leukemia) in sublethally irradiated NOD-SCID mice via intravenous or intrafemoral injection.	Intraperitoneal	Reduced leukemic repopulation	Jin, 2006
Mouse anti-CD44 antibody (IM 7)	CML (murine bone marrow transduced chronic myeloid leukemia) in lethally irradiated B6X129 F2 mice via intravenous injection	Co-injection with tumor implantation	Reduced induction of CML-like leukemia	Krause, 2006

(*continued*)

Table 20.1B (*continued*)

Acting agent	Tumor model	Injection method	Effect	Reference
Free hyaluronan				
HA-O(3–12 disaccharides)	B16F10 (murine melanoma) in BALB/c mice via subcutaneous retroscapular injection	Subcutaneous pump	Reduced primary tumor growth	Zeng, 1998
HA-O(3–10 disaccharides)	LX-1 (human lung carcinoma) in BALB/c mice via subcutaneous dorsal injection	Subcutaneous pump	Reduced primary tumor growth	Ghatak, 2002
	Tas3/St (murine mammary carcinoma) in A/jax mice via subcutaneous dorsal injection			
HA-O(2–6 disaccharides)	LM-8 (murine osteosarcoma) in C3H/He mice via subcutaneous dorsal flank injection	Daily intratumoral	Prevented distant metastasis, slightly reduced primary tumor growth	Hosono, 2007
HA-O(3–10 disaccharides)	C6 (rat glioblastoma multiforme) in Sprague–Dawley rats via intraspinal injection	Intratumoral	Inhibited primary tumor growth, increased apoptosis	Gilg, 2008
HMW-HA	Hs578T (human breast carcinoma) in CD1 mice via orthotopic mammary fat pad injection	Intratumoral	Regressed primary tumor growth in {1/3} of animals	Herrera-Gayol, 2002

peritoneal wall. When implanted in mice, these transfected cells caused fewer fatalities than either untransfected cells or cells that expressed mutant soluble CD44 (Peterson et al., 2000). This decreased primary tumor growth was linked, at least partially, to increased apoptosis (Yu et al, 1997). Primary malignant melanoma tumors which were comprised of cells transfected to stably overexpress soluble CD44 grew more slowly than tumors comprised of untransfected cells or cells transfected to express the non-HA binding mutant soluble CD44 (Ahrens et al., 2001).

Interference with CD44–HA Interactions by Antibodies

Metastasis formation may be treated with therapies other than those used to decrease primary tumor growth. In several studies involving the treatment of tumors with anti-CD44 antibodies, metastatic progression could be efficiently blocked, but such treatment had no effect on the primary tumor growth. Substantial decreases in metastasis were seen in a murine model of human melanoma (Guo et al., 1994), a rat pancreatic adenocarcinoma model (Seiter et al., 1993), and a murine model of T-cell lymphoma (Zahalka et al., 1995).

The timing of treatment was critical as antibodies were most efficacious when given either with tumor implantation or after tumor implantation but before metastasis. In a spontaneous, highly metastatic rat pancreatic adenocarcinoma model, treatment with anti-CD44 variant antibodies caused only transient relief if given after metastasis formation. When treatment was given before metastasis occurred, substantial decreases in colonization were observed (Seiter et al., 1993). Following intravenous treatment of a human melanoma mouse model there was no decrease in primary tumor growth, but a substantial decrease in metastasis. Although primary tumor growth could be inhibited if antibody treatment began shortly after cell implantation, primary tumors could not be treated once they were formed. Intravenous antibody treatment was only effective at preventing metastasis at this point (Guo et al., 1994). In a murine T-cell lymphoma model, lymph node invasion was prevented when treated by subcutaneous anti-CD44 antibodies soon after tumor cell implantation (Zahalka et al., 1995).

An anti-metastatic effect was also observed with a bispecific antibody which recognized both CD44 and a cell surface marker of the malignant B lymphocyte. For therapeutic efficacy, this antibody required both antigens to be present on the surface of the target cell. Adhesion was largely achieved by the high affinity of the antibody for the malignant B lymphocyte marker, so less binding to non-malignant lymphocytes occurred. Once associated with malignant cells the anti-CD44 antibody portion could cause anti-CD44 mediated metastasis blockage. The low affinity of the anti-CD44 portion prevented off-target toxicities to CD44

expressing cells elsewhere in the body. *In vivo* treatment using the bispecific antibody resulted in significantly decreased lymphoma cell dissemination to the lymph nodes, spleen, and bone marrow (Avin et al., 2004).

In vitro studies of several human glioma cell lines suggested that anti-CD44 antibodies prevented invasion by significantly decreasing adhesion (Merzak et al., 1994; Koochekpour et al., 1995; Radotra and McCormick, 1997; Wiranowska et al., 1998). *In vivo*, tumor size could be decreased 80% or more in rats with gliosarcoma and glioblastoma multiforme brain tumors by treating the tumors with anti-CD44 antibodies. *In vitro* studies of both of these cell types showed these antibodies caused CD44-specific, dose-dependent cell detachment (Gunia et al., 1999; Breyer et al., 2000) without significant cytotoxicity (Breyer et al., 2000). This suggested the antibodies were functional *in vivo* partly due to their ability to physically limit matrix association.

Other anti-CD44 antibodies were found to be effective largely due to their ability to induce anti-growth signaling pathways. Anti-CD44 antibodies promoted differentiation and apoptosis in human acute myeloid leukemia (AML) (Charrad et al., 1999; Song et al., 2004). Two anti-CD44 antibodies induced cell differentiation in a manner similar to that of high molecular weight HA (HMW-HA) and oligosaccharide HA (HA-O). However, a third antibody that recognized a different domain of CD44 did not induce differentiation, suggesting that the mechanism required a ligand-like association with CD44 (Charrad et al., 1999). Decreased proliferation and differentiation was seen, to varying degrees, in several human myeloid cell line models of AML subtypes. Very immature AML subtype cells could be induced to differentiate by a combination of anti-CD44 antibodies and retinoic acid, a drug used for treatment of only one subtype of AML (Charrad et al., 2002). One of the antibodies that induced differentiation also interfered with drug-induced apoptosis for several common chemotherapeutics in two myeloid cell lines. The antibody that was not effective at inducing differentiation caused no reduction of drug effectiveness, so the mechanism of differentiation may be antagonistic to the pathway sensitized for drug induced apoptosis (Allouche et al., 2000).

Intraperitoneal administration of antibodies in a human AML transplanted mouse model increased differentiation, caused loss of homing capacity of leukemic stem cells and reduced leukemic repopulation, possibly due to decreased AML engraftment (Jin et al., 2006). Anti-CD44 antibodies also decreased the presentation of chronic myeloid leukemia (CML) in lethally irradiated mice injected with CML-generating bone marrow. Although blockage of engraftment did not reach significance, antibody pretreatment of bone marrow resulted in a moderate increase in survival time. This suggested the antibody treatment may be successful for multiple types of CD44 presenting leukemia (Krause et al., 2006).

Interference with CD44–HA Interactions by HA

The nature of HA as a matrix component and a signaling molecule allows it to act as a pro-apoptotic agent (Ghatak et al., 2002; Alaniz et al., 2006; Hosono et al., 2007; Gilg et al., 2008). In lung, mammary and colon carcinoma cell lines, anti-growth effects were seen with HA-O but not with HMW-HA, chitin, or chondroitin sulfate (chitin and chondroitin are saccharides with similar structure to HMW-HA) (Ghatak et al., 2002). T-lymphoma cells underwent apoptosis when exposed to HA-O but not when exposed to HMW-HA (Alaniz et al., 2006). Anti-tumor effects were traced, in part, to the decrease in anti-apoptotic signaling (Ghatak et al., 2002; Alaniz et al., 2006; Gilg et al., 2008).

Both HMW-HA and HA-O are effective as *in vivo* therapeutic agents for the treatment of cancer. Daily intratumoral injection of HMW-HA in a murine xenograft breast cancer model resulted in complete tumor regression in 25% of animals studied (Herrera-Gayol and Jothy, 2002). HA-O, injected subcutaneously via a slow infusion pump, inhibited tumor growth *in vivo* in a murine melanoma model (Zeng et al., 1998), a murine osteosarcoma model, and a murine lung carcinoma model (Ghatak et al., 2002). Ghatak and colleagues delivered HA oligomers via an osmotic pump, prior to implantation, in subcutaneously injected lung and mammary carcinoma murine tumor models. In both models, treatment with HA-O reduced the weight of the resultant primary tumor compared to untreated controls (Ghatak et al., 2002). Intratumoral injection of octasaccharide HA, but not HMW-HA or tetrasaccharide HA, inhibited primary tumor growth and significantly decreased lung metastasis in a murine osteosarcoma model (Hosono et al., 2007). In a rat spinal cord glioma model, HA-O injected near the tumor site suppressed growth by down regulating Akt-related anti-apoptotic signaling (Gilg et al., 2008).

Exogenously added HA may also decrease cell growth by limiting matrix interactions independent of cell growth and survival (Ward et al., 2003). Addition of exogenous HA-O, but not chitin oligosaccharides, prevented glioma cell invasion through a reconstituted basement membrane which contained HMW-HA. Invasion was also inhibited by both anti-CD44 antibodies and overexpressed soluble CD44, suggesting the anti-invasive effect of HA-O was largely mediated through interruption of matrix HA–CD44 interactions (Ward et al., 2003). In this case, HA-O did not induce apoptosis or substantially influence cell migration in the absence of HMW-HA, potentially because this type of motility is CD44 independent (Ward et al., 2003).

The viability of HA as a stand alone anti-cancer treatment is unclear, as results from a similar study showed HMW-HA increased metastatic potential when co-delivered during cell implantation in a colorectal mouse

model (Tan et al., 2001). Furthermore, the consequences of introducing free HA of any size into the body at concentrations high enough to exhibit anti-tumor effects may result in side effects. These side effects may be based on HA's interaction with endogenous CD44 on other cell types, such as leukocytes within the epithelium of the skin (Mackay et al., 1994).

In summary, multiple types of therapeutics can disrupt the CD44–HA interaction, resulting in decreased tumor burden by physically shielding the tumors from the surrounding milieu, which prevents their spread, or actively engaging CD44 to induce anti-growth and apoptotic signaling.

HA-INDUCED SENSITIZATION TO CHEMOTHERAPEUTICS

The signaling pathways induced by HA–CD44 association may cause cells to become more sensitive to co-delivered chemotherapeutic agents (Misra et al., 2003; Cordo Russo et al., 2008). In drug resistant human mammary carcinoma cells, HA-O suppressed two kinase pathways: MAP kinase and phosphoinositide 3-kinase. Suppression of these pathways sensitized the cells to several commonly used chemotherapeutics: doxorubicin, taxol, vincristin, methotrexate, and BCNU (Misra et al., 2003). Efflux of doxorubicin from drug-resistant lymphoma cells was inhibited by treatment with HA-O (Cordo Russo et al., 2008). HA modulation of the signaling may lead to potentiated anti-cancer therapies, as in the case of HMW-HA co-administered with doxorubicin, but this augmentation has yet to be clinically established (Rosenthal et al., 2005).

Conflicting evidence suggests the administration of free HA may also be antagonistic in certain HA-drug combinations. In T-cells, HA oligomers of approximately 18 disaccharides up regulated P-glycoprotein, resulting in increased dexamethasone efflux (Tsujimura et al., 2006). Head and neck squamous carcinoma cells exposed to HMW-HA were less sensitive to three commonly used anti-cancer therapeutics: cisplatin, methotrexate and Adriamycin, in a manner that could be blocked by anti-CD44 antibodies (Wang and Bourguignon, 2006a; Wang and Bourguignon, 2006b). This suggests sensitization may be specific to the drug, HA length or cell type.

TARGETING CHEMOTHERAPEUTICS

Targeting Chemotherapeutics to CD44 with HA

Deregulation of CD44 maintenance in cancers often leads to its over-expression on the surface of tumor cells (Naor et al., 2002). Targeting cytotoxic drugs to CD44 can localize therapies to areas where CD44 is highly expressed. This targeting is advantageous for successful anti-tumor

activity and can limit off-target effects. The area of HA targeting therapies has recently been reviewed (Platt and Szoka, 2008; Yadav et al., 2008).

CD44-associated HA can be endocytosed (Luo and Prestwich, 1999; Luo et al., 2000) carrying conjugated cargo, such as drugs or drug carriers, into the cell. Cellular uptake increases therapeutic efficiency of HA conjugated drugs (Coradini et al., 2004a, b; Eliaz et al., 2004a). The polymeric nature of HA yields multiple functional groups for chemical conjugation. Attaching drugs to HA benefits anti-cancer therapies by increasing drug cytotoxicity to CD44 overexpressing cancer cells while decreasing toxicity to healthy cells.

Carboranes, used in boron neutron capture therapy, must be localized to the tumor at high enough concentrations to deliver a therapeutic dose of thermal neutrons. Carboranes conjugated to the side-chains of HMW-HA accumulated to a high concentration within human bladder carcinoma, colorectal adenocarcinoma, and ovarian adenocarcinoma cell lines due to CD44 specific uptake mechanism (Di Meo et al., 2007; 2008). So far, this approach has yet to be evaluated in animal models of cancer.

A number of cytotoxic anti-cancer drugs including paclitaxel (Luo and Prestwich, 1999; Luo et al., 2000; Auzenne et al., 2007), doxorubicin (Luo et al., 2002), sodium butyrate (Coradini et al., 1999; 2004a,b), mitomycin c (Akima et al., 1996), and epirubicin (Akima et al., 1996) had increased CD44 specific internalization and cell cytotoxicity when conjugated to HA.

HA–paclitaxel increased cytotoxic delivery to CD44 overexpressing cancer cell lines including breast, colon, and ovarian carcinoma (Luo and Prestwich, 1999; Luo et al., 2000). HA-conjugated paclitaxel was taken up in a dose-dependent manner that could be blocked by competition with anti-CD44 antibodies or HMW-HA but not chondroitin sulfate (Luo et al., 2000), a sulfated polymeric sugar composed of the same two saccharides as HA.

HA–drug conjugates treat cancer *in vivo*. Studies performed by Akima and coworkers showed potent anti-metastatic effects of HA–mitomycin c injected subcutaneously in a murine model of Lewis lung adenocarcinoma (Akima et al., 1996). HA–paclitaxel conjugates injected locally to the tumor increased the mean survival time in a human ovarian carcinoma xenograft mouse model (Auzenne et al., 2007). Sodium butyrate–HA conjugates injected intratumorally or subcutaneously reduced primary tumor growth and lung metastasis in a murine Lewis lung carcinoma model (Coradini et al., 2004a, b) and a murine melanoma model (Coradini et al., 2004b). However, HMW-HA conjugates injected intravenously exhibited a short half-life due to HA specific clearance mechanisms. Conjugates collected in the liver and spleen (Coradini et al., 2004b).

Targeting Chemotherapeutics to CD44 with HA Drug Carriers

Incorporating the targeting properties of HA into large carriers may improve CD44 expressing cell-specific uptake, prolong circulation time

and limit drug access to many organs that would normally be damaged by exposure to chemotherapeutic agents. Liposomes, drug carriers comprised of a phospholipid bilayer and an aqueous core, coated with HMW-HA (Peer and Margalit, 2004a, b) or HA-O (Eliaz et al., 2004a, b) showed specific uptake into CD44 expressing cells. HA coated liposomes can be created in two ways: conjugating HMW-HA to the surface via multiple site attachment or conjugating HA-O to individual lipids via a single connection at HA's reducing end. Either of these liposomes improved *in vitro* uptake of doxorubicin (Eliaz and Szoka, 2001; Eliaz et al., 2004a, b; Peer and Margalit, 2004a) in a manner that was shown to increase the potency of doxorubicin (Eliaz et al., 2004a) and mitomycin c (Peer and Margalit, 2004b) in CD44 expressing cells.

HMW-HA liposomes released mitomycin c more slowly than uncoated liposomes (Peer and Margalit, 2000; 2004b). *In vivo* HMW-HA bearing liposomes delivering either doxorubicin or mitomycin c increased the mean survival time in murine models of intraperitoneal ascites tumors, solid colon or pancreatic carcinoma tumors and metastatic melanoma (Peer and Margalit, 2004a, b). Mice with foot-pad colon carcinoma tumors treated with three doses of HMW-HA liposomes, encapsulating mitomycin c or doxorubicin, experienced long-term survival (Peer and Margalit, 2004a, b). Mice with metastatic lung melanomas also experienced long term survival when treated with doxorubicin containing HMW-HA liposomes (Peer and Margalit, 2004a).

HA targeted liposomes showed specific uptake in CD44 expressing cells. These carriers also have *in vivo* characteristics that improved tumor toxicity, such as prolonged circulation time, increased tumor accumulation, and sustained release parameters (Peer and Margalit, 2000; 2004a,b). Combining the tumor targeting characteristics of HA with the pharmacokinetic benefits of drug carriers appeared to efficiently treat tumors.

Nanoparticle systems can be constructed from ionic polymers, such as HMW-HA. HA nanoparticles were developed to improve the delivery of cisplatin. The physical solubility limitation of cisplatin was overcome by creating an ionic HA–cisplatin complex (Jeong et al., 2007). HMW-HA was partially degraded to low molecular weight HA (LMW-HA). An ionic interaction between cisplatin and HA formed nanoparticles of approximately 100–200 nm. The nanoparticles could be degraded by hyaluronidase to release cisplatin. The group did not investigate CD44 mediated cell uptake or test the anti-tumor activity of these compounds (Jeong et al., 2007).

Targeting Chemotherapeutics to CD44 with Antibodies

Anti-CD44 antibodies conjugated to radiolabels and cytotoxic drugs successfully target to CD44 (Heider et al., 1996). Antibody conjugates are

the most clinically advanced CD44 targeting therapy. Murine anti-CD44v6 monoclonal antibody conjugates were given to patients with squamous cell carcinoma of the head and neck. Patients receiving radiolabeled anti-CD44 antibodies experienced low anti-mouse immune responses. The conjugate accumulated preferentially in tumors and had a half-life of approximately 35 hours (Stroomer et al., 2000). Anti-CD44 antibodies conjugated to an anti-cancer drug were also well tolerated. No immune response was observed and the antibodies circulated for approximately 4 days (Sauter et al., 2007).

Humanized monoclonal antibodies against CD44v6 stabilized disease in refractory head and neck cancers when coupled to the radioisotope rhenium-186 (Verel et al., 2002; Borjesson et al., 2003; Colnot et al., 2003). Although the antibody–drug conjugate showed favorable disease stabilization in several patients with squamous cell carcinoma of the head and neck (Tijink et al., 2006), it showed unfavorable tumor to non-tumor ratios in patients with CD44 variant up regulated breast cancer (Koppe et al., 2004; Rupp et al., 2007). These trials were stopped due to fatal off-target toxicities within the skin. These effects show the importance of restricting the targeted carrier from healthy sites in the body that express moderate levels of CD44, such as the skin (Tijink et al., 2006).

CD44 MEDIATED CANCER IMMUNOTHERAPY

Although the interaction of CD44 and HA affords opportunities for delivery of therapeutic anti-cancer drugs, invoking an immune response against CD44 variants expressed on tumors could eliminate the need for systemic delivery of cytotoxic drugs and may result in a longer lasting effect.

Most cancer vaccines target tumors through stimulation of cytotoxic T-lymphocytes that recognize tumor specific antigens. One method to induce this type of T-cell response is to expose the T-cells to dendritic cells primed with antibody coated tumor cells (Pilon-Thomas et al., 2006). Dendritic cells exposed to CD44 expressing B16 melanoma cells coated with anti-CD44 antibodies were injected intravenously on three occasions to vaccinate mice. Sixty percent of mice immunized with these pulsed dendritic cells did not develop primary tumors after challenge with subcutaneous injection of CD44 positive metastatic melanoma cells (Pilon-Thomas et al., 2006). The number of metastatic lung tumors derived from intravenously injected metastatic melanoma cells also decreased upon treatment with dendritic cells. The dendritic cells were injected one day after the metastatic melanoma cells (Pilon-Thomas et al., 2006). Most of the control mice, that were either untreated or treated with unpulsed dendritic cells, formed some distant metastases in the gastrointestinal track or brain

(Pilon-Thomas et al., 2006). All mice injected with pulsed dendritic cells remained free of distant metastases.

Cytotoxic T-cells can be designed to recognize CD44 by genetically engineering a response into the T-cells (Hekele et al., 1996; Dall et al., 1997; 2005). This modification gives them the ability to lyse CD44 variant expressing cells. Murine cytotoxic T-cells transduced with a fusion gene, a single chain anti-CD44 variant antibody fused to a T-cell receptor signal transduction domain, gained the ability to recognize and lyse CD44v6-expressing pancreatic cells. Daily intravenous injection of 3×10^7 T-cells into mice bearing established subcutaneous rat pancreatic CD44v6-expressing carcinoma tumors significantly decreased tumor growth (Hekele et al., 1996). Daily intratumoral injection of 1×10^6 cytotoxic T-cells and the cytokine signaling molecule IL-2 resulted in consistent growth inhibition of established CD44v7/8 expressing cervical carcinoma tumors in a murine model (Dall et al., 2005). These cytotoxic T-cells were engineered to express a fusion construct of a single chain anti-CD44v7/8 antibody and a T-cell receptor signal transduction domain (Dall et al., 2005).

A bispecific antibody was developed that crosslinked a macrophage complement receptor and CD44. Macrophages are effector immune cells and, when associated with cancer cells, lyse them. An anti-CD44v6 anti-CR3 bispecific antibody increased macrophage–tumor cell interaction in a CD44v6 specific manner. However, when used *in vivo* this type of antibody was less effective than a control antibody which recognized only CD44v6. The bispecific antibody increased animal mortality when injected intraperitoneally in a rat adenocarcinoma model (Somasundaram et al., 1996). Macrophage mediated immune recruitment has yet to show robust anti-tumor activity.

Each of these immunotherapy methods successfully initiated a response from the host immune system directly against the tumor. Although these systems eliminate the need for cytotoxic drugs, their complexity and the fact that the treatment must be optimized on a patient to patient basis may limit their clinical usage.

GENE THERAPY BY DOWN REGULATION OF CD44 PRODUCTION

Genetic therapies which decrease the concentration of CD44 on the surface of the cell may also prevent metastatic spread of primary tumors (Harada et al., 2001; Subramaniam et al., 2007). CD44 expression was nearly completely down regulated by stably transfecting human colon adenocarcinoma cells with plasmids encoding antisense CD44 cDNA. These cells had a significantly reduced ability to bind HA *in vitro* (Harada

et al., 2001). When mice were injected with transfected cells, tumors developed more slowly than when they were injected with non-transfected cells. Intrasplenically injected cells developed no metastatic liver colonies and fewer peritoneal nodules (Harada et al., 2001).

Stable transfection of human metastatic colorectal carcinoma cells, with plasmids for CD44 variant specific siRNA, down regulated CD44v6 but not CD44 (Reeder et al., 1998). This transfection did not affect cell proliferation or HA binding ability *in vitro.* When transfected colorectal cells were allowed to grow as subcutaneous flank tumors, no difference in primary tumor growth was observed compared to untransfected cells (Reeder et al., 1998). However, the metastatic ability of the siRNA producing cells was drastically reduced. When the cells were injected into the spleen of nude mice these mice developed no metastatic liver tumors (Reeder et al., 1998).

In another study of human metastatic colon carcinoma, cells stably transfected to express siRNA against the transmembrane domain of CD44 showed decreased adhesion and decreased cell survival *in vitro.* Mice injected subcutaneously with siRNA transfected cells formed smaller tumors than mice injected with control carcinoma cells that expressed normal levels of CD44 (Subramaniam et al., 2007).

Tumors established from untransfected colon carcinoma cells were treated with direct injection of a polyethylenimine complex containing siRNA CD44 plasmid DNA. Mice were given twelve bi-weekly intratumoral injections beginning one week after tumor cell implantation. These mice experienced decreased tumor growth and one mouse had complete tumor regression; the study was continued into the seventh week. Long-term survival was not investigated (Subramaniam et al., 2007).

In each of the studies, stable transfection of cells was required to produce substantially decreased metastatic spread. Repeated intratumoral injection of plasmid DNA encoding siRNA provided some suppression of tumor growth (Subramaniam et al., 2007), but clinical siRNA therapy is not currently available. If simple robust delivery systems can be developed, the use of siRNA for therapeutic anti-cancer therapies may become a clinical option (Szoka, 2008).

GENE THERAPY BY PRODUCTION OF CYTOTOXIC GENES THROUGH CD44 REGULATION

Genetic deregulation of the CD44 system may promote cancer progression. Aspects of this deregulation, specifically up regulation of CD44 variants, can be exploited in anti-cancer therapeutics without attempting to alter CD44 levels. This is possible because cytotoxic genes activated by a CD44 variant-specific mRNA splicing mechanism produce

cytotoxic protein in cells that express the CD44 variant isotypes. Asman and colleagues cloned a prodrug converting enzyme (cytosine deaminase) under the control of the CD44v8/v9 splice domain (Asman et al., 1995). This recombinant CD44-cytosine deaminase fusion protein actively converted prodrug to drug (Asman et al., 1995). A similar approach placed alkaline phosphatase under the control of several different CD44 variant splice domains. Genes under the control of a v8/v9 domain were spliced in both CD44 and CD44 variant cells. Those genes controlled by a v9/v10 domain were only accurately spliced in CD44 variant expressing cells (Hayes et al., 2002; 2004). Prostate adenocarcinoma cells express CD44 variants 8, 9, and 10. When transfected with the v9/v10 alkaline phosphatase plasmid, the cells were sensitized to the prodrug etoposide phosphate (Hayes et al., 2002). Cells expressing CD44 but not the variant form were sensitive to etoposide, but not to the prodrug (Hayes et al., 2002).

These studies suggest that deregulation of the CD44 pathway at a genetic level can be usurped to express cytotoxic treatments at the tumor site. This may yield a treatment localized to areas of CD44 variant upregulation. However, some variants are endogenously expressed and may give rise to off-target effects. If efficient and robust gene carriers can be developed, this toxicity may be avoided by delivering the genes specifically to tumor sites.

CONCLUSION

This chapter describes six approaches which use the CD44–HA interaction to treat cancer in animal models and clinical settings. There are numerous opportunities for highly specific cancer therapy using drug conjugates, carriers, or antibodies that target drugs to CD44 or act to disrupt the interaction between CD44 and HA. Many of these methods can decrease tumor burden in animal models but have yet to show significant clinical utility. Further advances in the use of HA as a carrier or targeting molecule, antibodies as active agents, and methods of CD44 inactivation will have to be made before these treatments can be seen as clinically viable options.

Drugs conjugated to anti-CD44 variant antibodies are currently the most clinically explored therapy. However, these antibodies have severe off-target toxicities. With the current advances in siRNA delivery, downregulation of CD44 overexpression presents an attractive future avenue. The most obvious therapy for near-future clinical relevance is delivery of currently available cancer drugs via targeted carriers. These carriers may utilize HA or antibody targeting while still retaining long circulation and tumor specific accumulation. Furthermore, particles may be specifically

endocytosed by CD44 specific mechanisms, increasing drug potency. The many different approaches available for exploiting the CD44–HA interaction for anti-cancer treatments present opportunities for discoveries to develop and optimism that improved cancer therapies are on the horizon.

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CHAPTER

21

Hyaluronidase-2 and Its Role as a Cell-Entry Receptor for Sheep Retroviruses That Cause Contagious Respiratory Tract Cancers

A. Dusty Miller

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ONCOGENIC SHEEP RETROVIRUSES

Jaagsiekte sheep retrovirus (JSRV) causes pulmonary adenocarcinoma (also called sheep pulmonary adenomatosis or jaagsiekte) in sheep and goats (Palmarini et al., 1999). JSRV-induced tumors arise from epithelial cells in the lower airway, and tumor cells express markers of

type II alveolar and/or bronchiolar epithelial cells (Palmarini et al., 1995). Two strains of a closely related retrovirus called enzootic nasal tumor virus (ENTV) have been cloned from sheep (ENTV-1) (Cousens et al., 1999) and goats (ENTV-2) (Ortin et al., 2003) that share ~95% overall amino acid similarity with JSRV. ENTV can be found in the nasal fluid of animals with intranasal tumors, which eventually progress and cause severe cranial deformations and respiratory blockage, resulting in death (Vitellozzi et al., 1993). JSRV and ENTV can increase production of lung and nasal fluid and can spread by aerosolization of virus present in these secretions. JSRV and ENTV are present in many countries worldwide and have a significant economic and animal health impact. In addition, the disease induced by JSRV exhibits histological features similar to those of many human pulmonary adenocarcinomas, and study of adenocarcinoma induced by JSRV and ENTV may provide insights into the etiology of human lung cancer. While ENTV and JSRV do not appear to cause lung cancer in humans having occupational exposure to these viruses, this possibility has not been definitively excluded.

Until recently, oncogenic retroviruses were divided into those that cause cancer with long latency and do so by insertional activation of host oncogenes, and acutely transforming retroviruses that rapidly induce cancer as a result of virus acquisition and expression of host cell oncogenes. For example, Moloney murine leukemia virus induces leukemia over weeks to months by insertional activation of host cell oncogenes such as *lck* and *myc*, while the acutely-transforming Harvey murine sarcoma virus carries a mutant cellular *ras* oncogene and can acutely transform cells in culture and in animals (Rosenberg and Jolicoeur, 1997). JSRV and ENTV are examples of a small but growing new class of retroviruses that are acutely transforming and induce cancer as a direct result of expression of viral genes that show no relation to host cell genes. In the case of JSRV, cancer induction can occur in as little as 10 days in newborn sheep (Sharp et al., 1983), showing that it is acutely transforming, yet JSRV does not contain sequences related to mammalian genes. JSRV and ENTV are simple retroviruses (Fig. 21.1) that carry genes required for viral replication and that lack accessory genes typical of complex retroviruses or cell-derived genes typical of most acutely-transforming retroviruses. Analysis of the transforming activity of JSRV and ENTV in cell culture has revealed that the *env* genes of these viruses are necessary and sufficient to induce transformation (Dirks et al., 2002; Maeda et al., 2001; Rai et al., 2001). The primary role of Env in viral replication is to promote virus entry into cells following binding to specific cell-surface receptors, and it seemed likely that these receptors might play a key role in transformation as well.

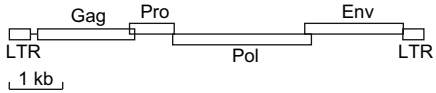

FIGURE 21.1 Genetic structure of JSRV and ENTV. The structure of the integrated DNA form of the retroviruses is shown. Long terminal repeat (LTR) sequences that function to initiate and terminate mRNA transcription are shown flanking the protein coding regions. Protein coding regions are: Gag, virion core polypeptide; Pro, protease; Pol, reverse transcriptase (polymerase) and integrase; Env, viral coat protein (envelope) required for cell entry. The reading frames of the protein coding regions are indicated by the elevation of the boxes, for example, the Pro and Env coding regions are in the same reading frame but Pol and Gag are in different reading frames. kb, distance in kilobases.

IDENTIFICATION OF HYAL-2 AS THE CELL-ENTRY RECEPTOR FOR JSRV AND ENTV

Retrovirus entry into cells depends on the presence of specific proteins that bind the viral Env protein and help trigger conformational changes in Env that lead to fusion of the virus and cell membranes and entry of the virus core into the cell. A wide variety of proteins have been found to serve as receptors for different retroviruses, based primarily on their ability to promote virus entry after expression in cells that are not naturally permissive for virus entry (Table 21.1). In most cases, a single protein suffices to render otherwise non-permissive cells susceptible to virus entry. Typically, these proteins promote virus binding, and may also promote virus fusion with the cell membrane. For other viruses (for example, HIV) there are distinct binding and fusion receptors that are required for virus entry. Retrovirus receptors are key determinants of the species and cell types that a retrovirus can infect, and thus are primary determinants of the host range and the type of disease induced by the virus.

To identify the cell-entry receptor for JSRV we used a retroviral vector that encodes human placental alkaline phosphatase (AP) and that was packaged into virions bearing the JSRV Env protein on the virion surface (Rai et al., 2000). In early experiments we found that this vector could transfer and express (transduce) the AP marker protein gene to sheep and human cells, but not to rodent cells, including those from mice, rats, and hamsters. This allowed us to develop a genetic screen to identify the human gene that when expressed in rodent cells would allow vector transduction. As target cells we used a set of 80 hamster cell lines carrying different fragments of DNA that had been produced by fusing hamster cells with irradiated human cells. This allowed us to identify the chromosomal location of the receptor within a few hundred kilobase pairs of DNA (Rai et al., 2000). We were lucky to find that this region had been cloned as a set of overlapping cosmid clones, and it was relatively straightforward to identify the gene encoding the receptor by testing

TABLE 21.1 Retrovirus Receptors

Retrovirus	Receptor	Type[a]	Function
Human immunodeficiency virus, simian immunodeficiency virus	CD4 and CXCR4, CCR5, others	TM1 TM7	Immune function G protein-coupled chemokine receptors
Feline immunodeficiency virus	CD134 and CXCR4	TM1 TM7	Immune function G protein-coupled chemokine receptor
Human T-cell leukemia virus	GLUT-1	TM12	Glucose transport
Ecotropic murine leukemia virus	CAT-1 (SLC7A1)	TM14	Basic amino acid transport
Gibbon ape leukemia virus, amphotropic murine leukemia virus, 10A1 murine leukemia virus, feline leukemia virus type B, woolly monkey virus	Pit1 (SLC20A1) or Pit2 (SLC20A2)	TM10-13 TM10-13	Phosphate transport Phosphate transport
RD114, type D simian retroviruses, baboon endogenous virus, human endogenous retrovirus type W	RDR (SLC1A5) or RDR2 (SLC1A4)	TM9-10 TM9-10	Neutral amino acid transport Glutamate and neutral amino acid transport
Xenotropic and polytropic murine leukemia viruses	XPR1	TM8	G protein-coupled signaling? Transport?
Feline leukemia virus type A	Thtr1	TM12	Thiamine transport
Feline leukemia virus type C	Flvcr	TM12	Heme export
Feline leukemia virus type T	Felix and Pit1 (SLC20A1)	soluble TM10-13	Env-like protein Phosphate transport
Pig endogenous retrovirus type A	Par-1 (GPR172A) or Par-2 (GPR172B)	TM10-11 TM10-11	G protein-coupled receptor G protein-coupled receptor
M813 murine leukemia virus	Smit1 (SLC5A3)	TM14	*myo*-inositol transport
Avian leukosis virus type A	Tva	TM1	LDL receptor-like protein
Avian leukosis virus types B, D, E	Tvb	TM1	Fas/TNFR-like receptor

Table 21.1 (*continued*)

Retrovirus	Receptor	Type[a]	Function
Avian leukosis virus type C	Tvc	TM1	Butyrophilin-like (immunoglobulin superfamily)
Avian leukosis virus type J	NHE1 (SLC9A1)	TM12	Na+/H+ antiporter
Mouse mammary tumor virus	Tfr1	TM1	Transferrin receptor
Jaagsiekte sheep retrovirus, enzootic nasal tumor virus	HYAL-2	GPI-anchored	Hyaluronidase (weak)

[a]TM indicates a transmembrane protein and the number after TM indicates the number of times the protein is predicted to span the membrane. GPI-anchored indicates a glycosylphosphatidylinositol-anchored membrane protein.

hamster cells for JSRV vector susceptibility following transfection of individual cosmids into the cells (Rai et al., 2001). This genetic analysis indicated that only one gene served as a receptor for JSRV, but to reinforce this conclusion, we tested all of the human hyaluronidase family members for receptor activity, and found no activity associated with human HYAL-1, HYAL-3, HYAL-4, or Spam1 (Rai et al., 2001). These data indicate that HYAL-2 is the only protein in the human genome that functions as a JSRV receptor.

We also tested hyaluronidase family members from other species for receptor function to determine if receptor function correlates with the ability of the JSRV vector to transduce cells from different species (Fig. 21.2). Indeed, mouse HYAL-2 functioned poorly as a receptor for JSRV vector cell-entry when expressed in mouse or hamster cells, consistent with the inability of the JSRV vector to transduce mouse cells (Rai et al., 2001). In contrast, sheep HYAL-2 functioned well as a receptor for JSRV vector cell-entry when expressed in mouse or hamster cells, consistent with the high susceptibility of sheep cells to JSRV vector transduction (Dirks et al., 2002). An intermediate result was obtained for rat HYAL-2, where overexpression of rat HYAL-2 in mouse, hamster, or rat cells rendered the cells susceptible to JSRV vector transduction, but rat cells are normally resistant to vector transduction (Liu et al., 2003a). Additional experiments showed that JSRV Env binds rat HYAL-2 less well than it does human HYAL-2, supporting the interpretation that higher levels of rat HYAL-2 are required to promote efficient JSRV vector transduction than are normally expressed on rat cells (Liu et al., 2003a). In conclusion, these experiments show that expression of a functional HYAL-2 protein is the primary determinant of JSRV Env-mediated virion entry into cells.

Using a retroviral vector encoding AP packaged into virions bearing either the ENTV or JSRV Env proteins on the virion surface, we found that

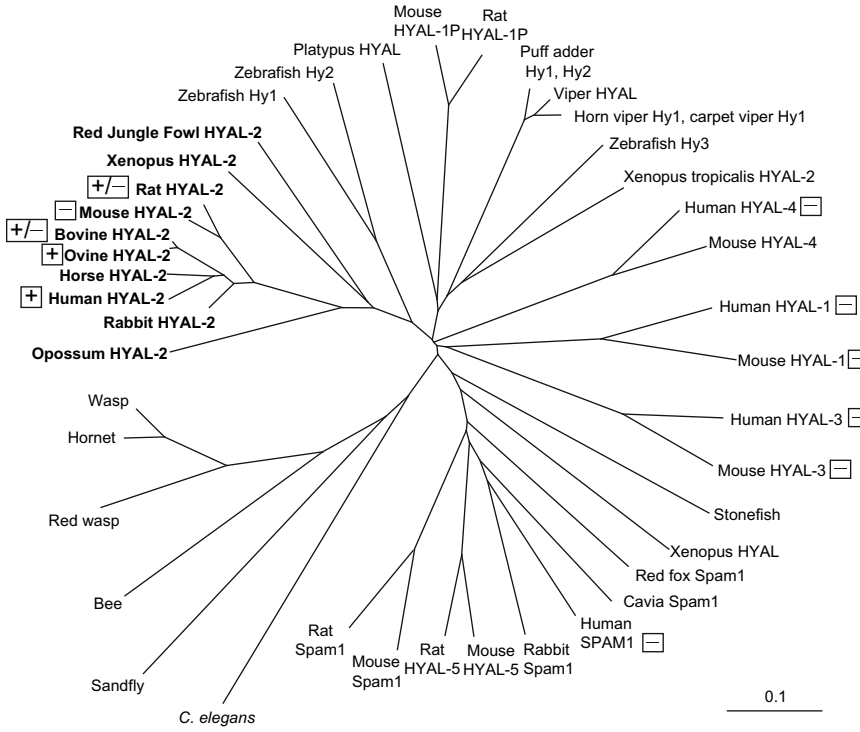

FIGURE 21.2 Receptor activity of HYAL-2 orthologs (bold) and paralogs. Proteins related to HYAL-2 obtained by BLAST search of GenBank are shown. Proteins that exhibit high receptor activity when expressed in cells that are normally non-permissive for JSRV and ENTV vector transduction are indicated by "+," those that exhibit moderate receptor activity are indicted by "+/–," and those that exhibit very low to no activity are indicated by "–" (Dirks et al., 2002; Duh et al., 2005; Rai et al., 2001; Van Hoeven and Miller, 2005).

ENTV Env promotes infection of a more restricted range of cell types than does the JSRV Env (Dirks et al., 2002). Given the similarity in ENTV and JSRV Env amino-acid sequences, we first tested whether ENTV Env might use HYAL-2 for cell entry as does JSRV Env. Indeed, both sheep and human HYAL-2 can serve as cell-entry receptors for virus bearing the ENTV Env. Interestingly, HYAL-2 expression was not sufficient to promote entry of the ENTV vector into all cells, indicating that other factors are important for ENTV Env-mediated entry into cells (Van Hoeven and Miller, 2005).

HYAL-2 LOCATION AND ENZYMATIC ACTIVITY

HYAL-2 was initially identified as a lysosomal hyaluronidase by addition of a green fluorescent protein (GFP) tag to the carboxy terminus of HYAL-2

and by showing that GFP fluorescence localized to lysosomes after expression of the hybrid protein in a rat glioma cell line (Lepperdinger et al., 1998). HYAL-2 exhibited low but detectable hyaluronidase activity with an acidic pH optimum in these experiments. However, later studies have conclusively shown that HYAL-2 is actually a glycosylphosphatidylinositol (GPI)-anchored cell-surface protein, consistent with its role as a cell-surface receptor for sheep retroviruses (Duh et al., 2005; Liu et al., 2003a; 2004; Miller et al., 2006; Rai et al., 2001; Van Hoeven and Miller, 2005). GPI-anchored proteins have an endoplasmic reticulum (ER) signal sequence at the amino terminus, which directs the proteins to the ER and is removed during protein translocation into the ER, and have hydrophobic carboxy termini that are replaced with a GPI anchor that tethers the proteins to the cell surface. The latter feature likely explains the original result indicating that HYAL-2 was a lysosomal protein — the GFP tag added to the carboxy end of HYAL-2 in those experiments was likely removed during GPI anchor addition and was sent to lysosomes for degradation, while the processed HYAL-2 was exported to the cell surface.

HYAL-2 exhibits very low hyaluronidase activity in comparison to HYAL-1 or Spam1 under all conditions analyzed to date. While the hyaluronidase activity of HYAL-2 could be detected in cells engineered to greatly overexpress HYAL-2 by infection with a vaccinia virus vector encoding human HYAL-2 (Lepperdinger et al., 1998), we have had difficulty detecting HYAL-2 activity in cells transduced with a retroviral vector that encodes human HYAL-2 (Rai et al., 2001). Therefore we studied a soluble preparation of HYAL-2 made by inserting a stop codon into the human HYAL-2 cDNA at the position of the GPI-anchor cleavage site, and by expressing this truncated protein in insect cells using a baculovirus vector (Vigdorovich et al., 2005; 2007). The endoplasmic reticulum signal sequence is properly removed from the translated protein, and without the hydrophobic tail and GPI-anchor signal sequence, the protein is secreted from the cells. This secreted form of HYAL-2 (sHYAL-2) corresponds exactly to native HYAL-2 expressed on the cell surface except that it lacks the GPI anchor. The sHYAL-2 protein appears to be a properly folded monomeric protein by size-exclusion chromatography and is stable in solution at 4°C for months. Initially, the hyaluronidase activity of purified sHYAL-2 appeared to have a neutral pH optimum (Vigdorovich et al., 2005), but we later showed that this was due to the presence of a very small amount of a highly active baculoviral hyaluronan lyase (Vigdorovich et al., 2007), and that the hyaluronidase activity of sHYAL-2 actually has an acidic pH optimum consistent with previous analysis of HYAL-2 activity in cultured cells. This activity could be greatly reduced by mutation of amino acid residues that correspond to the active site residues common to the bee venom and Spam1 hyaluronidases, indicating that the active site for hyaluronan digestion in HYAL-2 is similar to those of other hyaluronidases (Vigdorovich et al., 2007). However,

the hyaluronidase activity of sHYAL-2 is ~400-fold lower than that of Spam1 (Vigdorovich et al., 2007).

The availability of purified sHYAL-2 allowed us to further analyze the kinetics of hyaluronan degradation by HYAL-2 (Vigdorovich et al., 2005; 2007). Others have noted a 20 kDa intermediate of hyaluronan degradation that appeared to be uniquely associated with hyaluronan degradation by HYAL-2, and that this intermediate did not disappear even after prolonged incubation with HYAL-2 (Lepperdinger et al., 1998). We also find this intermediate following digestion of hyaluronan with sHYAL-2, but in contrast, this intermediate can be completely digested following prolonged incubation with sHYAL-2. Indeed, similar kinetics of hyaluronan digestion are observed for HYAL-1 and Spam1, with initial rapid digestion of hyaluronan to a 20 kDa intermediate followed by a 25-fold slower digestion of the 20 kDa form to smaller products. Thus digestion of hyaluronan by HYAL-1, HYAL-2 and Spam1 appears to follow similar biphasic kinetics involving a relatively stable 20 kDa intermediate corresponding to 50–60 disaccharide units.

Although purified HYAL-2 has low hyaluronidase activity, it is possible that other cellular proteins or cofactors might modulate HYAL-2 activity and/or be required for conversion of HYAL-2 into a more active enzyme. Indeed, a requirement for CD44 to promote acidification of the extracellular environment and activate the hyaluronidase activity of HYAL-2 has been described (Bourguignon et al., 2004; Harada and Takahashi, 2007). Given the long incubation times used for detection of hyaluronidase activity in these experiments, it still appears that HYAL-2 is a relatively weak hyaluronidase, although a direct comparison of HYAL-2 activities to a highly active hyaluronidase such as Spam1 was not performed. Perhaps in the local space adjacent to the cell only a small amount of hyaluronidase is required to mediate biologically relevant changes in hyaluronan properties and production of a highly active enzyme would be deleterious.

HYAL-2 ROLE IN SHEEP RETROVIRUS ONCOGENESIS?

Interaction of JSRV and ENTV Env proteins with human HYAL-2, location of the human HYAL-2 gene in the 3p21.3 lung cancer tumor suppressor locus, and the presumed role of HYAL-2 in metabolism of the extracellular matrix all pointed to a potential role of HYAL-2 in transformation by the sheep retrovirus Env proteins. Support for this hypothesis was provided by studies in the human bronchial epithelial cell line BEAS-2B (Danilkovitch-Miagkova et al., 2003). In these cells, HYAL-2 can bind to the RON receptor tyrosine kinase rendering it inactive. JSRV Env can transform the cells, and in cells expressing Env, Env associated with HYAL-2 and caused its degradation, releasing

RON from suppression by HYAL-2 and activating the Akt and mitogen-activated protein kinase oncogenic pathways. Most importantly, expression of a dominant-negative RON protein blocked Env transformation of the cells indicating that RON played a critical role in Env transformation.

On the other hand, JSRV and ENTV Env proteins cannot mediate virus entry into mouse cells and do not bind mouse HYAL-2 (Liu et al., 2003a), yet both Env proteins can transform cultured NIH 3T3 mouse fibroblasts (Liu et al., 2003a, b; Maeda et al., 2001). Furthermore, expression of either JSRV or ENTV Env in mouse lung can induce lung adenocarcinoma similar to that seen in sheep infected with replication-competent JSRV (Fig. 21.3) (Wootton et al., 2005; 2006a,b). These results indicate that mouse HYAL-2 plays no role in oncogenic transformation by either Env protein in mice. Whether HYAL-2 plays some role in sheep tumorigenesis is uncertain but an interaction of Env with HYAL-2 seems unlikely to be required based on the results in mice.

So how do the JSRV and ENTV Env proteins transform cells if not by interaction with HYAL-2? Several studies have shown that sequences in the cytoplasmic domain of the Env proteins are critical for transformation,

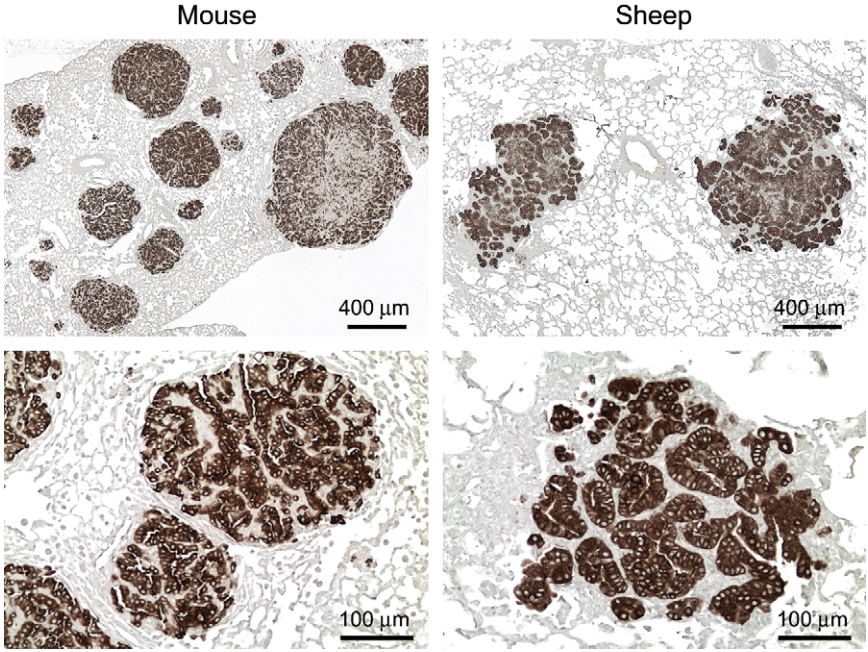

FIGURE 21.3 Lung tumors induced by Env expression in mice and JSRV infection in sheep. Fixed paraffin-embedded lung sections were stained for JSRV Env expression using a monoclonal antibody against Env which stains transformed lung cells in tumors. For more details see Wootton et al. (2006b).

and that oncogenic signaling occurs through the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways (Liu and Miller, 2007). The mechanisms by which the Env proteins activate these pathways are currently unknown.

HYAL-2 ROLE IN SHEEP PLACENTAL MORPHOGENESIS

Perhaps one of the most interesting findings relating to the interaction of sheep retrovirus Env proteins with HYAL-2 is the role of Env proteins synthesized from endogenous sheep retroviruses and HYAL-2 in placental morphogenesis in sheep. Mammals carry many copies of retroviruses in their genomes. Sheep carry ~20 copies of endogenous retroviruses related to JSRV and ENTV, but the Env proteins synthesized from these viruses are either nonfunctional or contain mutations that render the Env proteins non-transforming. However, some of these Env proteins can still interact with HYAL-2, and in this case, can mediate fusion not between virions and cells but between cells. It has been hypothesized that endogenous retroviruses play a role in mammalian reproduction, particularly in placental morphogenesis, because intact retroviral Env genes are expressed in the syncytiotrophoblasts of human and mouse placenta and can elicit fusion of cells in culture. The importance of endogenous sheep retrovirus Env expression during pregnancy in sheep was confirmed by administration of morpholino antisense oligonucleotides to block Env expression *in utero*, which resulted in termination of pregnancy (Dunlap et al., 2006). This study dramatically confirms that retroviruses are not simply pathogens but can contribute in a positive way to mammalian evolution, and shows that HYAL-2 plays a critical role in sheep reproduction. Further work is necessary to decipher other potential functions of HYAL-2 in mammals.

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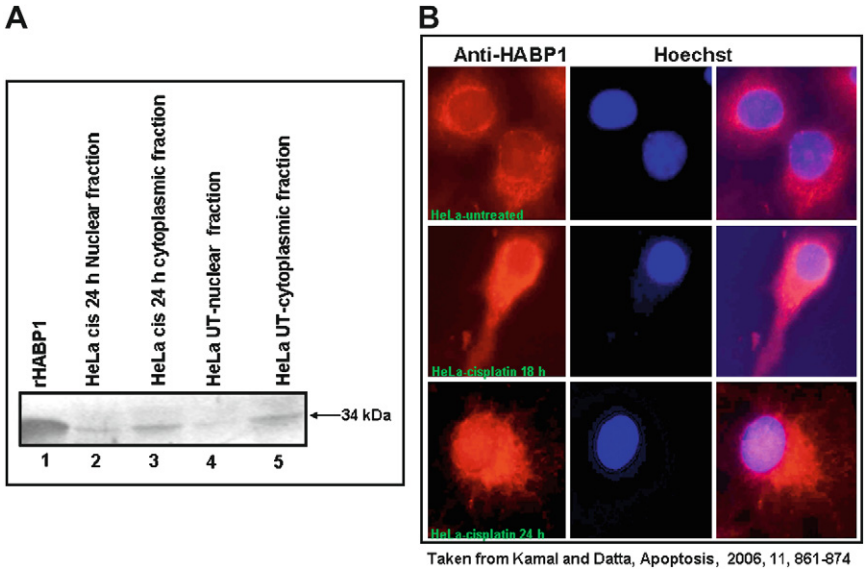

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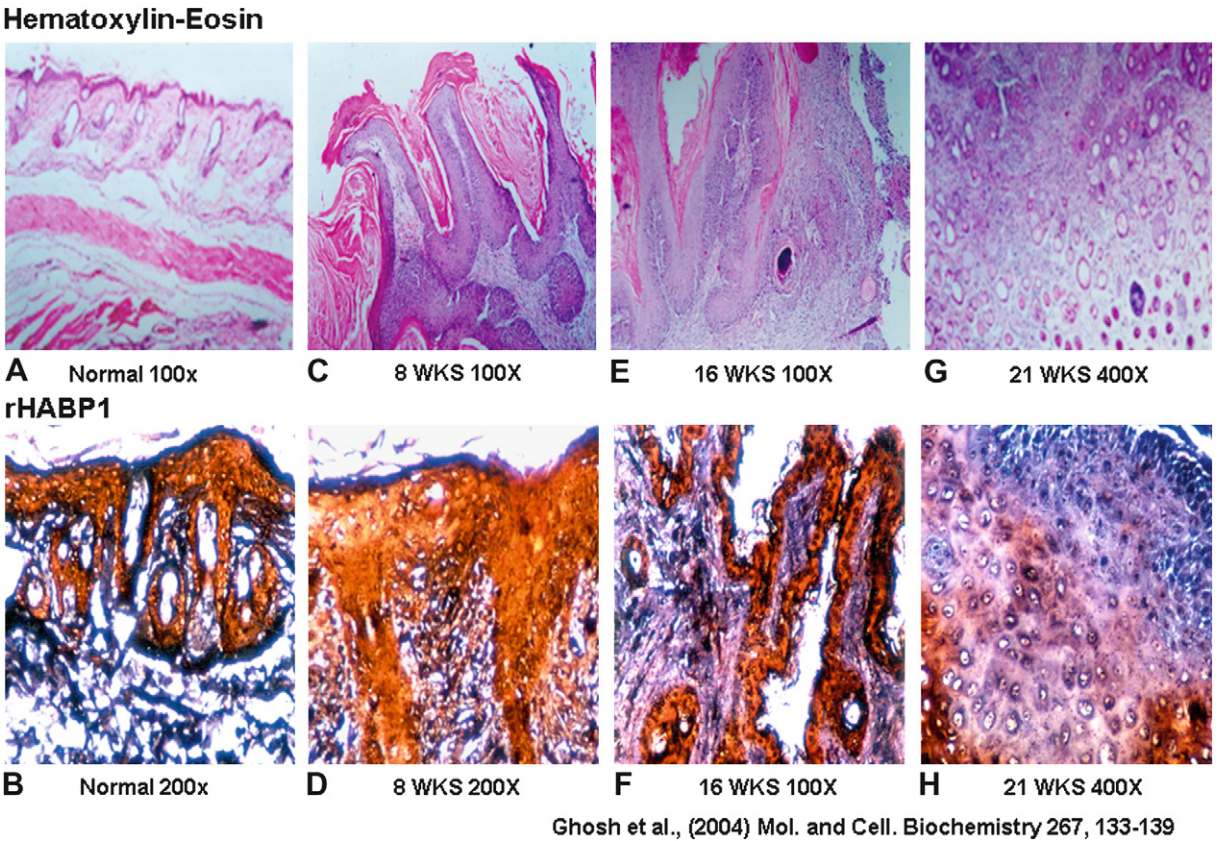

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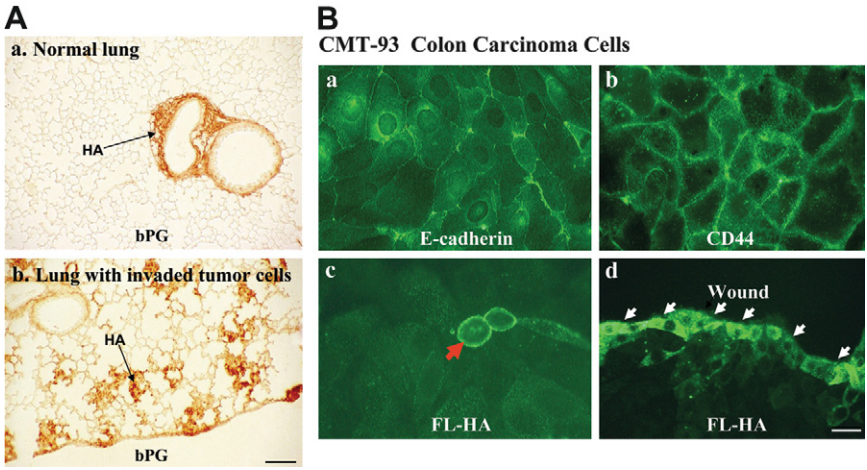

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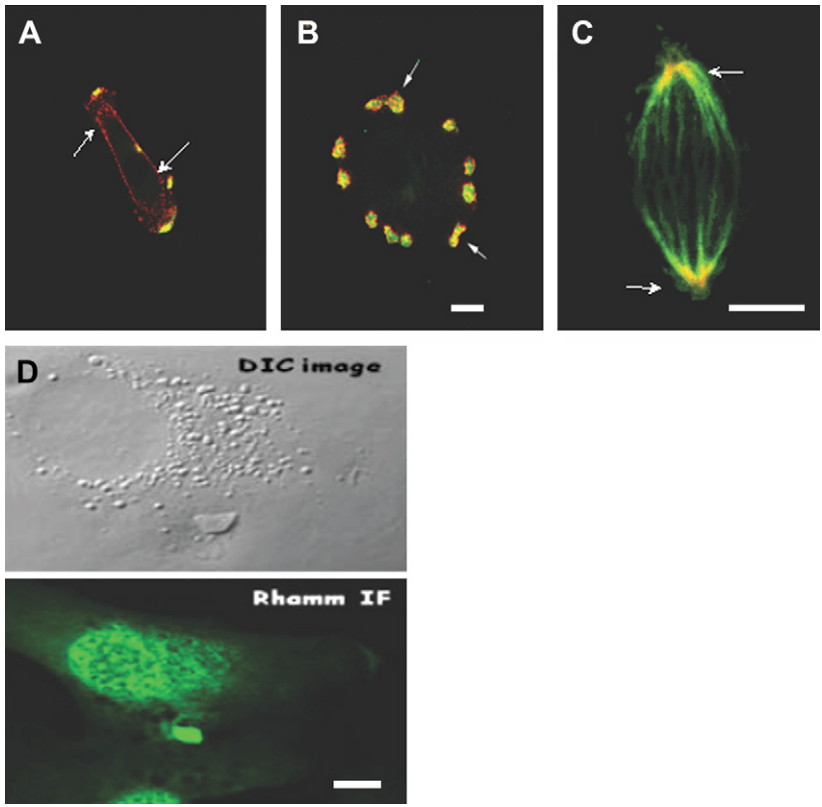

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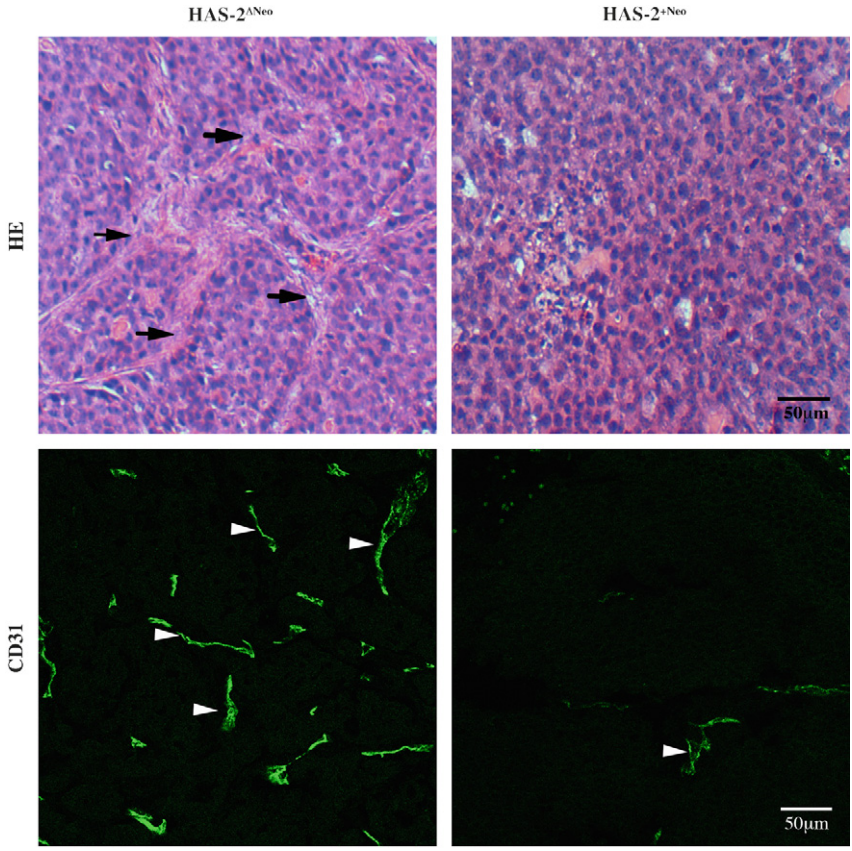

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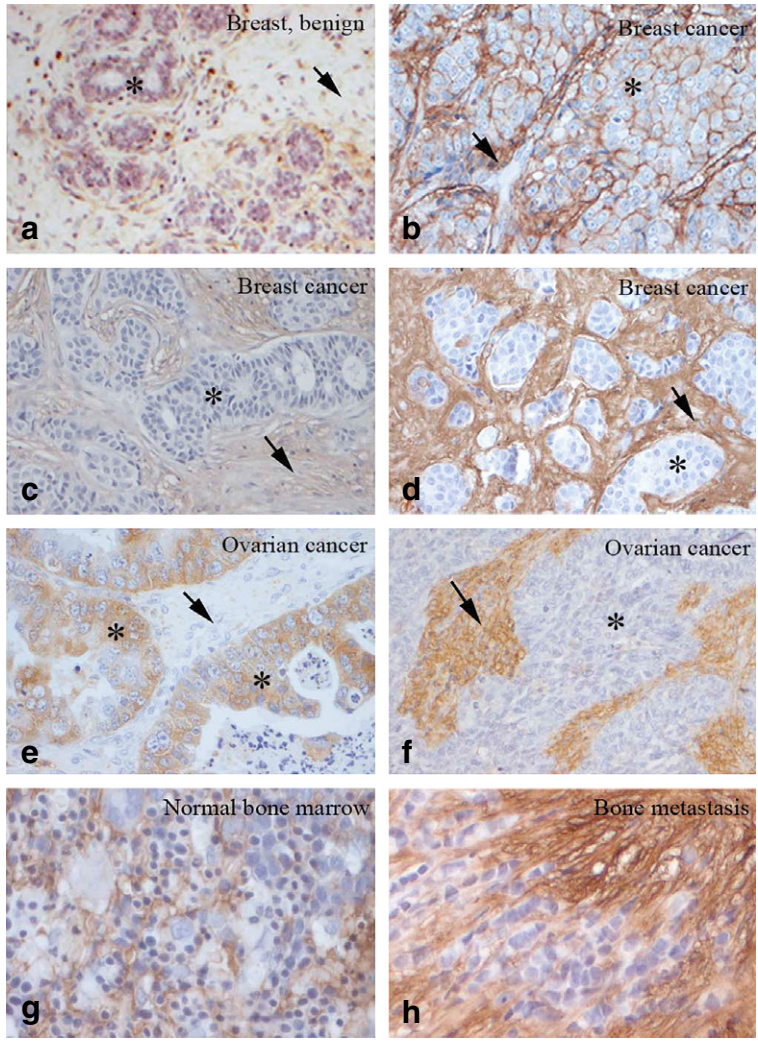

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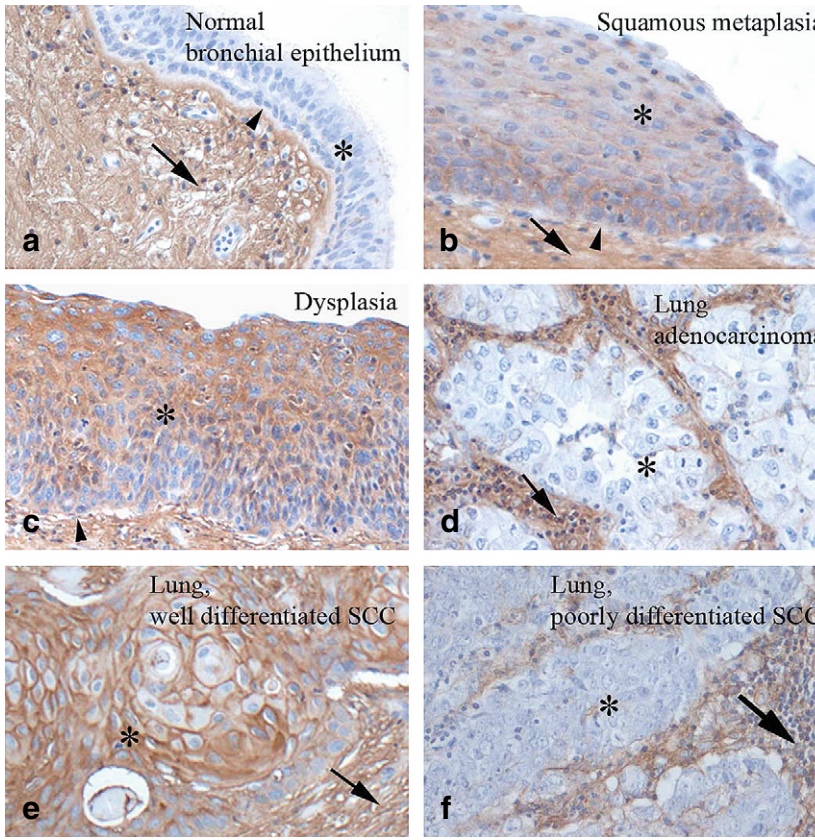

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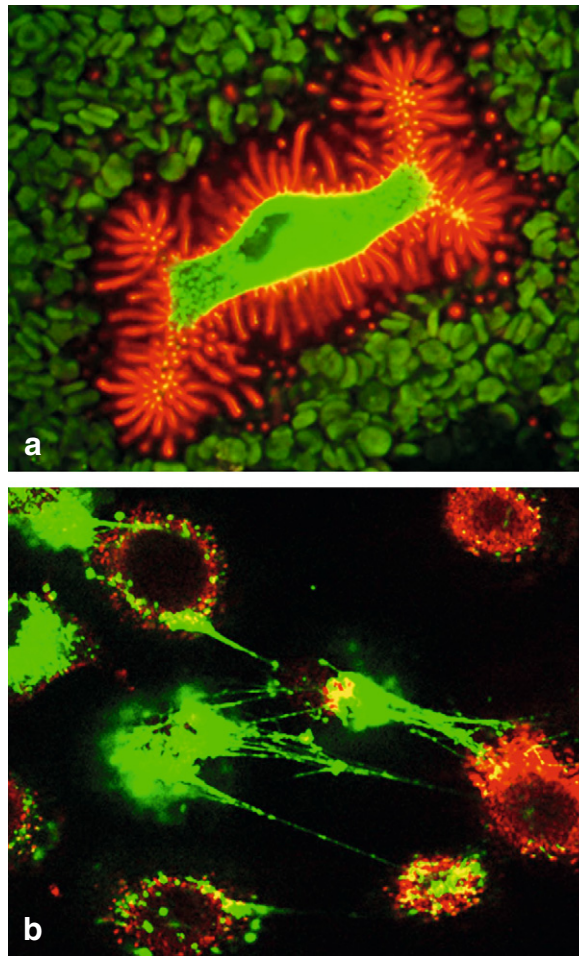

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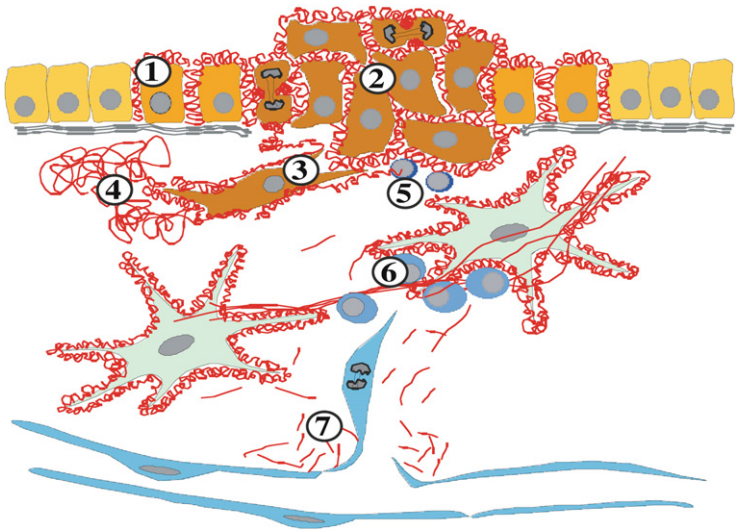

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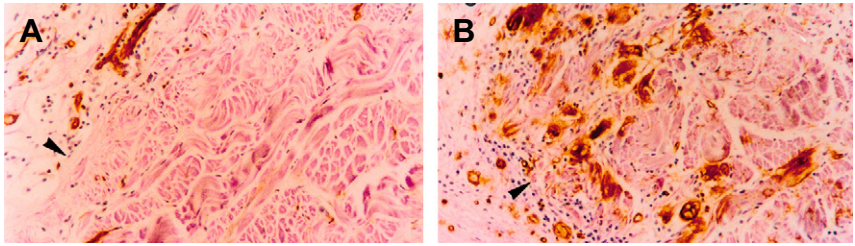

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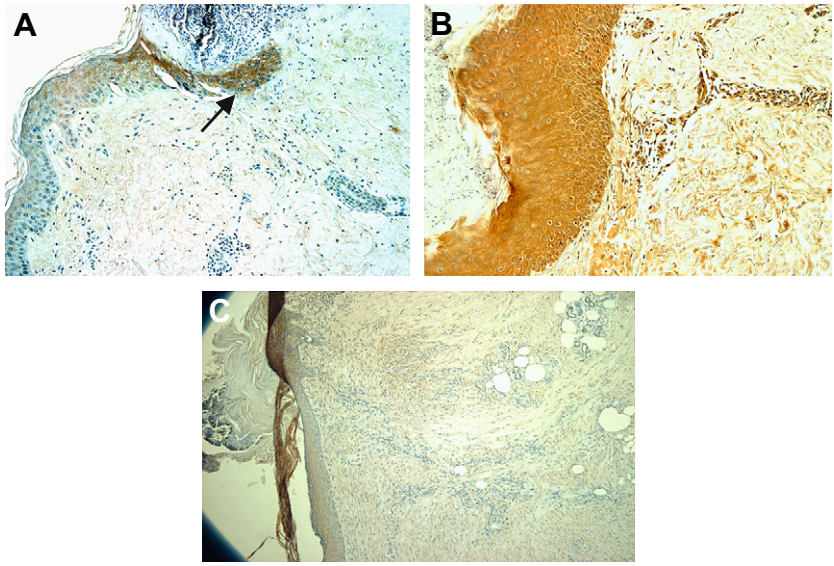

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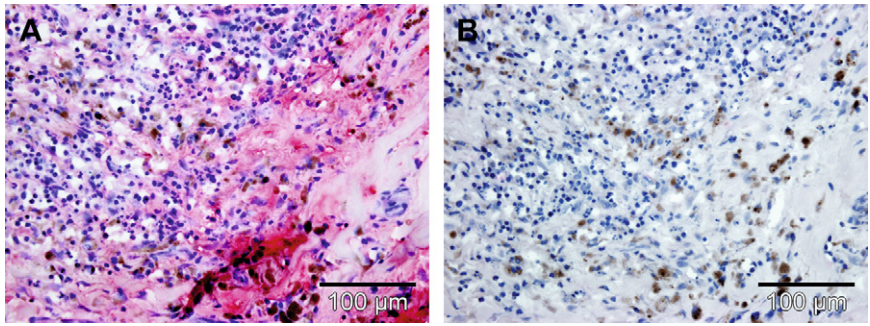

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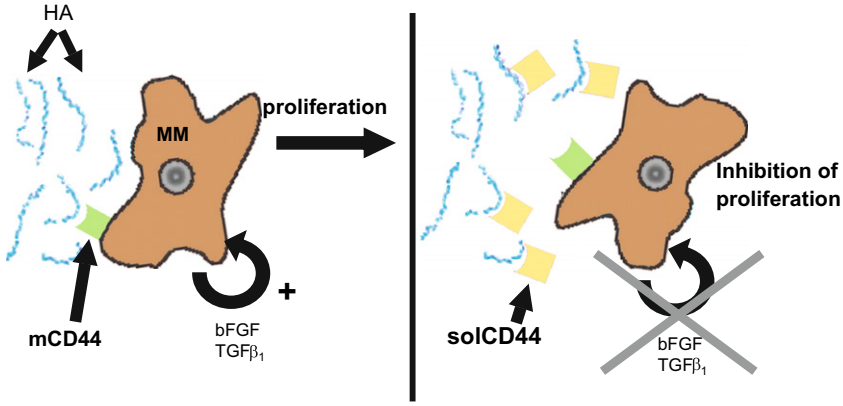

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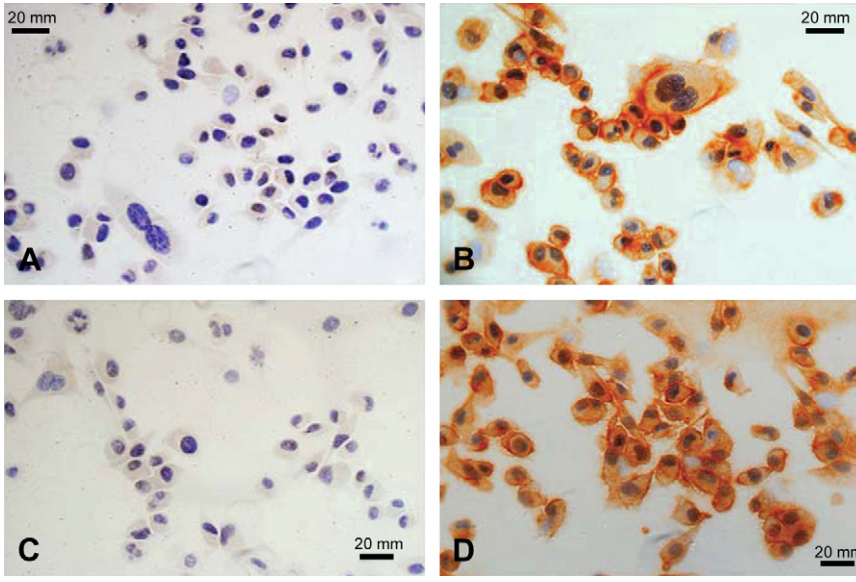

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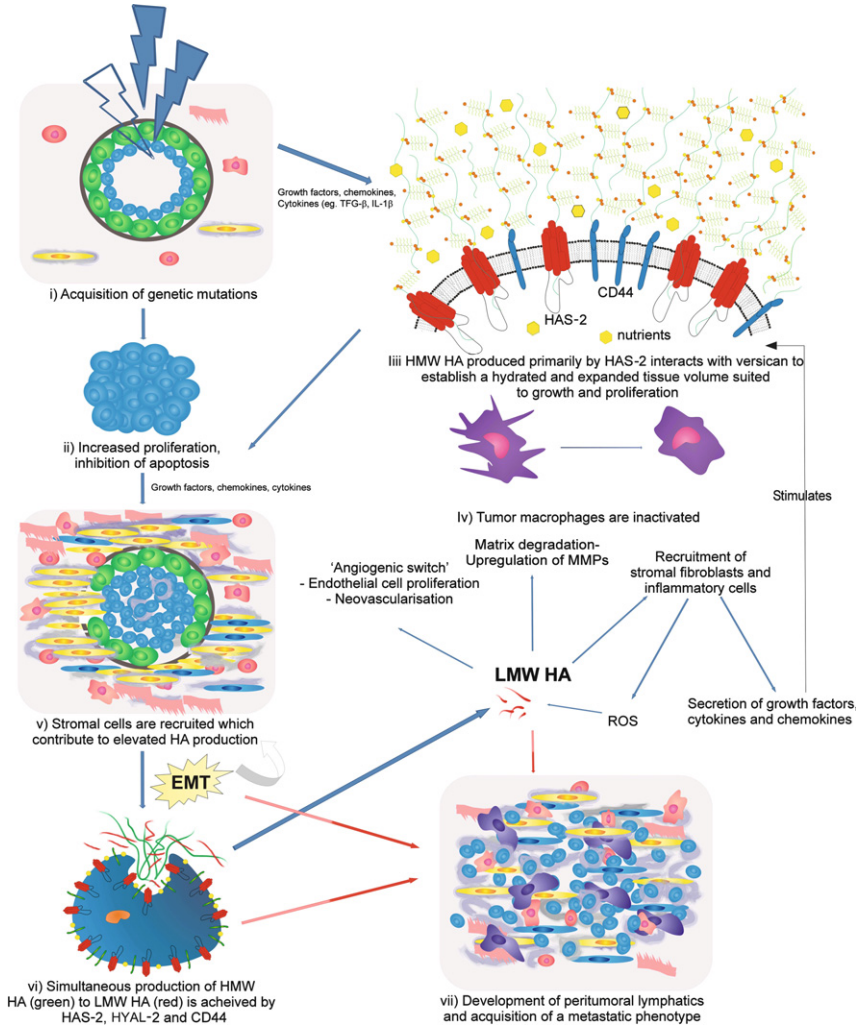

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