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The Biochemistry of Cancer Dissemination

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ABSTRACT: The progression of a tumor cell from one of benign delimited proliferation to invasive and metastatic growth is the major cause of poor clinical outcome of cancer patients. Recent research has revealed that this complex process requires many components for successful dissemination and growth of the tumor cell at secondary sites. These include angiogenesis, enhanced extracellular matrix degradation via tumor and host-secreted proteases, tumor cell migration, and modulation of tumor cell adhesion. Each individual component is multifaceted and is discussed within this review with respect to historical and recent findings. The identification of components and their interrelationship have yielded new therapeutic targets leading to the development of agents that may prove effective in the treatment of cancer and its metastatic progression.

KEY WORDS: invasion, metastasis, angiogenesis, proteolysis, adhesion, migration.

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I. INTRODUCTION

The hallmark of neoplastic proliferation is the loss of growth regulation. This alone does not constitute a full definition of malignancy. Benign neoplasia is characterized by localized, circumscribed growth that may have vascular boundaries or retention of some growth regulation signals. In contrast to malignant neoplasia, cells are not only growth dysregulated, but they have unmasked their invasive potential. These cells have gained the ability to dissociate from the primary tumor cell mass and to invade into surrounding tissues from which they then migrate to distant sites. Thus, malignancy and metastatic dissemination require both the capacity for dysregulated proliferation and local invasion.

Metastatic dissemination of cancer rather than the primary tumor is primarily responsible for treatment failure and death in cancer patients. Metastases may be present in more than 70% of patients at the time of their initial diagnosis (Schirrmacher, 1985; Sugarbaker, 1981). This suggests that the process of invasion and dissemination is not a late one, but one that occurs much earlier in the process of tumor progression. Figure 1 shows an example of this time line, demonstrating the presence of detectable and undetectable primary and metastatic disease at the time the patient seeks medical attention

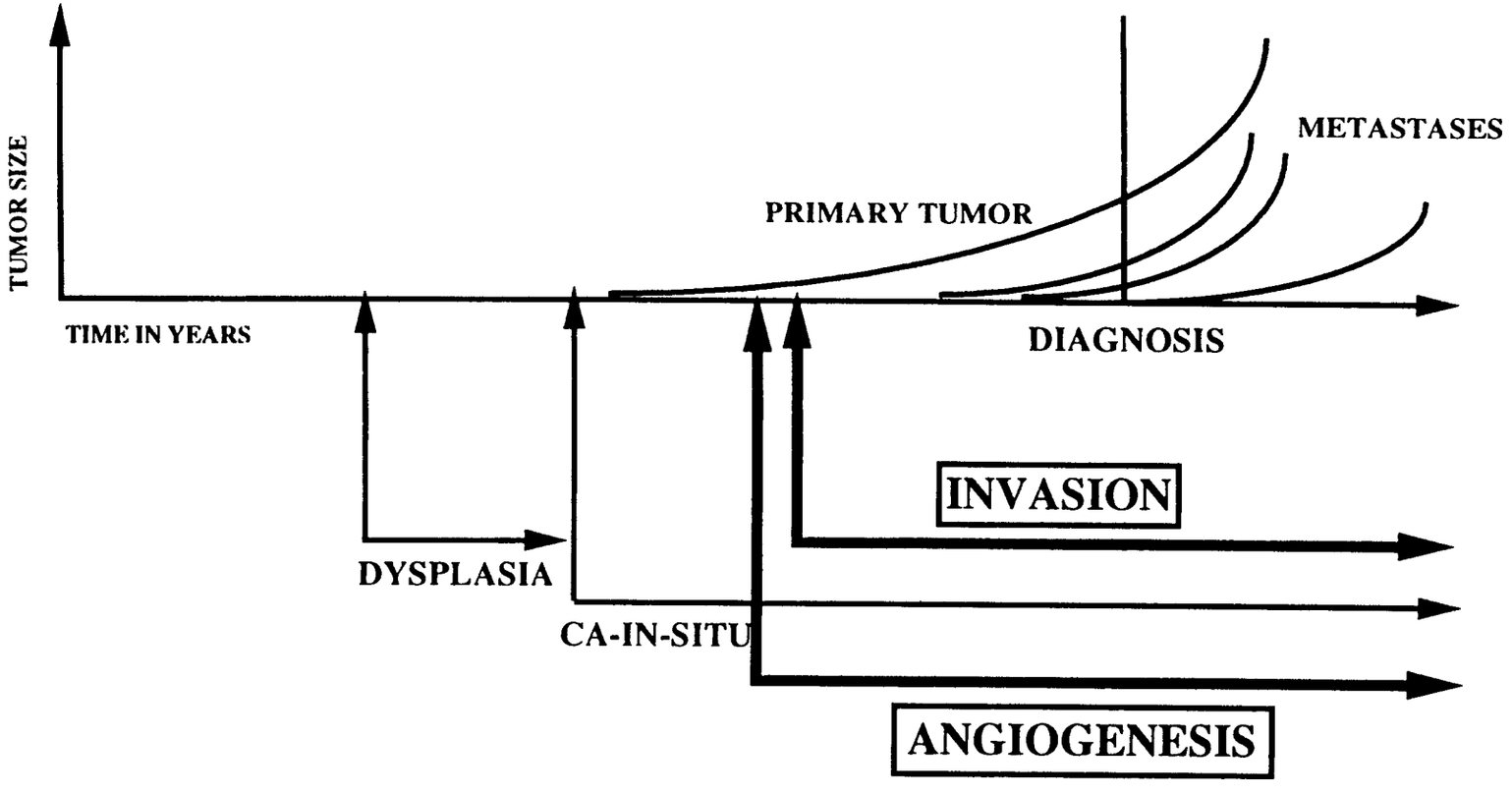

FIGURE 1. Time line of tumor progression. At the time of diagnosis, there are detectable and undetectable primary and metastatic tumors. At this time, the invasive process has already been initiated. Furthermore, angiogenesis occurs prior to the onset of the invasive potential as neovascularization is required for the successful metastatic spread.

(Kohn and Liotta, 1995). Further, it illustrates that the initiation of neovascularization and invasion long precedes tumor diagnosis. As shown, for most malignancies the onset of tumor cell invasive potential occurs in concert with or shortly after the initiation of primary tumor neovascularization. Tumor-stimulated angiogenesis is necessary for tumor access to the vasculature for metastatic spread, as well as for local nutrition. It has been calculated that the majority of metastases from breast carcinomas, for example, are initiated when the primary tumor is less than 0.125 mm^2, a size at which neovascularization may only be beginning (Fidler and Hart, 1982).

The ability to cure primary malignancies with local intervention, surgery or radiation, is very limited, with less than one third of patients falling into the curable category. Overt metastatic disease can be detected in almost half of well-staged patients at diagnosis with occult metastatic disease present at diagnosis in another 25% of patients. Occult metastatic disease is especially problematic in that it may remain dormant for many years after treatment of the primary tumor (Holmgren et al., 1995). These patterns may be changing with improved screening, surveillance, and diagnosis. For example, both breast cancer and prostate cancer can now be picked up at earlier stages where the frequency and severity of metastatic dissemination is less. While this may create a lead time bias for survival studies, it also demonstrates that early, nonmetastatic disease is a detectable and treatable point along the continuum of malignancy and stresses the importance of improved diagnostic tools and techniques. Experimental evidence has indicated that these dormant malignant cells can reactivate and grow rapidly after initiation of neovascularization. The capacity for local and disseminated invasion results in multifocal metastatic sites further complicating development of successful therapeutic interventions. Recent strides in understanding the molecular basis of invasion and metastasis are yielding new targets for therapeutic intervention that may be used early in the development and detection of cancer.

Several key observations regarding the biology of the tumor can be made under direct and microscopic inspection of tumor masses. Benign tumors may have sharply demarcated borders as a result of capsule formation or pseudo-encapsulation by compression of local normal tissue. Neovascularization becomes necessary to allow tumor to grow beyond a critical mass supplied by nutrient diffusion and therefore is not a hallmark of an invasive, malignant tumor. In contrast, malignant tumors may have poorly demarcated borders as a result of their front of actively invading cells.

A critical barrier to tumor cell dissemination is the basement membrane. This avascular and acellular structure is the scaffolding on which epithelial cells and endothelial cells build. Carcinomas, derived from epithelial cell origin, must invade by crossing the basement membrane that defines its organ of origin. All tumors must invade endothelial cell basement membranes to gain access to the vasculature for subsequent dissemination. General and widespread changes occurring in the organization and integrity of the basement membrane were defined historically by pathologists as the histologic marker of the transition from *in situ* to invasive carcinoma.

Studies of the human breast have provided an example of the progressive change from benign breast pathology to disseminated malignant breast cancer. Benign proliferative disorders of the human breast such as fibroadenoma, intraductal hyperplasia, and fibrocystic disease all have been shown to have a continuous basement membrane separating the breast epithelium from the local stroma. Noninvasive or *in situ* carci-

noma of the breast is defined by growth dysregulation with nuclear atypia of the breast epithelium, no interruption of the ductal or lobular basement membrane, with invasive potential yet to be clinically demonstrated. In contrast, infiltrating breast carcinoma is marked by multiple defects in the organization and continuity of the basement membrane (Barsky et al., 1983). This is underscored by the fact that 20 to 25% of the patients with stage I lymph-node negative breast cancer, less than 2 cm primary tumor, may die of metastatic disease. Defects in the basement membrane may also be observed at sites of hematologic or lymphatic vasculature entry or exit and at sites of dissemination such as parenchymal organ metastases. This pattern is not limited to breast cancer but has been shown to hold true in other cancers where the progression from benign through hyperplastic to invasive and malignant can been followed usually, such as in colon cancer and cervical cancer.

Acquisition of the invasive and malignant phenotype is a developmental process requiring gain-of-function and loss-of-function changes. Progress in the molecular characterization of invasion and metastasis has identified a number of genes and gene products that through gain or loss can drive metastatic potential. Invasion and metastasis are not monogenic phenotypes, although experimental approaches using selected genes can reconstitute or abrogate metastasis, providing important molecular tools with which to dissect the behavior (Table 1). Gain-of-function is illustrated by transfection of activated H-*ras* into primary rat embryo fibroblasts, wherein transfection yielded both tumorigenic and metastatic phenotypes in xenograft studies (Pozzatti et al., 1986). Experimental gain-of-function can also be shown by induction of the metastatic phenotype by overexpression of matrilysin (Wilson et al., 1997) or inhibition of inhibitors by downregulation of TIMP-2 (Ray et al., 1994; Ray et al., 1995). Loss-of-function, or metastasis suppression, has been demonstrated for several genes, for example, nm23 (Steeg et al., 1988) and E-cadherin (Vleminckx et al., 1991). In these studies, loss of gene expression has been correlated with increased invasiveness and metastatic potential, and replacement or augmentation of gene expression has resulted in suppression of the invasive phenotype. E-cadherin is an extracellular adhe-

TABLE 1
Altered Expression/Mutation of Several Molecules Functionally Associated with the Development of Metastasis

Molecule	Normal	Malignant	Postulated role	Ref.
p53	+[a]	–[b]	Regulates angiogenesis via regulation of thrombospondin	Dameron et al., 1994
*nm*23	+	–	Metastasis suppressor	Steeg et al., 1988
mts-1	–	+	Metastasis/motility inducer	Ford et al., 1994
Matrilysin	–	+	Matrix metalloprotease	Wilson et al., 1997
TIMP-2	+	–	Metastasis suppressor	Stetler-Stevenson et al., 1989
E-cadherin	+	–	Loss of cell-cell adhesion	Hoffmann et al., 1995
APC	+	–	Metastasis suppressor	Rubinfeld, 1995; Su, 1993
ras	–	+	Metastasis inducer	Pozzatti et al., 1986

[a] (+) Expressed or wild-type genes.
[b] (–) Nonexpressed or mutated genes.

sion molecule that helps maintain the architecture and integrity of the organ. Loss of E-cadherin is associated with loss of cell-cell contact and an increase in motile and metastatic potential (Hoffmann et al., 1993). Coordination of the function and/or loss of gene products in the malignant cells drives the final invasive phenotype.

A. Metastasis

Metastasis is a dynamic process in which the cell, under both autocrine and paracrine stimulation, leaves its primary environment and travels either locally or distantly within the body, after which it forms a proliferative focus. Successful metastasis requires invasion, angiogenesis, and proliferation. Tumor cells of metastases can themselves initiate further metastases. Each cell executes its invasive program individually and may respond differently to identical stimuli, depending on its molecular make up. The steps of metastasis are not unique to malignancy but constitute a normal program for many physiologic events. After appropriate stimuli, cells begin the invasive process by losing their normal cell-cell adhesive contacts and separating physically from their neighbors. Cells next use their powers of migration and local proteolysis to move within the organ or to locally degrade stroma or basement membrane to facilitate migration.

A key event in malignant invasion and metastatic dissemination is the ability of the empowered tumor cells to enter the circulation. This requires adhesion to the outside of the vascular basement membrane, local degradation of the matrix, and migration through the damaged basement membrane and between endothelial cells to enter the vasculature. This triad of adhesion, proteolysis, and migration constitutes invasion. However, without angiogenesis and proliferation, it cannot yield metastasis. The efficiency of the invasive and metastatic cell is very poor. It has been calculated that millions of cells are shed daily into the circulation by invasive tumors (Fidler and Hart, 1982; Liotta et al., 1974). However, only 0.01% of circulating cells successfully establish metastases. It was initially hypothesized that this was due to immune surveillance as well as mechanical sheer forces acting on the individual or clumped malignant cells. The high frequency of tumor cell(s) embolization within the first downstream capillary bed after experimental inoculation of tumor cells or physiologically within an invasive tumor or organ bed in part supports the adherence and trapping theory. It has been shown through intravital videomicroscopy studies that greater than 80% of injected cells can extravasate within 24 h, however, not all these cells will form colonies, indicating that growth after extravasation is a key stage of metastatic control (Koop et al., 1995).

Many patterns of tumor dissemination follow common anatomic routes. Lung metastases from limb and lung tumors, liver metastases from tumors shedding into the portal circulation, bone metastases from breast and prostate cancers, and brain metastases from lung primary tumors can be explained by first capillary exposure (Table 2). However, not all patterns of metastasis fit this flow-oriented explanation. The ability of gastric cancer to place drop metastases on the ovarian surface and ovarian cancer to metastasize to breast suggest that there are other directional factors at work. Evidence for chemoattractant cytokines that may provide homing signals for metastasizing tumor cells indicates that organ selectivity may be a directional event and the common patterns of metastasis observed may result from biochemical signals as well as mechanical simplicity.

Invasion is also a normal physiologic process; none of the mechanisms used by

TABLE 2
Frequency of Metastatic Sites

	≤10%	10 to 30%	30 to 50%	50 to 70%	≥70%
Breast		Kidney, skin, brain	Adrenal	Liver, bone, lung	Lymph nodes
Bladder	Brain, skin	Kidney, bone	Adrenal, lung		
Cervix	Skin, brain	Bone, kidney	Adrenal, lung		
Colorectum	Skin	Brain, kidney, lung	Bone, adrenal, liver	Lymph nodes	
Kidney	Skin, bone	Brain, kidney	Liver	Lung	
Lung	Lung	Kidney, distant nodes	Adrenal, brain	Bone	Liver, local lymph nodes
Melanoma		Kidney	Adrenal, brain, bone, skin	Lung, liver, nodes	
Ovary	Brain, skin, kidney	Bone, adrenal	Lung, liver, nodes		
Prostate	Brain, skin	Kidney, adrenal	Liver, lung	Bone, nodes	

Adapted from Weiss, L., 1992 and references therein.

malignant cells during metastasis are unique to malignant cell behavior. Invasion is a critical physiologic component of the immune system, implantation and development, wound healing, neurite outgrowth, and neovascularization, among others (Folkman, 1992; Folkman, 1971; Kohn and Liotta, 1995, and references therein; Liotta et al., 1974; Liotta et al., 1991). For example, in inflammation or infection, circulating monocytes must adhere to endothelial cells, translocate into the extravascular space, and then migrate to their site of action as tissue macrophages. Embryo implantation into the wall of the uterus uses the same repertoire of adhesion and proteolysis proteins used by invasive cancer cells. During placental development, trophoblasts have the ability to invade the endometrial stroma and maternal blood vessels. Migration of embryonic stem cells during organogenesis and limb bud formation also uses the same cellular machinery found in the invasive cells. Normally quiescent endothelial cells acquire an invasive phenotype during angiogenesis, breaching their own basement membrane and interstitial stroma during the development of new capillary buds. Local tissue invasion by endothelial cells during the process of blood vessel formation is regulated by intracellular and stromal signals, yielding organized capillary trees. These cells return to a quiescent and noninvasive phenotype when the angiogenic stimulus ceases. Some regulation persists in the development of new capillary networks in the physiologic invasion of neovascularization resulting in a vascular network within the tumor.

Angiogenesis is a critical step in the establishment of tumor metastasis. The process of tumor neovascularization is a result of a host-tumor interaction. Capillary networks around tumor are required for expansion of the tumor area beyond its local capillary. The initiation and establishment of new vessels is mediated by a balance of angiogenic and angiostatic signals that are secreted by the tumor as well as stromal tissue (Liotta et al., 1991; O'Reilly et al., 1994). Like invasion, angiogenesis occurs during normal and pathologic processes. It is limited in time and restricted in location.

Pathologic but nonmalignant neovascularization is seen in collagen vascular and chronic inflammatory diseases, proliferative retinopathy, and in response to blood vessel thrombi or emboli causing vessel occlusion (Folkman, 1996). In these normal and pathologic conditions, neovessels that are formed are normal in caliber and generally have an intact basement membrane. Neovessels formed under malignant stimuli frequently are more leaky, with decreased endothelial cell contact, larger fenestrations, and a more poorly formed basement membrane. The etiology of these differences have not been elucidated yet.

II. THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) defines boundaries between tissue compartments and organ parenchyma and is composed of two forms: basement membrane and underlying interstitial stroma. Basement membrane specifically separates epithelium and endothelium from mesenchymal tissue. It is found encompassing epithelial islands such as ducts and glands, blood vessels, bundles of muscle, and components of the nervous system (Vracko, 1974). The main structural components of the basement membrane are glycoproteins, such as laminin, collagens, entactin, fibronectin, and proteoglycans such as heparan sulfate and chondroitin sulfate (Chung and Durkin, 1990; Timpl et al., 1984). These components interact with one another to create a non-fibrillar continuous network in the basement membrane composed predominantly of collagen type IV (Yurchenco, 1994). Underlying this layer in tissues and the lymphatics is the interstitial stroma, which is composed of glycosaminoglycans, glycoproteins, collagens, and elastin. The collagenous network of this layer is composed of the interstitial collagens, type I, II, and III (Kadler, 1994). Composition of the ECM varies according to local environment. Each cell type synthesizes and deposits a unique array of matrix proteins to guide specialized functions for the normal physiology of the organ. The ECM molecules form a continuous passive, impermeable layer around tissues that becomes focally permeable to cell movement during select physiological processes such as tissue remodeling and wound healing (Timpl and Dziadek, 1986). During events such as cellular transformation (Hayman et al., 1982; Siegal et al., 1981) and the progression to invasiveness (Barsky et al., 1983), tumor cells selectively reorganize their local basement membrane so that they can disseminate to other locations.

Collagens and elastin are two predominant proteins that provide the structural framework of the ECM. Together they create a tensile, through collagen, yet flexible, through elastin, structure (Kleinman et al., 1981). Interspersed in this framework are a variety of other proteins with structural and/or specialized functions. Proteins of the ECM can act as ligands for cellular receptors, creating specialized cell-matrix links called focal contacts (Burridge et al., 1988). Signals can then be transduced into the cell in response to formation of these select contacts to modulate a variety of cellular functions. The ECM also protects and sequesters cytokines and growth factors through interactions with a variety of molecules such as heparan sulfate and collagen (Taipale and Keski-Oja, 1997). For example, heparan sulfate interacts with both basic fibroblast growth factor, FGF-2, and acidic FGF, FGF-1, which are potent angiogenic factors. This interaction has been shown to provide two functions. First, FGF-2 is sequestered to provide a rich source of growth factor that is protected from proteolytic degradation. Second, FGF-2 can be presented specifically to FGF receptors by heparan sulfate present on

the cell surface (Miao et al., 1996; Richard et al., 1995). Collagens have been shown to contribute to the growth-promoting activities of platelet-derived growth factor, PDGF, at fibroproliferative lesions through short-lived and specific interactions (Somasundaram and Schuppen, 1996). The interactions of tumor cells, growth factors, and proteolytic enzymes with the ECM are critical during the invasive process. Thus, the components of the ECM are discussed to understand these biological processes.

A. Collagens

The collagens are a large and diverse family of at least 19 members that are localized to the ECM. Collagens share structural features that include a triple helical region, non-helical regions, and globular domains (Bork, 1992). The globular domains contribute to the molecular assembly of collagen, while the non-helical regions that are interspersed in the helical domains contribute to flexibility. Collagens are formed from three chains that exist either as homotrimers or as heterotrimers. A highly repetitive arrangement of Gly-X-Y repeats is characteristic of collagen (X = proline and Y = hydroxyproline). Repetition of this tripeptide sequence results in the suprastructure of the three chains as a triple helix, the characteristic rod shape of collagen through supramolecular interactions that include helices interacting with helices and a variety of posttranslational modifications (Bork, 1992). Despite their genetic similarities, the collagens differ quite considerably in structure due to differences in the size and nature of their globular domains, the length of their triple helix and the localization of abundant noncollagenous modules such as von Willebrand's factor motifs, transmembrane domains, thrombospondin domains, and complement factor motifs (Brown and Timpl, 1995).

Collagens provide mechanical stability and strength by interacting with one another or with other ECM components. Collagen IV forms a homologous lattice by self-assembly that provides scaffolding for the other ECM proteins. Fibrils of collagen I and III interact with proteoglycans and glycoproteins to add resilience to structures such as cartilage and tendon. Collagens have been shown to stabilize a variety of cell-matrix interactions mediated by other matrix components as well, such as proteoglycans and laminin. Collagen expression may be regulated by cell differentiation. For example, chondrocytes express collagen type I, type IX, and type XI but switch to collagen type I when they differentiate (Vornehm et al., 1996).

The fibrillar collagens I, II, III, V, and XI are the predominant components of the interstitial stroma (Kadler, 1994). These collagens form cross-striated fibrils of 67 nm periodicity through alignment of their triple helical domains and subsequent cross-linking of parallel fibers (Hulmes et al., 1995). This structure is due in part to a deviant triple helical domain. Typically, the collagen triple helix is formed by the association of adjacent Gly-X-Y repeats; however, the fibrillar triple helix contains multiple Gly-X-Y repeats that contain clusters of charged and phobic residues in the X and Y positions. Collagens I, II, and III provide tensile strength to connective tissue. Collagen I is the most abundant collagen found in skin, bone, and tendon. Collagen II is found predominantly in cartilage and in connective tissue during early development. Collagen III is a major component of blood vessels and is found within the parenchyma of internal organs (Kleinman et al., 1981). Collagens V and XI associate with collagens I and II, respectively, generating collagen fibrils with smaller diameter that are referred

to as microfibrils (Wu and Eyre, 1995). In cartilage matrix, type XI collagen forms homopolymers through head to tail cross-linkage between adjacent molecules and then establishes links to collagen type II through the N-terminus of collagen type XI. Similarly, type V collagen is arranged with type I collagen in bone (Keene et al., 1995). Collagen V and XI are buried within the interior of the co-fibrillar assembly. Both of these collagens have been found to contain sites important in cell adhesion and in binding of heparin (Fichard et al., 1995).

Basement membrane collagens, predominantly type IV, VI, VIII, and X, do not form fibrils but are cross-linked into a three-dimensional network composed predominantly of collagen IV (Glanville, 1982). N-terminal ends of collagen type IV tetramers align in parallel and in anti-parallel fashion followed by cross-linking or dimerization of two most adjacent C-terminal ends to create an independent, insoluble structure (Siebold et al., 1988). This network has been shown to interact with a laminin network through several bridging proteins (Yurchenco, 1994). Collagen types VIII and X also form networks, although because they contain multiple Gly-X imperfections in the Gly-X-Y repeats, they form small triple helical domains and are called short-chain collagens. Collagen type VIII is found in sheet-like structures in Descemet's membrane of the cornea. It is also secreted by skin keratinocytes, corneal epithelial, and endothelial cells, lens epithelial cells, and mesenchymal cells surrounding cartilage (Mann et al., 1990; Maragaki et al., 1992). Type X collagen occurs in calcifying cartilage zones (Brown and Timpl, 1995).

Collagen type VI forms a thin-beaded filamentous, flexible network. The beads result from the lateral association or end-end aggregation of the tetramers stabilized by unique short cysteine-rich segments (Kuo et al., 1995). Collagen VI anchors structures such as nerves, blood vessels, and collagen fibers into surrounding connective tissues and is found in skin, cartilage and the basement membrane of nerves, vessels, and fat cells (Keene et al., 1988). Collagen type VII is a major component of anchoring fibrils, specialized structures that extend in complex branches from the basement membrane into the tissue epithelial cell layer (Sakai et al., 1986). In the ECM, the anchoring fibrils also appear to insert into dense patches called anchoring plaques. Entrapped within these anchoring fibrils and plaques are collagen banded fibers, microfibrils, and other stroma-matrix macromolecules such as fibronectin, providing important cell-matrix interactions (Keene et al., 1987).

A group of collagens identified by signature collagenase-resistant col1 structures (Shaw and Olsen, 1991), types IX, XII, XIV, XVI, are called fibril-associated collagens with interrupted triple helices (FACIT). The C-terminal col1 regions are characterized by a highly conserved pair of cysteine residues and by imperfections in the triple helix, Gly-X-Gly-X-Y and Gly-X-Y-X-Y, that are located at similar positions relative to the cysteines (Dublet and van der Rest, 1991). FACITS share at least two triple helical domains separated by non-helical segments (Yamagata et al., 1991). They appear to associate with cross-striated fibrils composed of fibrillar collagens and do not form homopolymers. Collagen type IX is an unusual collagen molecule with one of its subunits consisting of a covalently linked glycosaminoglycan of either chondroitin sulfate or dermatan sulfate, attached to a region of the collagen containing a tetrapeptide sequence, Gly-Ser-Ala-Asp. Collagen type IX decorates and is covalently linked to the surface of fibrils and is found in fetal cartilage. Type IX collagen is present in hyaline cartilage in association with covalently linked collagens II and XI (Keene et al., 1995). It may contribute to the stabilization

of the network of thin fibers of the ECM of cartilage by interactions of its triple helical domains with fibrils (Muller-Glauser et al., 1986).

Collagen XIV contains two RGD sequences in a col2 domain, a second collagenase resistant structure that is found in some FACIT members (Yamagata et al., 1991). The RGD sequence, Arg-Gly-Asp, is a primary cell interaction site that is shared by many matrix molecules of the ECM. Collagen XIV and XII function to modulate the deformability of the ECM by binding between fibrils and linking tissue fibrils to one another. These collagens interact directly with multiple ECM components such as heparan sulfate proteoglycan, collagen VI, and dermatan sulfate and may mediate interactions between the collagen fibrils and matrix macromolecules (Keene et al., 1991). Collagen XIV is found in embryonic tissue, lung, heart, muscle, skin, stomach, tendon, around hair follicles, and within dense connective tissue such as dermis, tendons, and the stroma of lung, liver, and blood vessels. It is a newly described member of the FACIT class (Castagnolo et al., 1992). Collagen XII is found in dense connective tissue such as tendons, ligaments, and the perichondrium. The novel collagens are type XIII through type XIX. These molecules have been cloned and sequenced (Mayne, 1993).

B. Fibronectin

Fibronectin is a modular, multifunctional glycoprotein that is found in plasma, liver, and the ECM. *In vivo*, fibronectins are found in body fluids, loose connective tissue matrices, and most basement membranes. Physiologic sources of fibronectin are fibroblasts, endothelial cells (Ruoslahti and Vaheri, 1974), and a plasma form produced primarily by hepatocytes (Hynes, 1985). *In vitro*, many cells such as fibroblasts, endothelial cells, chondrocytes, macrophages, and mammary epithelial cells have been shown to synthesize fibronectin (Mohri, 1996). Fibronectin is an asymmetric molecule containing several globular domains that are formed by the disulfide linkage of two 225-kDa subunits resulting in a 450-kDa protein (Skorstengaard et al., 1985). It is encoded by one gene that is alternatively spliced to generate a diverse population of molecules (Schwarzbauer et al., 1983).

Fibronectin functions through several distinct binding domains to promote cell adhesion and matrix assemblage in the ECM. The primary cell-binding domain is an RGD cell attachment sequence. A major heparin-binding site has been identified in the C-terminus of fibronectin and may play a role in the organization of the ECM (Hayashi and Yamamda, 1982; Ruoslahti et al., 1981). A collagen- or gelatin-binding domain was identified through binding to gelatin-affinity columns and to interstitial collagens *in vivo* (Balian et al., 1980; Mosher et al., 1980). Additionally, the C-terminal end of fibronectin interacts with proteoglycans mediating the interactions between the cell and the ECM (Woods et al., 1986). Also, fibronectin has been shown to be an essential component in mediating cell-attachment functions of collagen. The interactions between collagen, proteoglycan, and fibronectin are important in matrix assembly and in development of the structural organization of the ECM.

C. Laminin

Laminin is a basement membrane glycoprotein that mediates a variety of functions such as cell adhesion, cell migration, neurite

outgrowth, cellular proliferation, and basement membrane assembly (Beck et al., 1990). Laminin was first isolated from the matrix of the EHS mouse tumor (Timpl et al., 1979) and consists of three distinct but related major chains, B1, B2, and A, that assemble into a cruciform molecule composed of three short arms and one long arm (Beck et al., 1990; Paulsson et al., 1985; Sasaki et al., 1988). Laminin is now known to consist of a family of proteins that are composed of variantly expressed chains that generate seven different heterotrimeric laminins that vary in size composition and structure (Timpl and Brown, 1994). Functionally, the role of these isoforms may be related directly to their binding affinities (Brown et al., 1994; Delwel et al., 1994). Each of the laminin chains contains a 570 amino acid α-helical, coiled-coil domain in the C-terminus and a globular domain in the N-terminus. Interspersed between these ends are a variety of structural motifs, such as EGF-like repeats, globular-domains, alpha-helical regions, and protein binding sites, that have been elucidated by cloning and sequencing the domains (Olsen et al., 1989; Sasaki et al., 1988). Laminin assembly initiates after association of the coiled-coil domains to form a triple helix that constitutes a major portion of the long arm. The laminin chains are heavily glycosylated by N-linked oligosaccharides, increasing the mass and complexity of this molecule (Arumugham et al., 1986). Laminin plays an important role in supramolecular assembly of the basement membrane. Laminin is synthesized and deposited into an independent network in the basement membrane that becomes interconnected through the interaction of entactin (Chung and Durkin, 1990) or alternatively nidogen (Mann et al., 1989) to a separate network formed of collagens. The short arms were found *in vitro* and *in vivo* to mediate the interactions required for basement assembly (Yurchenco, 1994).

Multiple functional domains were identified within laminin by proteolytic analysis. (Deutzmann et al., 1988; Rao et al., 1982). Proteolytic fragments of laminin bind to a variety of ECM components, such as collagen IV, heparan sulfate, perlecan, and entactin. Several cell interaction sites also have been identified. A major cell-binding domain, RGD, has been located at the center of the laminin molecule (Tashiro et al., 1991). Additional regions of the molecule are thought to be essential for cell adhesion activity. YIGSR, Glu-Iso-Gly-Ser-Arg, of the B1 chain was identified due to its role in mediating cell attachment and chemotaxis (Aumailley et al., 1987; Graf et al., 1987). A third region defined by the sequence IKVAV, Iso-Lys-Val-Ala-Val, is involved in migration and neurite outgrowth (Tashiro et al., 1989).

D. Proteoglycan Molecules

The proteoglycan family of ECM proteins perform a variety of adhesion functions in the ECM, such as binding to matrix macromolecules and growth factors (Cao and Pettersson, 1993) facilitating cell-matrix interactions (Murdoch et al., 1992), and direct interactions with β1 integrins (Battaglia et al., 1993). They are composed of glycosaminoglycan side chains attached to a polypeptide core molecule. Glycosaminoglycan side chains include heparan sulfate (hspg), dermatan sulfate (dspg), chondroitin sulfate (cspg), and keratin sulfate (kspg). Side chain function can be inhibited in culture by the inclusion of chlorate, a sulfate inhibitor, resulting in an impaired ability to form laminin-proteoglycan complexes and to assemble a proper ECM (Brauer et al., 1990). These side chains were also shown to be important in proteoglycan self-assembly, which is im-

portant in modulating tissue thickness and resilience (Yurchenco et al., 1987). Proteoglycans with heparan sulfate side chains are the most common proteoglycan in the ECM and only recently have molecules containing galactosaminoglycan side chains been identified. These are a hybrid perlecan molecule with hspg/dspg side chains, and bamacan, basement membrane-chondroitin sulfate proteoglycan (Couchman and Woods, 1996).

Two distinct groups of ECM proteoglycans have been identified. The first group, large aggregating molecules, mediate a variety of matrix component interactions and specifically provide tissues with resistance to compressive forces. The best known members of this group are aggrecan, versican, and perlecan. The latter two molecules are found in the ECM of most tissues. Perlecan, a 600-kDa modular protein that is bound by three heparan sulfate chains attached to a 400-kDa core protein, was shown to be one of the most abundant proteoglycans in the basement membrane (Hassell et al., 1980). Perlecan encodes a signal peptide followed by five to seven globular domains that are connected by rod structures that resemble the short arms of laminin (Laurie et al., 1988), giving perlecan the appearance of a string of pearls under the electron microscope (Paulsson et al., 1987). The heparan sulfate chains in perlecan are attached to its N-terminus and give the molecule an asymmetric structure (Noonan et al., 1991). In perlecan, as well as in all proteoglycans, the core protein sequences Ser-Gly-X-Gly specifically guide side chain attachment to the Ser residue (Bourdon et al., 1987).

When perlecan was isolated, a small 130-kDa hspg was also found. This small proteoglycan was formed of four 29-kDa side chains attached to a 10-kDa core polypeptide and appeared like a star when viewed by electron microscopy (Paulsson et al., 1987). It was initially thought to represent a proteolytic degradation product of perlecan. Biochemical analysis demonstrated that in fact this was a distinct type of proteoglycan with a core region consisting of a leucine-rich region, nine repeats of the motif LXXLXLXXNXL, a cysteine-rich region, a C-terminal disulfide loop, and an N-terminal glycosaminoglycan attachment site.

There are four well-characterized members of this family of small proteoglycans that are found in the extracellular matrix, decorin, a single chain substitution cspg/dspg, biglycan, a double side chain substitution cspg/dspg, fibromodulin, a kspg, and lumican, a four side chain substitution kspg. These proteoglycans mediate proper matrix formation by interacting with matrix proteins such as collagen. Decorin is expressed in all connective tissues rich in collagen type I or type II, such as blood vessels, dermis, bone, and cartilage (Bianco et al., 1990) and interacts with both collagens type I and type II, modulating the speed of collagen fibrillogenesis and the structure of the collagen fibrils (Kresse et al., 1994). Decorin and fibromodulin share similar expression patterns and both bind fibrillar collagens, although through unique interactions (Hedbom and Heinegard, 1993). Targeted disruption of these interactions result in abnormal collagen fibril morphogenesis and fragile skin (Danielson et al., 1997). Biglycan generally is not expressed in cells that express decorin, such as skeletal myofibrils and differentiating epithelial cells (Bianco et al., 1990). Biglycan has been found co-localized with collagen in dermis and bound to purified collagen type I. It has not been found bound to collagen *in vivo* (Schonherr et al., 1995).

Syndecans are a family of cell surface proteoglycans that mediate cell-ECM and cell-cell interactions. These transmembrane proteins encode glycosaminoglycan attachment sites in the extracellular region for cell-matrix and cell-cell interactions through heterotypic and homotypic interactions. Syn-

decan-null B cells form large aggregates in suspension culture when mixed with cells transfected with syndecan-1. Syndecan-transfected cells bind to a receptor present on the untransfected cells. The presence of phosphorylation sites in the cytoplasmic domain of syndecan and the discovery that these proteins mediate interactions with cytoplasmic proteins suggest that these proteins are components of signaling pathways (Stanley et al., 1995).

III. INVASION

The three dynamic steps of invasion are adhesion, invasion, and migration. Adhesion occurs through the interaction between tumor cells and tumor cells, stromal cells, and endothelial cells as well as with the interstitial stroma and basement membrane. These interactions occur both within the organ of origin of the tumor or metastasis and within the vasculature. Tumor and normal cells may have a variety of adhesion receptors that may guide both organ-specific and invasion-associated behaviors. Proteolysis is required in order to traverse extracellular matrix barriers in the interstitial stroma and basement membrane. Tumor cells may either induce production of proteases from surrounding cells through secretion of stimulatory cytokines or protease substrates, they may produce and secrete proteases themselves, or they may produce factors that inhibit or activate existing local proteases. Both normal and tumor cells can secrete protease inhibitors, so the net local proteolytic activity is a balance between protease production and activation and availability of endogenous inhibitors. Migration is the induction of forward motion in the form of a pseudopodial protrusion followed by translocation and retraction of the trailing cell body. This movement is influenced by paracrine and autocrine attractants and may direct tumor cell homing to select organ sites of metastasis. Similar to proteolysis, migration may also be a net balance between scatter-inducing factors, directional attractants, and local inhibitory influences. Together, these three forces drive the invasive process, physiologic or malignant.

A. Adhesion

A critical component of invasion is the ability of tumor cells to interact with their environment to grab traction during migration and to stabilize proteolytic substrates during local degradation of matrix (Liotta et al., 1991). Cell adhesion, similar to invasion as a whole, is a dynamic process involving homotypic or heterotypic cellular interactions, cell interactions with local stroma, and dissolution of these contacts. Adhesion can function in a positive way to stabilize tissue structure and organization under normal conditions or negatively to facilitate dissemination. In the context of invasion, adhesion can be broken into a continuous circle of parts: attachment, spreading, and detachment. The outcome of adhesion is then regulated by the type and location of attachment, direction of spreading, and mechanism of detachment. For example, homotypic interactions through cell adhesion molecules and cadherins stabilize tissue integrity, whereas loss of these cell surface proteins has been shown to be associated with increased metastatic potential (Behrens, 1993). Heterotypic interaction between tumor cells and endothelial cells is involved in the entry and exit of tumor cells from the vasculature. Direction and organization of cell spreading and reach may be regulated by cell interaction with extracellular matrix

proteins, such as the basement membrane glycoproteins, through cell surface receptors, including the integrin family (Hynes, 1992). Activation of these cell surface receptors passes outside-in signals to the cell and directs the cell behavior. Key components of the attachment and adhesion machinery are discussed.

1. Integrins

The integrins are a family of transmembrane glycoproteins that are expressed by the cell as αβ heterodimers (Hynes, 1992). There are 16 distinct α subtypes and 8 β subtypes as well as alternative splice variants of the subunits that form this family. The α chain ranges from 120 to 185 kDa in size and consists of two polypeptide fragments joined by a disulfide bond. The α chain N-terminus contains several calcium binding domains through which it interacts noncovalently with the β chain. The β subunit ranges from 90 to 110 kDa in size and contains several cysteine-rich regions in its N-terminus that have been shown to be involved in ligand binding and ligand specificity (Shih et al., 1993). The integrins mediate a variety of cell-ECM and cell-cell interactions.

Integrin-ECM ligands include a variety of molecules such as collagen type IV, collagen type I, laminin, tenascin, fibronectin, vitronectin, von Willebrand's factor, and thrombospondin (Nicolson, 1991). Cell-cell interactions are mediated through heterophilic association as seen in the α4β7 interaction with mucosal endothelial addressin cell molecule-1 (MAdCAM-1) and αEβ7 interaction with E-cadherins. αlβ2 and αmβ2 interact with ICAM-1 and ICAM-2, leukocyte-expressed cell adhesion molecules, CAMs. Binding affinities of the receptors are determined by the αβ chain association but can be classified in a general sense by the β chain. The β1 subfamily of receptors interact with at least 10 α subunits and primarily mediate adhesion interactions with the ECM. Some of the β1 receptors also mediate direct cell-cell adhesions, such as α4β1, which can mediate homotypic interactions in addition to matrix interactions. The β2-containing receptors have been found in contact with at least three unique α subunits, αx, α1, and αm. They interact with ICAM-1 and ICAM-2 and are important in neutrophil migration. These integrins are sometimes referred to as the leukocyte receptors. The β3 subunits interact with two α subunits and mediate ECM interactions predominantly with fibrinogen, vitronectin, von Willebrand's factor, fibronectin, collagen, and the RGD peptide. The remaining β integrins have single α subunits specificities (reviewed in Clark and Brugge, 1995).

Integrin-ligand interactions are accompanied by activation and clustering of integrins on the cell surface and transduction of this activation into intracellular signal transduction pathways that mediate a variety of intracellular events. Integrin signaling depends on the dynamic formation of cellular focal contacts or focal adhesions. Focal adhesions are specialized sites in which cytoskeletal proteins are concentrated, which regulate cell shape and migration and create a framework for the association of important signaling molecules (Horowitz et al., 1986; Otey et al., 1990; Schwartz, 1992). Protein phosphorylation, mobilization of calcium, and GTP exchange are common signals involved in propogation of integrin-mediated information (Burridge et al., 1992; Dash et al., 1995; Kohn et al., 1995).

FAK is a common protein in integrin-mediated focal adhesions activated by a variety of ligands, such as fibronectin, laminin, collagen type IV, and vitronectin, as well as through anti-integrin antibodies (Guan and Shalloway, 1992; Schaller et al.,

1992). FAK is a cytoplasmic tyrosine kinase that after activation and phosphorylation binds to and may phosphorylate other signaling proteins in continuation of the signal transduction cascade. One such partner protein is paxillin, involved in cytoskeletal reorganization (Tremblay et al., 1996) and further downstream is activation of *src* and mitogen-activated protein kinase (MAPK) and their respective signaling systems. Activation of MAPK occurs after integrin occupation and capping-mediated signaling, accompanied by translocation of MAPK from the cytosol to the nucleus in response to cell adhesion to fibronectin, laminin, and RGD peptides (Burridge et al., 1992; Morino et al., 1995). Recently, an alternative pathway has been discovered that activates MAPK in a FAK-independent manner by ILK, an integrin-linked kinase (Weaver and Roskelley, 1997). β2-integrin activation has been shown to induce tyrosine phosphorylation of phospholipase Cγ-1 which is recruited to focal contacts after integrin engagement (Kanner et al., 1993). Activation of phospholipase Cγ-1, results in hydrolysis of membrane phospholipid, phosphatidylinositol bisphosphate to yield diacylglycerol, an activator of protein kinase C isoforms, and inositol trisphosphate that mediates release of internal calcium stores (Cole and Kohn, 1994; Whitfield, 1992).

Integrins mediate a variety of functions, such as wound healing, cell differentiation, homing of tumor cells, and apoptosis. Collagen type I in its native form can be bound by two integrins, α1β1 and α2β1, through interactions with the Asp-Gly-Glu-Ala tetrapeptides of collagen. Denaturation, as might happen during wounding, results in exposure of cryptic Arg-Gly-Asp sites in collagen type I that can bind to $\alpha_v\beta3$ expressed by melanoma cells. This arrangement may allow the cell to bind collagen even during tissue remodeling or infiltration. During differentiation of breast cells, integrin-induced gene expression is responsible for directing lactoferrin and casein gene expression. Also, integrin-induced tissue structure rearrangement results in a modulation of whey acidic protein (Weaver and Roskelley, 1997). Liver cells are preferentially colonized during metastasis by lymphomas. It has been found that homing of tumor cells is determined by the ability of the expressed receptors to interact with the microenvironment of the target organs. $\alpha_v\beta3$ integrin has been shown to suppress apoptosis to lead to cell survival. Melanoma cells expressing $\alpha_v\beta3$ had a higher survival rate when compared with a variant melanoma cell line lacking this receptor (Montgomery et al., 1994).

Integrins, such as $\alpha_v\beta3$, α6, and β1, are involved in the regulation of adhesion interactions important in tumor metastasis (Table 3). Benign tumors of the head, neck, and skin down-regulate α6 integrin expression, whereas high levels of α6 are detected in metastatic tumors (Friedrichs et al., 1995). Furthermore, patients survival from these tumors correlates with loss of expression of α6 expression. Suppression of α6 expression by ribozyme treatment or treatment of metastatic cells with antibodies directed against the α6 subunit resulted in abrogation of adhesion, invasion, and experimental metastasis (Blood and Zetter, 1993; Ruiz et al., 1993). Melanoma variants lacking $\alpha_v\beta3$ were found to be less tumorigenic than $\alpha_v\beta3$-containing cells. Tumorigenicity was restored after addition of $\alpha_v\beta3$ (Felding-Habermann et al., 1992). $\alpha_v\beta3$ interactions with PeCAM-1 on vascular endothelial cells are essential for extravasation. Blocking these interactions with RGD peptides or anti-α_v or anti-β3 inhibited metastatic formation to the vascular endothelium (Piali et al., 1995). Increased expression of several of the β1 integrin receptors correlated with aggressiveness of tumor cells for many tumor types. Transfection of the α2β1 integrin into a poorly metastatic rhabdomyosarcoma cell line

TABLE 3
Altered Integrin Expression from Normal to Metastatic Phenotype

Tumor Tissue/cell type	Integrin	Expression	Ref.
Lung adenocarcinoma	$\alpha2\beta1$, $\alpha3\beta1$	Dec	Clarke et al., 1997
	$\alpha_v\beta3$	Inc	Clarke et al., 1997
Renal carcinoma	$\alpha3\beta1$	Dec	Jenq et al., 1996
Oral squamous cell carcinoma	$\alpha_v\beta6$	+	Jones et al., 1997
	$\alpha6\beta1$	+	Zhang et al., 1996
Prostate carcinoma	$\alpha4\beta1$	–	Haywood-Reid et al., 1997
Melanoma	$\alpha_v\beta3$	Inc	Albelda et al., 1990
	$\alpha6\beta1$, $\alpha3\beta1$	Inc	Giancotti et al., 1994
Glioblastoma	$\alpha_v\beta3$	Inc	Gladson et al., 1994
	$\alpha3\beta1$	Inc	Giancotti et al., 1994
Head/neck carcinoma	$\alpha_v\beta3$, $\alpha6\beta1$	Inc	Wolf et al., 1992

Note: (Dec) decreased expression, (Inc) increased expression, (+)/(–) switched on/off.

enhanced production of metastatic colonies in nude mice and enhanced adhesion of cells to the ECM components laminin and collagen *in vitro* and *in vivo* (Chan et al., 1991).

Several integrins have been found to be correlated inversely with metastatic potential. Qian and colleagues demonstrated an inverse correlation of $\alpha4\beta1$ expression with invasive potential of the murine B16 melanoma or CHO cell lines transfected with $\alpha4\beta1$. Treatment of the melanoma cells with anti-$\alpha4$ mouse antibody resulted in an increase in invasion (Qian et al., 1994). Decreased expression of $\alpha5\beta1$, classically considered the fibronectin receptor, also is correlated inversely with tumorigenicity of transformed cells, wherein stable transfection of CHO cells with $\alpha5\beta1$ inhibited their ability to migrate, form colonies in soft agar, and to establish tumors in nude mice, suggesting a metastasis suppressor function for this integrin subtype (Giancotti and Ruoshlati, 1990; Plantefaber and Hynes, 1989).

2. Laminin Receptors

Several laminin-binding proteins have been cloned and characterized, including the high-affinity 67-kDa receptor (67LR), members of the galectin family, and integrins. 67LR was first identified in breast cancer biopsies and was purified from B16 murine melanoma cells (Rao et al., 1983) and murine fibrosarcoma cells (Malinoff and Wicha, 1983). It has now been identified in numerous normal human epithelial and endothelial cell lines and in pulmonary macrophages. 67LR has been shown to be highly expressed in metastatic, invading cells. On tumor cells,

the 67LR has been shown to be increased in number, disorganized in distribution, and unoccupied (Wewer et al., 1986). In contrast, normal cells have 67LR on their basal surface in contact with basement membrane. 67LR mediates adhesion between laminin and tumor cells and endothelial cells that can be blocked by monoclonal antibodies to 67LR (Wewer et al., 1987). Expression of 67LR has been correlated with tumor invasiveness (Castronovo et al., 1990) and poor prognosis (Martignone et al., 1993). Expression of 67LR is significantly higher in cancer cells than in normal cells, and a strong correlation was shown between expression of 67LR at the cell surface and the metastatic potential of breast and colon cancer cells (Castronovo et al., 1992; Cioce et al., 1991; Mafune et al., 1990; Yow et al., 1988).

A 37-kDa precursor of the 67LR has been cloned and identified by pulse-chase experiments (Castronovo et al., 1991), Northern analysis (Rao et al., 1989), primer extension (Wewer et al., 1986), and immunohistochemistry (Castronovo et al., 1991). The 37LR has been shown to dimerize to form 67LR. Expression of both 67LR and 37LR precursor have been shown to be regulated by exposure to laminin (Romanov et al., 1995). A region of 67LR, peptide G, was identified that blocks 67LR binding to laminin by blocking binding to the YIGSR region of the laminin B1 chain (Castronovo et al., 1991). It has been proposed that 67LR may stabilize laminin interactions with $\alpha6\beta4$ as 67LR was co-immunoprecipitated with an antibody to $\alpha6$. In addition to this physical association, the expression of $\alpha6\beta4$ and of 67LR are co-regulated (Ardinin et al., 1997).

Galactoside-binding lectins, HLBP14 and HLBP31, have been shown to bind to the poly-*N*-acetylgalactosamine residues of laminin (Castronovo et al., 1992; Castronovo et al., 1992). HLBP14 shares immunological similarity 67LR and 37LR, suggesting a common functional domain. Down-regulation of HLBP31 in colon cancer has been correlated with metastatic potential. Galactosyl-transferase recognizes extracellular galactosaminoglycan-type oligosaccharides found on laminin A chain and specifically mediates cell spreading on laminin (Arumugham et al., 1986; Shur, 1982).

Several members of the $\beta1$ and $\beta4$ family of integrins mediate cell binding to laminin through the C-terminal globular domain of the laminin A1 chain. $\alpha6\beta1$ associates with peptides of the C-terminal globular domain of the laminin A1 chain; these peptide sequences as well as antibodies against $\alpha6$ or $\beta1$ inhibited adhesion on laminin (Nakahara et al., 1996). $\alpha3\beta1$ has been shown to bind to the globular domain of the laminin A chain at a site different from the region bound by 67LR, peptide G, or YIGSR (Gehlsen et al., 1992). Synthetic peptides to this region support cell attachment but inhibit cell adhesion to laminin-coated surfaces. Laminin-binding integrin $\alpha7\beta1$ on melanoma cells binds specifically to laminin, was not found in normal melanocytes, and may be involved in the transition to metastasis (Kramer et al., 1991).

3. Cadherins

Cadherins are transmembrane glycoproteins that mediate calcium-dependent cellular interactions (Kadler, 1994). There are several members of this family, including (E) epithelial, (N) neural, (P) placental, as well as many newer members including (Ksp) kidney-specific protein, (OB) osteoblast, (VE) vascular endothelial, and (PB) pituitary gland, brain cadherins, the desmogleins and desmocollins. Each member regulates cell adhesion of particular cell types or is active during different developmental stages (Marrs and Nelson, 1996). The extracellular

domain of cadherin encodes a receptor recognition site that contains an His-Ala-Val sequence, HAV, that mediates cadherin-protein interactions (Byers et al., 1992). Binding at this site is modulated by a nearby Ca^{2+} binding site that contains numerous negatively charged amino acids (Hatta et al., 1988). The intracellular cadherin cytoplasmic domain is the most highly conserved region and functions to transduce receptor activation into the cell. This domain contains multiple protein binding sites and has been found in complex with a variety of cytoplasmic and cytoskeletal proteins, such as actin (Ozawa et al., 1990). Analysis of the cytoplasmic domain also demonstrated the importance of this domain in cell adhesion functions (Nagafuchi and Takeichi, 1988).

E-cadherins, the most extensively studied of the cadherins, was originally designated ovumorulin. Phosphorylation of E-cadherin was found to be associated with compaction of the mouse embryo (Sefton et al., 1992). It is expressed on the basolateral surfaces of epithelial cells, particularly at intracellular complexes that are involved in cell-cell communication (Boller et al., 1985). These complexes, called adherens junctions, are characterized by dense structures formed of actin filaments in tight association with cytoskeletal proteins such as α-actinin, vinculin, radixin, plakoglobin, and actin (Tsukita and Tsukita, 1989). E-cadherins also are found in complex with a family of distinct but related cytoplasmic proteins that include α, β, and γ catenin and armadillo; γ catenin is also known as plakoglobin. Catenin interactions with cadherins provides cadherins with a link to the actin-based cytoskeleton. β-catenin binds directly to a 25 amino acid sequence in the cytoplasmic domain of E-cadherin and also associates directly within the N-terminus of α-catenin (Jou et al., 1995). A second type of cadherin-catenin complex has been identified. In this complex, plakoglobin mediates the interaction between cadherin and α-catenin (Butz and Kemler, 1994; Hinck et al., 1994).

The cadherin-catenin complexes are linked to the cytoskeleton through direct interactions between α-catenin and α-actinin in N- and E-cadherin-expressing cells (Knudsen et al., 1995). These interactions have been shown to require α-catenin expression and thought to be directly mediated by a region of α-catenin with high homology to vinculin, called the talin-binding domain (Herrenknecht et al., 1991). Without α-catenin, cells cannot mediate cellular adhesion. For example, poorly aggregating PC9 cells transfected with α-catenin were transformed into tightly aggregating cells (Hirano et al., 1992). Adherens junctions and cadherins, similar to focal adhesions and integrins, have been found to mediate cellular function by transducing the adhesion interactions into cellular and organizational changes. Src tyrosine kinase has been detected in association with the adherens junctions (Tsukita et al., 1991). Tyrosine phosphorylation of β-catenin and E-cadherin by *src* has been shown to decrease cell-cell adhesions (Behrens, 1993) and tyrosine kinase inhibition results in the reformation of adherens junctions (Volberg et al., 1992). Activated *ras*-transformed MCF7-10A cells are characterized by less-developed adherens junctions and increased focal adhesions due to an increase in the phosphorylation of β-catenin and $p120^{cas}$ accompanied by a decreased binding of β-catenin to E-cadherin. Inhibition of β-catenin phosphorylation resulted in a return to normal phenotype (Kinch et al., 1995).

Several studies have shown a correlation between tyrosine phosphorylation of E- and N-cadherin and decreased adhesion, increased migration, and increased invasiveness (Behrens, 1993). Specifically, E-cadherin has been demonstrated to function as a metastasis suppressor molecule in several cell lines. Noninvasive MDCK cells acquired

an invasive phenotype after treatment with antibodies that blocked E-cadherin (Burridge et al., 1988). This was measured by enhanced migration through collagen I gels and invasion in a chick fragment model. Hoffman et al. demonstrated that a reduction in cell adhesiveness observed during neoplastic progression of rat ovarian surface epithelial, ROSE, cells was linked to decreased expression of E-cadherin. This suggested that the loss of cadherin-mediated cell adhesion was an important step during progression of the transformation of ovarian surface epithelium to ovarian carcinoma (Hoffman et al., 1993). E-cadherin expression does not correlate with invasive phenotype in all cell models. Some breast cancer cell lines that were E-cadherin negative were only weakly invasive, suggesting a different etiology (Maher et al., 1985). When E-cadherin was transfected and expressed into invasive breast cancer MCF7-10A cells, they exhibited the ability to aggregate, but morphology was unaltered and the cells remained invasive. Notably, these cells had reduced expression of plakoglobin and less phosphorylation of β-catenin when compared with less invasive MCF7 human breast cancer cells.

The role of β-catenin in cancer progression has been demonstrated recently. Under normal conditions, β-catenin binds to the tumor suppressor gene adenomatous polyposis coli, *APC*. Mutations in this gene are linked to progression of colorectal and gastric tumors (Rubinfeld et al., 1995; Su et al., 1993). APC-β-catenin interactions promotes APC hyperphosphorylation, resulting in targeted degradation of β-catenin (Korinek et al., 1997; Morin et al., 1997). Mutations in either APC or β-catenin inhibits the degradative process of β-catenin leading to its association with the transcription factor T-cell factor or lymphoid enhancer factor (TCF/LEF). β-catenin-LEF is found in complex with DNA and has been demonstrated to result in apoptosis. Detection of β-catenin in the serum of patients with melanoma and colon cancer has been suggested to serve as a prognostic indicator of cancer progression (Rubinfeld et al., 1997).

4. Cell Adhesion Molecules

The family of cell adhesion molecules, CAMs, are transmembrane glycoproteins defined by their tissue of origin. This family includes V-CAM, vascular endothelial, PeCAM, platelet endothelial, I-CAM, intracellular, L-CAM, liver, C-CAM, carcinoembryonic antigen homology (CEA), and N-CAM, neural (Gallin et al., 1985; Murray et al., 1984; Obrink, 1991; Xie and Muller, 1993). CAMs mediate a variety of cell-cell or cell-substratum adhesion interactions (Cole and Glaser, 1986). By immunohistochemistry, they have been shown to define discrete borders between cells. CAMs also play important roles in morphogenesis, histogenesis, and neurite outgrowth (Obrink, 1991).

The CAMS are multidomain proteins that consist of an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The extracellular domain consists of multiple immunoglogulin-like homology units (Ig) and fibronectin III repeats (FNIII). Due to their frequency of Ig repeats, the CAMS are known as members of the immunoglobin family. Both Ig and FNIII motifs have been identified in cell-cell interactions. N-CAM and homologues, TAX-1, Ng-CAM, and Nr-CAM, mediate neuronal cell-cell interactions for neuronal development, neurite outgrowth, neuronal migration, and axonal growth (Daniloff et al., 1994; Grumet et al., 1991). The N-CAM extracellular domain is formed of five Ig units and three FNIII repeats, the third of which has been found to be essential for the localization of the CAMS to cell-cell con-

tacts. The extracellular FNIII domains in Tax-1 were found to be necessary for adhesion and to induce cell aggregation (Tsiotra et al., 1996). PeCAM-1, which is expressed by most immune cells, platelets, monocytes, neutrophils, and all vascular endothelial cells, mediates adhesion through the second of six Ig domains (Sun et al., 1996).

The CAM cytoplasmic domain was demonstrated to play a role in organizing cells during embyogenesis and development. L-CAM was initially identified in chick liver but is also found in epithelial cells and mediates calcium-dependent adhesion interactions (Sorkin et al., 1988). Hybrid receptors composed of the extracellular and transmembrane domain of L-CAM complexed with the cytoplasmic domain of N-CAM inhibited the ability of transfected and untransfected cells to sort from one another but did not block cellular aggregation (Jaffe et al., 1990). A role for the cytoplasmic domain in cell adhesion has been suggested from studies with C-CAM, expressed in liver, epithelial cells, platelets, and granulocytes (Obrink, 1991). The cytoplasmic domain of C-CAM is 71 amino acids long and contains a 10 amino acid region that is important for adhesion (Lin et al., 1995). A tetrapeptide sequence, GSDH, found in this 10 amino acid region has been suggested to effect the interaction of C-CAM with cytoplasmic proteins, thus regulating cellular interactions.

The cytoplasmic domain may provide other functions such as in transmitting signals from the extracellular domain. An antigen-receptor homology domain that is necessary for signal transduction in B-cells has been identified (Cambier and Campbell, 1992). The cytoplasmic protein calmodulin (Edlund et al., 1996) and the neural proteoglycan neurocan (Rauch et al., 1992) have been found in association with the CAM cytoplasmic domain. Neurocan, a 500-kDa chondroitin sulfate proteoglycan in rat brain, interacts with N-CAM and Ng-CAM to decrease homophilic adhesions and disrupt cell aggregation as well as to inhibit neurite outgrowth (Margolis et al., 1996). Similar interactions between calmodulin and C-CAM were established in which the binding of calmodulin resulted in a down-regulation of homophilic self-association of C-CAM (Edlund et al., 1996).

In a variety of cases, CAM expression is associated with the malignant phenotype. V-CAM mediates interactions with the β1 integrin, VLA-4 and is believed to function as a tumor adhesion receptor in melanoma. V-CAM-mediated adhesion of melanoma cells to activated endothelium through VLA-4 (Rice and Bevilacqua, 1989). ICAM-1 is detected in advanced human melanomas but not on benign melanocytes and is correlative with the progression of metastasis in melanoma (Johnson et al., 1989). I-CAM functions in mediating lymphocyte adhesion. I-CAM expression has also been found to be induced by several cytokines, such as TNF-α in melanoma cells and IL-6 in human breast cancer cells (Hutchins and Steel, 1994). Increased expression of I-CAM-1 was also detected in endothelial cells after treatment with TNF-α and IFN-γ, whereas PeCAM-1 expression was down-regulated under the same conditions (Stewart, 1996). PeCAM-1 mediates a variety of leukocyte-endothelial interactions, such as $\alpha_v\beta3$- PeCAM-1 interactions involved in transendothelial migration (Piali et al., 1995).

5. Selectins

Selectins are members of the immunoglobin family that mediate interactions between leukocytes and vessel wall, such as at sites of tissue injury and inflammation. The selectins have been shown to promote vascular adhesion by mediating neutrophil, monocyte, and lymphocyte rolling along the

venular wall. There are three family members with differential expression by leukocytes, L, platelets, P, and vascular endothelium, E (Springer and Lasky, 1991). Selectins have an extracellular domain with a type C N-terminal lectin domain, an EGF-like domain, repeats with homology to complement binding protein domains, a transmembrane section, and a short cytoplasmic tail (Tedder et al., 1995). The N-terminal lectin binds ligands by interacting with sialylated and fucosylated carbohydrate molecules. Examples of these proteins are glycoprotein membrane receptors of the sialomucin adhesion family that function to act as a scaffolding to present selectin carbohydrate ligands in a clustered and tissue-specific manner.

Selectin-ligand bonds have high mechanical strength with high off-on rates that can mediate events such as rolling in response to the hydrodynamic drag of the vasculature. A high hydrodynamic threshold is required for adhesion interactions mediated by L-selectins, suggesting that the L-selectins mediate the initial attachment of leukocytes to the endothelium (Finger et al., 1996). The E-selectins facilitate the entry of leukocytes into inflamed tissues and have been found to promote angiogenesis during tumor growth and wound repair. Soluble, recombinant E-selectin has been shown to induce endothelial cell chemotaxis *in vitro* and to stimulate angiogenesis *in vivo*. Selectin moieties have also found expressed on tumor cell surfaces. These moieties were found to mediate the adhesion of tumor cells to platelets during blood-borne metastasis.

6. CD44

CD44 is a widely expressed cell surface glycoprotein that serves as an adhesion molecule in cell-substrate and cell-cell interactions. The molecule has been shown to have a variety of functions, including the binding of extracellular matrix hyaluronic acid, fibronectin and collagens (Ishii et al., 1993; Jalkanen and Jalkanen, 1992; Peach et al., 1993), a role in the catabolism and production of hyaluronate (Culty et al., 1992), lymphocyte homing and T cell activation (Tarin and Matsumura, 1993), and cell migration and tumor metastasis (Lesley et al., 1993). The numerous functions and interactions in which CD44 is involved can be related to its complex structure. The CD44 gene encodes 20 exons, of which at least 10 have been shown to be variably expressed due to alternative splicing of the RNA (Gunthert et al., 1995; Screaton et al., 1993). These 10 alternatively spliced exons each can be inserted into a single site situated in the extracellular portion of the molecule, creating CD44 variants (CD44v) (Jackson et al., 1992).

The standard form of CD44 (CD44s), sometimes referred to as the hemopoietic form, does not contain alternatively spliced exons. It forms a 35-kDa core protein that is modified by posttranslational glycanation with chondroitin sulfate or heparan sulfate, yielding a protein of 85 to 90 kDa. The extracellular amino terminus of CD44s has significant homology with the hyaluronate binding region of the cartilage link and proteoglycan core proteins and is responsible for binding hyaluronic acid (Stamenkovic et al., 1989). Birch et al. showed that human melanoma cells expressing high levels of CD44s had an increased motility, homotypic aggregation, and adhesion to hyaluronate, as well as an increased invasion potential *in vivo* (Birch et al., 1991). Similarly, monoclonal antibodies towards CD44s significantly decreased adhesion and *in vitro* invasion of a human glioma cell line. Transfection of antisense oligonucleotides against CD44s had a similar effect (Merzak et al., 1994). It has been postulated that CD44s binding hyaluronic acid localized on peritoneal mesothelial cells may play a role in the peritoneal metastasis

of ovarian tumors (Gardner et al., 1996). The expression of CD44s has also been associated with poor prognosis in gastrointestinal lymphoma and colorectal and breast cancer (Ichikawa, 1994; Joensuu et al., 1993; Joensuu et al., 1993).

Expression of variant CD44 (CD44v) isoforms in some tissues has been related to tumor progression and metastatic potential (Zoller, 1995). Gunthert et al. demonstrated that CD44v sharing the v6 exon promote the metastatic spread of pancreatic carcinoma cells from a subcutaneous injection site in an isogenic model (Gunthert et al., 1991). Transfection of nonmetastasizing cancer cells lacking the v6 isoform with CD44v6 containing variants gained the potential to colonize the lungs in an experimental animal model (Seiter et al., 1993). Injection of antibodies specific for the v6 epitope was shown to prevent the growth of the transfectants in the draining lymph nodes and reduced the formation of metastases. Expression of CD44v6 isoforms have been associated with adverse prognosis in colorectal cancer, and the occurrence of both CD44s and CD44v9 on the surface of gastric cancer cells was related significantly to higher tumor-induced mortality and a shorter survival time (Streit et al., 1996). In contrast, repressed expression of CD44 has been associated with malignant transformation of tumors of squamous cell origin (Salmi et al., 1993) and Tanabe et al showed that overexpression of CD44s in colon carcinomas resulted in reduced tumorigenicity (Tanabe et al., 1995). Recently, CD44 was postulated to be a metastasis suppressor gene for prostate cancer (Gao et al., 1997).

B. Proteolysis

Degradation of and migration through extracellular matrix barriers such as the basement membrane is a complex process that requires the production, release, and activation of a number of extracellular matrix-degrading enzymes. Inappropriate overexpression of one or more of these enzymes has been shown to occur in almost all cells of the tumor-host microenvironment, for example, tumor cells, fibroblasts, and recruited macrophages (Gottesman, 1990). Although this review addresses tumor cell production of proteolytic degradation, the component that has been attributed to other host cells should not be overlooked. Stromal fibroblasts have been shown to produce a number of proteases that are important to the metastasis of tumor cells, similarily, macrophages and neutrophils, recruited into the tumor, also play a prominant role in extracellular matrix remodeling leading to the dissemination of the tumor cells (Jiang et al., 1995; Dabbous et al., 1995; Gregoire et al., 1995; McGary et al., 1997; Welch et al., 1989; and references therein). However, the unbridled production of degradative enzymes by the tumor cells and the surrounding host cells would not be expected to result in invasion, as extensive lysis of matrix components required for tumor cell traction during migration would also occur. Thus, the invading tumor cell uses proteolysis in a highly organized manner, both spatially and temporally.

Localized degradation of the basement membrane requires the expression of many proteolytic enzymes that have a variety of substratum specificity. Degradation of the basement membrane is not solely dependent on the amount of proteolytic enzymes present but on the balance of activated proteases and their naturally occurring inhibitors. The metastasis field has progressed from establishing a correlation between proteolysis and malignant progression to the actual blockade of a number of these proteases to prevent invasion and metastasis. A positive association with tumor aggressiveness has been shown for a variety of degradative enzymes,

including heparanases (Nakajima et al., 1991), seryl- (Reich et al., 1988), thiol- (Sloane and Honn, 1984), and metal-dependent enzymes (Liotta, 1979).

1. Plasminogen Activator System

Plasminogen activators (PA) are serine-specific proteases that convert inactive plasminogen to active plasmin, a trypsin-like enzyme that has broad substrate specificity. Plasmin degrades a variety of proteins, including fibrin, fibronectin, type IV collagen, vitronectin, and laminin. It also has the ability activate latent collagenases and pro-plasminogen activators (Gottesman, 1990) (Figure 2). Plasminogen activator exists in two forms, tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA) (Liotta, 1979). The two forms of plasminogen activator have different biological functions. tPA is the main plasminogen activator in plasma where it plays a vital role in intravascular fibrinolysis (Gottesman, 1990), whereas u-PA is primarily involved in cell-mediated proteolysis during macrophage invasion, wound healing, embryogenesis, invasiveness, and metastasis (Conese and Blasi, 1995; Dano et al., 1985; Fazioli and Blasi, 1994).

u-PA is a glycoprotein that is secreted as a single chain pro-enzyme. Activation of the pro-enzyme is achieved by limited proteolysis to produce active u-PA. Proteases that have been shown to activate uPA include plasmin, kallikrein, trypsin, thermolysin, factor XIIa, and the cathepsins (Conese and Blasi, 1995). In addition, nerve growth factor γ, another serine protease, can also cleave pro-uPA at the plasmin cleavage site, both in solution and at the cell surface (Wolf et al., 1993). Active uPA is a two chain, M_r 54,000 molecule consisting of a light A chain and a heavy B chain. The light chain contains the amino terminus of the uPA molecule and forms the receptor binding or growth factor domain and a single kringle unit. The catalytic domain is contained in the heavy chain at the carboxy-terminal region (Saksela and Rifkin, 1988). Two active forms of u-PA have been identified, M_r ~54,000 and M_r ~33,000, the latter being composed mainly of the heavy chain catalytic domain.

Active uPA and plasmin play a direct role in the degradation of extracellular matrix components and thereby modulate cell-cell and cell-extracellular matrix interactions. In addition, they have been shown to have an indirect function in the degradation of the extracellular matrix by their ability to activate pro-collagenases and degrade tissue inhibitors of metalloproteases (TIMPs) (Figure 2) (DeClerck and Laug, 1996). uPA has been shown to activate directly latent growth factors such as the precursor form of hepatocyte growth factor/scatter factor (pro-HGF/SF) and indirectly latent transforming growth factor-β (pro-TGF-β) through activation of plasminogen (Naldini et al., 1992; Odekon et al., 1994). The ability of uPA to activate such growth factors suggests not only a role in modulating extracellular matrix degradation but also in tumor cell migration and proliferation. Further regulation of migration may be facilitated by uPA degradation of fibronectin, a component known to be a strong chemoattractant for tumor cells (Webber and Waghray, 1995).

High levels of uPA have been observed in both human tumors and cell lines (Billstrom et al., 1995; Heiss et al., 1995; Kuhn et al., 1994). In bladder cancer, tumor progression and recurrence were associated with high u-PA content (Hasui et al., 1996). Breast cancer expressing high u-PA levels had an increased risk for early recurrence and had poor prognosis (Duffy et al., 1990), whereas in primary breast cancer, metastasis-free survival could

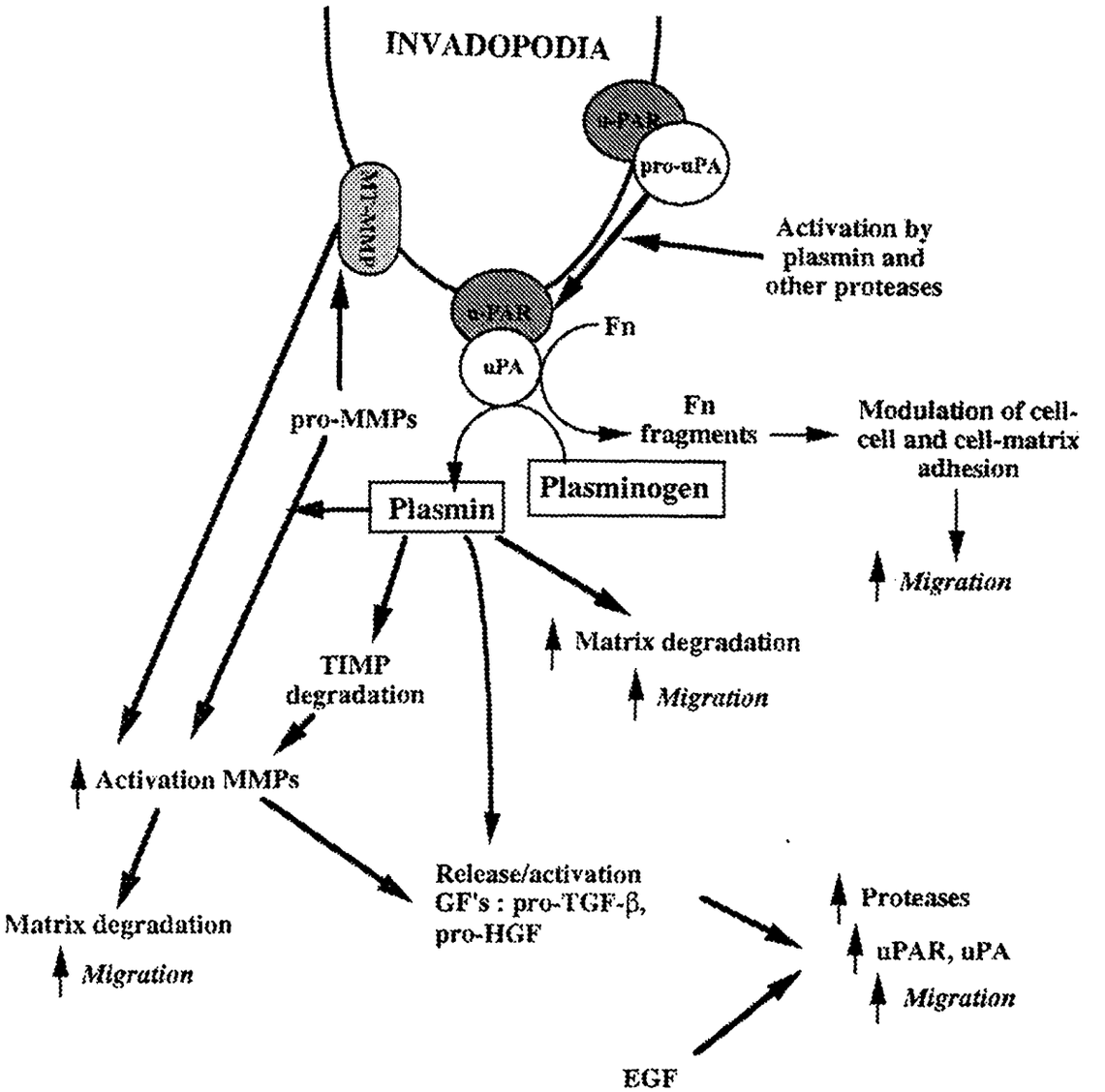

FIGURE 2. Schematic diagram illustrates the multiple effects of the plasminogen activator system in matrix degradation, migration, growth factor, and protease activation. **Fn**, fibronectin; **EGF**, epidermal growth factor; **TGF-β**, transforming growth factor β; **HGF**, hepatocyte growth factor; **MMP**, matrix metalloprotease; **MT-MMP**, membrane type-metalloprotease; **uPA**, urokinase plasminogen activator; **uPAR**, urokinase plasminogen activator receptor.

be predicted by uPA levels (Spyratos et al., 1992). In addition, a greater number of cells were positive for u-PA in advanced, invasive lung carcinomas than in low-grade lung tumors (Oka et al., 1991). Moser et al. showed that production of u-PA in normal ovarian epithelial cells was 17- to 38-fold lower than that found in ovarian carcinoma cells (Moser et al., 1994). The DU-145 human prostate cancer cell line has been shown to express five times more extracellular, secreted u-PA activity than normal prostatic epithelial cells (Waghray and Webber, 1995). It has also been demonstrated that stimula-

tion of PC3 human prostatic carcinoma cells with epidermal growth factor (EGF) not only increases their u-PA expression but also increases the invasive ability of the cells (Jarrard et al., 1994).

Further evidence of the role of u-PA in tumor cell invasion has been provided by the specific inhibition of the enzyme by anticatalytic antibodies and inhibitors. Ossowski demonstrated that nude mice inoculated with a human squamous cell carcinoma developed invasive tumors expressing high levels of u-PA (Ossowski et al., 1991); anticatalytic monoclonal antibodies against the enzyme inhibited invasion. Anti-uPA also has been shown to block human HEP-3 cell invasion in the chick chorioallantoic membrane assay and murine B16F10 melanoma cell metastasis following tail vein injection (Estreicher et al., 1989; Ossowski and Reich, 1983). In addition to the inhibition of u-PA by monoclonal antibodies, specific inhibitors of the enzyme have been shown to reduce invasion. For example, retinoic acid was shown to reduce secreted u-PA activity in a prostate carcinoma cell line, and it was further shown that the invasion of these prostatic carcinoma cells *in vitro* was reduced (Waghray and Webber, 1995; Webber and Waghray, 1995).

uPA has been shown to bind to the cell via surface specific receptors (Nielsen et al., 1988; Stoppelli et al., 1986). The presence of high-affinity binding sites on several different cancer types, including ovarian carcinoma, colon carcinoma, and glioma, has been demonstrated (Casslen et al., 1991; Ossowski et al., 1991). The mature uPA receptor, 55 to 60 kDa, is a highly glycosylated single-chain polypeptide made up of three homologous repeats of approximately 90 residues, of which the amino-terminal end is involved in binding (Roldan et al., 1990). The uPA receptor, uPAR, is attached to the plasma membrane by a covalent linkage to a glycerophosphatidyl-inositol glycan (GPI) moiety. The presence of this GPI anchor allows the receptor to be released from the plasma membrane by phosphotidylinositol-specific phospholipase C (PI-PLC) (Ploug et al., 1991). Glycosylation of the receptor is important in its intracellular transport and maturation but has also been shown to affect the affinity of the receptor for uPA (Moller et al., 1993; Picone et al., 1989).

Both u-PA and its inactive zymogen (pro-uPA) bind with high affinity to uPAR. Expression of uPAR is up-regulated by a variety of growth factors and tumor promoters, including TGF-β, EGF, HGF/SF and phorbol ester (Lund et al., 1995; Lund et al., 1991; Pepper et al., 1992; Picone et al., 1989). The uPA receptor does not internalize bound active uPA (Stoppelli et al., 1986); in this way uPAR provides tumor cells with a means of generating localized protease activity at the cell surface, such as at the invasive front (Kristensen et al., 1990). Receptor-bound pro-uPA can be converted to the enzymatic active form by plasmin and/or cell surface-associated cathepsin B or cathepsin L (Goretzki et al., 1992; Kobayashi et al., 1992). Evidence that uPAR plays a role in the activation of pro-uPA was shown in U937 cells. Receptor bound pro-uPA activation generated by cell-associated plasmin activity was approximately 20-fold higher than that generated by plasmin in solution (Ellis et al., 1989). Furthermore, blocking the amino terminal domain of uPAR with specific monoclonal antibodies prevented uPAR bound uPA activation, indicating that the receptor is central to the potentiation of the u-PA-mediated system of plasmin generation (Ronne et al., 1991).

The importance of uPAR in the invasion of tumor cells has been shown in studies in which the interaction of u-PA with its receptor was blocked. Crowley et al. demonstrated that the expression of mutant u-PA in PC3 human prostate cancer cells resulted in a dominant negative suppression of

metastasis in nude mice xenografts. This inhibition occurred through the displacement of active u-PA from its receptor by the inactive mutant enzyme (Crowley et al., 1993). Increased levels of uPAR have also been found in a number of brain tumors suggestive of its involvement in efficient activation of pro-uPA and confinement of uPA activity on the cell surface of invading brain tumor cells (Mohanam et al., 1994). Pedersen et al. demonstrated increased uPAR expression in ovarian tumors was associated with short overall survival of patients (Pedersen et al., 1994).

In addition to its expression on tumor cells, uPAR has also been shown to be expressed by other cell types in the tumor microenvironment. Ohtani et al. demonstrated that stromal cells as well as tumor cells expressed uPAR in human adenocarcinoma specimens. uPAR-positive cells were most abundant at the invasive margin and suggested that a variety of non-malignant host cells play an important role in the plasmin-mediated breakdown of the extracellular matrix at the invasive margin (Ohtani et al., 1995). The interplay of uPA and its receptor, uPAR, play an important role in direct and indirect extracellular matrix degradation, thus potentiating invasive events. The action of uPA can be counteracted by several naturally occurring inhibitors of the uPA/plasmin system.

2. uPA Inhibitors

The principal plasminogen activator inhibitors are members of the SERPIN (SERine Protease INhibitor) family, plasminogen activator inhibitor-1 (PAI-1), plasminogen activator inhibitor (PAI-2), and the recently discovered protease nexin 1(PN-1) (Potempa et al., 1994). Interaction between active uPA and the inhibitors results in its inactivation and cleavage of the inhibitor (Andreasen et al., 1990). Receptor bound active uPA is accessible to and its activity inhibited by PAI-1, PAI-2, and PN-1 (Ellis and Dano, 1991). However, unlike the uPAR:uPA complex, which remains stable at the cell surface, the uPAR:uPA:inhibitor complex is quickly internalized by the cell and degraded.

Plasminogen activator inhibitor 1 (PAI-1) is a 52-kDa glycosylated protein that has specificity for uPA. It is secreted as an active antiprotease, which is quickly converted to the latent form if no binding of the inhibitor occurs. In addition to binding uPA in solution and the receptor bound form, PAI-1 is also known to bind to vitronectin, allowing PAI-1 to remain in an active form from which it can then inactivate uPA at the cell surface (Ciambrone and McKeown-Longo, 1990). PAI-1 is produced primarily by endothelial cells, however, a number of other cell types, including tumor cells, have been shown to produce the protein (Andreasen et al., 1990; Dano et al., 1994).

PAI-1 has been shown to inhibit the adhesion of myeloid cells, fusion of myoblasts, and migration of endothelial cells. Inhibition of endothelial cell migration indicates a role for PAI-1 in blocking neoangiogenesis (Fazioli and Blasi, 1994; Montesano et al., 1990; Odekon et al., 1992). Cancer cells that overexpress PAI-1 have been shown to have a decreased ability to degrade extracellular matrix and a reduction in their invasive potential (Cajot et al., 1990). A recent study showed that PAI-1 negative gastric tumors had a significantly higher incidence of liver metastases and a poorer prognosis than PAI-1 positive tumors (Ito et al., 1996). In contrast to these findings, other studies have demonstrated that high levels of uPA and/or PAI-1 in breast cancer (Duffy et al., 1990; Grondahl-Hansen et al., 1993; Janicke et al., 1991) and advanced ovarian cancer (Kuhn et al., 1994) are markers of a poorer prognosis. Elevated antigen levels of uPA and of PAI-1 in tumor extracts of ovarian,

breast, colon, and lung cancer have correlated with increased incidence of relapse, shorter overall survival, and increased degree of invasion (Schmitt et al., 1995). This apparent contradiction between studies may be explained in that highly vascularized tumors have a higher propensity for metastatic spread and thereby a poorer prognosis. Due to this vascularization, a higher number of endothelial cells are present that produce higher amounts of PAI-1, and therefore increased PAI-1 correlates with poorer prognosis. The net balance between the inhibitor and the protease is a determinant of the degradative ability of the tumor cells. Therefore, an increase in the inhibitor may not always result in a reduction of active uPA, as uPA production may also be upregulated by the tumor cells.

Plasminogen activator inhibitor 2 (PAI-2) is a glycosylated, secreted 60-kDa protein that can accumulate as a 47-kDa intracellular protein, is released after cell death. Although it is a less-efficient inhibitor of uPA than PAI-1, it is more specific. This inhibitor has been shown to be produced by a variety of cell types that include monocyte/macrophages, fibroblasts, and tumor and endothelial cells (Kruithof et al., 1995). Increased PAI-2 has been shown in breast cancer extracts compared with extracts of benign breast tumors (Foucre et al., 1991; Sumiyoshi et al., 1992). PAI-2 concentrations were found to be lower in breast carcinomas with lymph node involvement than those without, whereas for PAI-1 the opposite was found (Bouchet et al., 1994; Foekens et al., 1994). Extracts from pulmonary adenocarcinoma with lymph node involvement had lower levels of PAI-2 than node-negative lung carcinomas (Nagayama et al., 1994). Tissue levels of PAI-2, uPA, and PAI-1 in gastric cancer were found to be higher than in control tissues and remote lymph node involvement were characterized by higher PAI-1 and lower PAI-2 levels (Nakamura et al., 1992). PAI-2 expression in pancreatic cancer was significantly lower in carcinomas with peritoneal metastases with higher PAI-2 levels being associated with a higher survival rate (Takeuchi et al., 1993). Although several studies have shown a correlation with high uPA and PAI-2 levels associated with a poor prognosis in advanced ovarian cancer and colorectal tumors (Chambers et al., 1995; Ganesh et al., 1994); generally, higher levels of PAI-2 are associated with better prognosis and less disseminated disease.

As anticipated by these results and the specificity of PAI-2 for uPA, the inhibitor has been shown to inhibit cancer cell invasion and metastasis. Exogenously added PAI-2 inhibited plasminogen-dependent degradation of collagen IV, proteoglycan, and glycoprotein by cancer cells (Baker et al., 1990; Reiter et al., 1993). Suramin, which enhances PAI-2 production in human renal cell carcinoma cells, inhibited metastasis after renal subcapsular implantation in nude mice (Marutsuka et al., 1995). Intraperitoneal injection of PAI-2 into nude mice bearing A431 cell xenografts resulted in the decrease of tumor mass (Shinkfield et al., 1992). Intratumoral injection of the urokinase inhibitor *p*-aminobenzamidine into SCID mice with subcutaneous DU145 tumors also decreased tumor size (Billstrom et al., 1995).

Protease nexin-1 (PN-1) is a recently discovered inhibitor of uPA, tPA, plasmin, and thrombin. It is a 45-kDa protein that has the ability to bind to extracellular matrix proteins and heparin. Although it inhibits uPA, it does not have the capacity to bind pro-uPA. As with PAI-1 and 2, receptor bound active uPA in complex with PN-1 is rapidly internalized and degraded (Conese et al., 1994). Collagen IV decreases the formation of complexes between PN-1 and uPA or plasmin (Donovan et al., 1994). Therefore, matrix components may influence the availability of PN-1 and its inhibition al-

lowing control of degradation. Further studies are needed to understand the importance of PN-1 to the metastatic process.

3. Cathepsins

There is increasing evidence for the role of the cysteine proteases cathepsins B and L in cancer progression. Cathepsin B is a lysosomal acid hydrolase that has a broad range of endopeptidase activity against substrates as myosin, actin, proteoglycans, fibronectin, laminin, and the non-helical portions of type IV collagen. Although lysosomal, cathepsin B activity has been found in association with the plasma membrane fraction of tumor cells and in the conditioned cell media from tumor cell cultures (Sloane and Honn, 1984; Sloane et al., 1986). Cathepsin B is not only important due to its ability to degrade extracellular matrix, but as it has the ability to convert inactive pro-uPA, soluble and tumor cell receptor bound forms, into enzymatically active uPA (Kobayashi et al., 1991).

Pietras and co-workers measured cathepsin B activity in the serum of patients with gynecological malignancies and found a significant increase in cathepsin B activity in patients with advanced squamous cell carcinoma of the uterine cervix. Minimal elevations were observed in women with lower stage disease, dysplasia, and controls (Pietras et al., 1979). They also observed that advanced stage adenocarcinomas of the ovary and endometrium were associated with markedly elevated levels of enzyme activity, up to 2- to 3-fold higher than seen in early stage patients. Cathepsin B activity was observed in papillary carcinoma of the thyroid to be 15-fold higher than that found in normal adjacent tissue. Increased mRNA levels of cathepsin B were also seen in tumor samples compared with normal and nonneoplastic diseased tissue of the thyroid (Shuja and Murnane, 1996). Van der Stappen and colleagues demonstrated in a model of progression from colorectal adenoma to carcinoma that there was an 8-fold increase in the presence of the pro-form of cathepsin B in the conditioned medium. In addition, mature enzyme was only detected in cell lines that had an increased malignant potential (Van der Stappen et al., 1996). It has also been shown in colorectal carcinomas that cathepsin B expression correlates with tumor progression and shortened patient survival (Campo et al., 1994). A two- to sevenfold elevation of cathepsin activity was found in the metastatic B16-F10 cell line when compared to the B16-F1 line which displays lower metastatic potential *in vivo* (Sloane et al., 1982).

Like cathepsin B, cathepsin L exhibits a high capacity to degrade extracellular matrix proteins, such as collagen, laminin, and elastin (Chauhan et al., 1991). Its proteolytic activity is said to be higher than that of cathepsin B. In addition, similar to cathepsin B, cathepsin L is a strong activator of pro-uPA (Goretzki et al., 1992). Joseph et al. described a high correlation between the mRNA level of cathepsin L with the metastatic potential of c-Ha-*ras* transformed cell lines (Joseph et al., 1988). Its activity was markedly increased in papillary carcinoma of the thyroid by 9-fold (Shuja and Murnane, 1996).

Cathepsin D, an aspartyl protease, is also a lysosomal acid protease (Rochefort et al., 1990). It has documented mitogenic activity on estrogen-depleted MCF-7 human breast adenocarcinoma cells in culture, and its secretion is constitutive in hormone-independent breast cancer cell lines (Rochefort et al., 1987). Recently, Losh et al. have shown that cathepsin D expression has a significant prognostic value for overall survival in endometrial cancer (Losch et al., 1996). It has also been shown to correlate with tumor

aggressiveness in patients with early cervical squamous cell carcinoma (Kristensen et al., 1996) and has been associated with metastasis in breast cancer (Rochefort, 1992). *In vitro* studies by Johnson et al. showed no correlation of cathepsin D levels and invasive behavior in MCF-7 cell line subclones expressing different levels of the protease (Johnson et al., 1993), suggesting that in breast tumor cells its association with tumor progression was not due to degradation of extracellular matrix components. Liaudet and colleagues proposed that cathepsin D overexpression may stimulate cell proliferation of micrometastases in nude mice by inactivating a secreted growth factor inhibitor (Liaudet et al., 1995). Thus, Cathepsin D may facilitate the growth of tumor cells at secondary distant sites through the inactivation of growth factor inhibitors or by the activation of growth factors and interaction with growth factor receptors (Garcia et al., 1996; Jiang et al., 1994). However, active cathepsin D may also play an indirect role in extracellular matrix remodeling by its ability to activate pro-cathepsin B and receptor bound pro-uPA (Van der Stappen et al., 1996; Jiang et al., 1994). Further investigations are required to define its exact involvement.

4. Cathepsin Inhibitors

The cysteine and aspartyl proteases can be regulated by their interaction with endogenous inhibitors. Endogenous cysteine and aspartyl protease inhibitors are divided into three families, stefins, cystatins, and kininogens, all which belong to the cystin superfamily (Calkins and Sloane, 1995). These inhibitors block the active site of the proteases in a noncovalent and reversible manner. Alterations in the cysteine protease inhibitor/cysteine protease ratio has been postulated to contribute to the malignant progression of tumors. A decrease in the concentration and/or the activity of the inhibitors may contribute to this imbalance favoring the malignant phenotype (Kane and Gottesman, 1990).

Several studies have focused on experimental systems to determine the importance of cysteine protease inhibitors in tumor progression. Van der Stappen observed an inverse correlation between the amount of cathepsin protease inhibitor secreted into the media and the tumorigenicity of colorectal cells that represented progressive steps in the transformation of adenoma to carcinoma (van der Stappen et al., 1991). Cathepsin protease inhibitor was also observed to have decreased activity in metastatic *ras*-transformed NIH 3T3 cells when compared with their non-transfected counterparts (Chambers et al., 1992). In a similar study, Sloane et al. demonstrated that a decrease in cathepsin protease inhibitor was associated with *ras* transfection and metastatic potential in rat embryo fibroblast cell lines (Sloane et al., 1992). The high ratios of the cysteine proteases to cysteine protease inhibitors found between normal and tumor tissue and cell lines suggests that metastatic cells may not produce enough cysteine protease inhibitor to regulate the cysteine protease activity (Chambers et al., 1992; Rozhin et al., 1990).

In two thirds of tested human breast carcinoma specimens, lower stefin and cystatin cathepsin protease inhibitor activity was found when compared with normal tissue. Low cathepsin protease inhibitor is associated with poorly differentiated breast carcinomas (Lah et al., 1992; Lah et al., 1992). However, in one study of colorectal carcinoma, no difference was observed between tumor tissue and that of normal (Sheahan et al., 1989). Few paired studies have been performed to be able to draw any general observations.

The best-studied cathepsin protease inhibitor is stefin A. At low concentrations,

stefins markedly decreased the stimulated motility of both human melanoma cells and W256 carcinosarcoma cells. Its expression is decreased in malignant human tumors (Jarvinen et al., 1987); for example, it is less abundant in cancer of the uterine cervix than in normal cervix (Eide et al., 1992). Eighty percent of breast cancer samples tested had decreased stefin A transcript levels when compared with matched normal breast tissue (Lah et al., 1992). Isolation of stefin A from human sarcoma revealed that the protein had a decreased inhibitory capability against cathepsin B. The authors postulate that this may suggest a decreased activity due to a protein modification at the primary structure. Thus, cysteine proteases and their inhibitors may play a direct role in the development of a migratory phenotype (Boike et al., 1992). Studies of the role of cathepsin inhibitors in malignant progression continue.

5. Matrix Metalloproteases

Matrix metalloproteases (MMPs) are a family of neutral metalloenzymes that depend on the metal ion, Zn^{2+}, for their activity. The metalloproteases are secreted as latent proenzymes that require activation through the proteolytic cleavage of an amino terminal domain. Once activated, this family of proteases function optimally at pH levels in the physiological range. They are active against most components of the basement membrane, and the family includes the only enzymes capable of cleaving and denaturing fibrillar collagen. MMPs are produced and secreted from a wide variety of cell types and have been found to be present at the sites of both physiologic and pathologic tissue and matrix remodeling (Coussens and Werb, 1996; Matrisian, 1992).

The matrix metalloproteases are regulated at a number of levels, including transcriptional regulation by growth factors and cytokines, posttranscriptional regulation due to changes in mRNA stability, posttranslational regulation by activation of the secreted latent form, and inhibition by endogenous inhibitors known as the tissue inhibitors of metalloproteases (TIMPs). These levels of regulation allow tight control of active MMP under normal physiological conditions. However, in pathological conditions, such as metastasis, the opportunity for dysregulation is enhanced.

Conserved functional and structural domains give similar mechanisms of latency, activation and proteolysis to the members of the MMP family (Birkedal-Hansen et al., 1993). The greatest homology of the family is at the catalytic site (VAAHEXGHXXGXXH), which binds the zinc atom and the amino-terminal activation locus (PRCGXPDV). The carboxy-terminal region of the MMPs possess a hemopexin/vitronectin-like domain that has been shown to be important in substrate and inhibitor binding (Sanchez-Lopez et al., 1993). Matrilysin (MMP-7) is the only member that does not possess such a domain. Members of the family can be divided into five general subclasses based on their substrate specificity: stromelysins, interstitial collagenases, membrane type-MMPs, gelatinases, and elastase.

The stromelysin family consists of four related gene products, stromelysins 1, 2, and 3 (53-57 kDa), and matrilysin at 28 kDa (Liotta et al., 1980; Matrisian, 1992; Murphy et al., 1993). These enzymes have a broad range of preferred substrates, including laminin, fibronectin, and the noncollagenous domain of type IV collagen. A strong positive correlation between the overexpression of stromelysin 1 and clinical tumor invasion has been demonstrated for breast cancer (Wolf et al., 1993). Stromelysin 3 has been shown to be expressed in most invasive human carcinomas but rarely in sarcomas and other nonepithelial tumors (Rouyer et

al., 1994). Stromelysin-3 mRNA expression has been shown to be specifically localized to the stroma of breast, colon, basal cell, and head and neck cancers (Basset et al., 1990; Basset et al., 1993; Muller et al., 1993; Newell et al., 1994; Wolf et al., 1992). The highest levels of stromelysin 3 gene expression was observed in tumors exhibiting a high degree of local invasiveness or those that had a poor prognosis (Chenard et al., 1996).

Matrilysin (MMP-7) is another member of the stromelysin family and has been shown to be expressed in a variety of tumors, including breast, prostate, colon, stomach, lung, and skin (Wilson and Matrisian, 1996). Matrilysin is the only MMP for which exclusive tumor cell-associated MMP production has been shown. No production of matrilysin by stromal elements has been demonstrated in gastric cancer, colon cancer, breast cancer, and prostate cancer (McDonnell et al., 1991; Pajouh et al., 1991; Wolf et al., 1993). Additionally, matrilysin was shown not to be present in normal colon mucosa, whereas it was highly expressed in gastric and colon carcinoma (Newell et al., 1994; Yamamoto et al., 1994). Studies of matrilysin in SW620 colon tumor cells showed that its expression correlated with the ability of the cell to invade artificial basement membrane substrata *in vitro* and to metastasize to the liver in xenograft models. Antisense transfection of matrilysin knocked out its expression and reduced the tumorigenicity and subsequent metastasis to the liver of the highly metastatic SW620 cells. Additionally, transfection of wild-type or activated forms of matrilysin into the parental, nonmetastatic SW480 colon tumor cells, that do not express endogenous matrilysin did not increase *in vitro* invasion but did increase tumorigenicity (Witty et al., 1994). Matrisian and colleagues have shown recently that matrilysin is expressed in 88% of adenomas arising in *min* mice, transgenic mice bearing a nonsense mutation in the APC gene giving rise to multiple intestinal neoplasia. Molecular knockout of matrilysin in the *min* mouse demonstrated that matrilysin-deficient mice developed 60% fewer intestinal adenomas. These results suggested that matrilysin plays an important role in the development of benign lesions in this model system (Wilson et al., 1997). Therefore, matrilysin may play a role in the growth and progression of tumor cells, possibly by the release of sequestered growth factors in the extracellular matrix during matrix remodeling.

The interstitial collagenases consist of interstitial collagenase (MMP-1), neutrophil collagenase (MMP-5), and collagenase-3 (MMP-13), all of which degrade the triple helical domain of collagen types I, II, III, and X found in the interstitial stroma (Liotta et al., 1981; Matrisian, 1992; Stetler-Stevenson et al., 1993). Interstitial collagenase expression has been documented in lung carcinomas, colorectal tumors, and squamous cell carcinomas of the head and neck among others (Gray et al., 1992; Hewitt et al., 1991; Muller et al., 1991; Urbanski et al., 1992). Recently, it has been shown that interstitial collagenase expression correlates with poor prognosis in colorectal cancer (Murray et al., 1996). Recently, collagenase-3 has been cloned from human breast carcinoma tissue, and observations suggest that it may be important in the invasion of breast cancer cells (Freije et al., 1994).

The membrane type-MMPs have been discovered recently and are the first to possess a transmembrane domain that localizes their activity to the cell membrane. At present, four members of the MT-MMP subfamily have been identified (Sato et al., 1994; Takino et al., 1995; Will et al., 1995; Puente et al., 1996). MT1-MMP and MT2-MMP are cell membrane-bound activators of the pro-form of gelatinase A (MMP-2) (Sato et al., 1994; Takino et al., 1995). Increased MT1-MMP expression has been observed in invasive cervical carcinoma and lymph node metastases compared with its expres-

sion in noninvasive lesions, and it has been associated with cervical cancer cell invasiveness (Gilles et al., 1996). Increased levels of MT1-MMP expression have been observed in malignant astrocytomas, metastatic lung carcinomas, and head and neck cancers (Yamamoto et al., 1996; Yoshizaki et al., 1997).

The gelatinases, gelatinase A (MMP-2) and gelatinase B (MMP-9), also known as the type IV collagenases, are another subgroup of matrix metalloproteases (Garbisa et al., 1987; Liotta and Stetler-Stevenson, 1990). They are distinguishable by immunologic, molecular, and biochemical criteria, but not by substrate specificity. The preferred substrates for these enzymes include collagen types I and IV, vitronectin, fibronectin, and gelatin. MMP-2 and MMP-9 are secreted as proenzymes and are 72 and 92 kDa in size, respectively. Activation of the enzymes occurs by the proteolytic cleavage of the pro-peptide domain. This can be achieved by other proteases such as cathepsin B and plasmin (Eeckhout and Vaes, 1977; Paranjpe et al., 1980), and now the MT-MMPs have been shown to activate pro-MMP-2 at the cell surface (Cao et al., 1995). It has been observed that TIMP-2 and TIMP-2:MMP-2 complex bind to the cell surface via an, as-yet unpurified TIMP-2 receptor (Emmert-Buck et al., 1995). It has been proposed that MT-MMP or a MT-MMP-associated protein serves as the cell surface receptor for TIMP-2. The activated MT1-MMP:TIMP-2 complex can in turn bind and activate pro-MMP-2. Alternatively, pro-MMP-2:TIMP-2 binds to the cell surface through TIMP-2, after which pro-MMP-2 can be activated by MT-MMP. Stoichiometric quantities of TIMP-2 are required for this mechanism, and increased concentrations of TIMP-2 interfere with the process (Cao et al., 1996; Strongin et al., 1995).

Cytokines, growth factors, and signal transduction regulators have been demonstrated to modulate the expression, production, and/or function of the matrix metalloproteases as well as many other proteases involved in the metastatic cascade (Brown et al., 1990; Mauviel, 1993; Reynolds, 1996). Inflammatory cytokines such as interleukin-1 and tumor necrosis factor-α (TNF-α) are known to strongly induce the expression of MMP-9, stromelysin-1, and interstitial collagenase. A postulated mechanism of induction is by increasing the expression of members of the family of AP-1 transactivator transcription proteins that bind to the TPA-responsive element (TRE) and initiate transcription of some MMP promoters. Induction of those MMPs also occurs with exposure to TPA. The MMP-2 promoter does not have this element and thus, TPA does not induce the expression of this MMP (Brown et al., 1990; Frisch and Morisaki, 1990).

TGF-β has been shown to increase MMP-2 and MMP-9 secreted activity in mammary adenocarcinoma cells, cervical carcinoma cells, and endothelial cells, among others (Kohn et al., 1995; Welch et al., 1990). Newly discovered MMP-13 expression has also been shown to be enhanced by TGF-β and TNF-α (Johansson et al., 1997). EGF has been shown to increase the *in vivo* and *in vitro* metastatic potential of a variety of tumor cell types, postulated to be as a result of induction of MMP secretion (Holting et al., 1995; Otani et al., 1994; Price et al., 1996).

Growth factors not only enhance protease production by tumor cells but have been shown to effect other cell types such as stromal fibroblasts. For example, platelet-derived growth factor (PDGF) has been shown to stimulate collagenase expression by human fibroblasts (Circolo et al., 1991). Additionally, tumor cells have been demonstrated to elicit enhanced protease expression from surrounding host cells, such as fibroblasts. Biswas and colleagues initially described the tumor cell-derived collagenase-stimulatory factor (TCSF), now termed extracellular matrix metalloprotease inducer (EMMPRIN) (Biswas et al., 1995). This in-

ducer is present on the cell surface of several human tumor cell types *in vitro* and *in vivo*, and stimulates the production of interstitial collagenase, stromelysin-1, and MMP-2 in human fibroblasts (Guo et al., 1997; Kataoka et al., 1993). Thus, MMP induction in the surrounding stroma enhances the ability of the tumor cells to degrade the local extracellular matrix.

In addition to MT-MMPs, localization of the degradative ability of MMP-2 has been shown to be due to the interaction with the cell surface integrin $\alpha_v\beta3$. Brooks et al. demonstrated that expression of $\alpha_v\beta3$ on cultured melanoma cells enabled their binding to MMP-2 in the active form, allowing cell-mediated collagen degradation (Brooks et al., 1996). Previous to this finding, increases in $\alpha_v\beta3$ expression in melanoma cells correlated positively with tumor progression (Gehlsen et al., 1992; Hart et al., 1991; Seftor et al., 1992). Seftor and colleagues demonstrated that MMP-2 was modulated via differential expression of $\alpha_v\beta3$ and $\alpha5\beta1$ during human melanoma cell invasion (Seftor et al., 1993). The receptor has also been shown to be involved in tumor cell motility and thus regulates both tumor cell matrix degradation and motility allowing for directed cellular invasion.

MMP-2 activity has been shown to correlate with metastatic activity of B16 murine melanoma cells (Liotta et al., 1980). Induction of the malignant phenotype by activated H-*ras* transfection was shown to enhance production of collagen-degrading activity (Pozzatti et al., 1986). A similar effect was seen in *ras*-transfected bronchial epithelial cells in which mRNA levels and enzyme activity was shown to have a close correlation with the invasive and metastatic properties of the cells (Ura et al., 1989). Down-regulation of type IV collagenolytic activity by retinoic acid treatment of human melanoma cells has been correlated with a loss of the invasive phenotype. Studies of the mechanism of this effect have revealed that retinoic acid treatment of human melanoma cells results in a reduction of the steady state levels of MMP-2 mRNA (Hendrix et al., 1990). Studies of human tumors have shown that MMP-2, is increased in multiple tumor types, such as breast cancer, colon cancer, thyroid cancer, and pulmonary adenocarcinomas (Campo et al., 1992; D'Errico et al., 1991; Levy et al., 1991; Urbanski et al., 1992). Campo et al showed that invasive carcinoma of the ovary stained positively for MMP-2, whereas benign ovarian cysts had no enzyme detectable. In addition, secreted MMP-2 activity has been demonstrated from ovarian carcinoma cells *in vitro* and *in vivo* and in ascites of ovarian cancer patients (Davies et al., 1993; Kohn et al., 1992; Moser et al., 1994). Although MMP-2 is localized to the tumor cells of colon cancers, *in situ* hybridization studies revealed that stromal elements surrounding the tumor cells were responsible for the production (Levy et al., 1991; Pyke et al., 1993). MMP-9 has been shown to be associated with inflammatory cells of tumors as well as tumor cells of the skin and lung (Canete-Soler et al., 1994; Pyke et al., 1992).

6. Metalloproteinase Inhibitors

The invasive potential of the tumor cell is determined by the net balance of the active protease content and the availability of endogenous inhibitors. MMPs are inhibited by a family of endogenous inhibitors known as the tissue inhibitors of metalloproteases (TIMPs) (Stetler-Stevenson et al., 1993). The balance between the levels of activated MMPs and free TIMPs determines the net MMP activity, altering this equilibrium affects the process of invasion. There are three well-defined members of the TIMP family. Recently, a murine TIMP-4 has been isolated,

although it is still to be determined if a human homologue of this new member of the TIMP family exists (Leco et al., 1997). TIMPs are natural inhibitor proteins, produced by either the host cells or by the tumor cell itself. They have been shown to block both the latent and the active metalloproteases (Goldberg et al., 1989; Murphy et al., 1981; Stetler-Stevenson et al., 1989), and, recently, they have been shown to be active in tumor cell growth (Corcoran et al., 1996; Nemeth et al., 1996).

Inhibition of the metalloproteinase activity may not be the only biological function of the TIMPs. TIMP-1 has been shown to stimulate the proliferation of the human erythroleukemia cell line K562 *in vitro* (Avalos et al., 1988), as well as that of Raji Burkitt's lymphoma cells, gingival fibroblasts (Hayakawa et al., 1992), and keratinocytes (Bertaux et al., 1991). TIMP-2 has also been shown to stimulate proliferation of Raji cells and fibroblasts in serum-free conditions (Corcoran and Stetler-Stevenson, 1995; Hayakawa et al., 1994). TIMPs also have antiangiogenic activity. Both TIMP-1 and TIMP-2 have been demonstrated to inhibit angiogenesis in the chick chorioallantoic assay (Albini et al., 1994; Murphy et al., 1993; Ohba et al., 1995). It has also been shown that TIMP-2 but not TIMP-1 or the progelatinase A:TIMP-2 complex can inhibit basic fibroblast growth factor (FGF-2)-induced stimulation of endothelial cell proliferation independent of its ability to inhibit MMP activity (Murphy et al., 1993).

TIMP-1, the first member of the TIMP family to be described, is a glycoprotein with an apparent molecular size of 28.5 kDa. It forms a complex of 1:1 stoichiometry with activated interstitial collagenase, activated stromelysin, and activated MMP-9 (Carmichael et al., 1986; Docherty et al., 1985; Herron et al., 1986; Khokha et al., 1989). The same cells that produce interstitial collagenase are capable of synthesizing and secreting TIMP-1 (Herron et al., 1986). Thus, the net collagenolytic activity for these cell types is the result of the balance between secreted activated enzyme concentrations and TIMP-1 concentrations. Studies have shown an inverse correlation between TIMP-1 levels and the invasive potential of murine and human tumor cells. Furthermore, natural and recombinant TIMP-1 have been shown to prevent tumor cell invasion of human amnion *in vitro*, and to block metastasis formation in animal models (Alvarez et al., 1990). Suppression of the tumorigenic as well as the metastatic potential of B16-F10 melanoma cells in mice was observed in cell clones overexpressing TIMP-1 (Khokha et al., 1994). Martin et al. further demonstrated that in transgenic mice that overexpress TIMP-1 in the liver, SV-40 T antigen-induced hepatocellular carcinoma development was blocked. High TIMP-1 levels did not only inhibit the later stages in tumor development, that is, growth and angiogenesis, but also the events associated with tumor initiation (Martin et al., 1996).

Stetler-Stevenson and colleagues have isolated, purified, and cloned the second member of the TIMP family, TIMP-2, located on chromosome 17q25 (Stetler-Stevenson et al., 1989; Stetler-Stevenson et al., 1992). TIMP-2 is a 21-kDa nonglycosylated protein that selectively forms a complex with the latent proenzyme form of gelatinase A/MMP-2. TIMP-2 has 37% identity and overall 66% homology to TIMP-1 at the amino acid level. TIMP-1 and TIMP-2 are differentially regulated at the level of mRNA expression; TGF-β reduces expression of TIMP-2 and increases TIMP-1 message (Goldberg et al., 1989; Stetler-Stevenson, 1990; Stetler-Stevenson et al., 1989). TIMP-2, unlike TIMP-1, inhibits the type IV collagenolytic activity and the gelatinolytic activity associated with the latent and active MMP-2 and the active forms of the other MMPs. Overexpression of TIMP-1 and

TIMP-2 in a rat bladder carcinoma cell line, LMC 19, suppressed primary tumor growth, local invasion, and metastasis to the retroperitoneal lymph nodes. It also significantly inhibited extravascular growth of pulmonary tumor emboli (Kawamata et al., 1995). Treatment of HT1080 cells with 8-bromo-cAMP induces the expression of TIMP-1 and TIMP-2, correlating with the suppression of the invasive phenotype of the HT1080 cells (Tanaka et al., 1995).

Cell culture studies using cell lines that produce a variety of collagenase family enzymes as well as both TIMP-1 and TIMP-2 have shown that TIMP-2 preferentially interacts with MMP-2. Thus, like interstitial collagenase activity, net MMP-2 activity may depend on the balance between the levels of activated MMP-2 and TIMP-2. Furthermore, binding of TIMP-2 to the latent form of MMP-2 may block MMP-2 activation by serine proteases such as plasmin. Binding of TIMP-2 to the active enzyme also abolishes its activity. In view of the 1:1 molecular stoichiometry, active proteolysis will only take place if the number of local MMP-2 molecules is greater than the number of TIMP-2 molecules. TIMP-2 inhibition of MMP-2 may not only be involved in extracellular matrix degradation, but has been shown to modulate the adhesion and morphology of a melanoma tumor cell line. Inhibition of endogenous MMP-2 resulted in an increase in cellular adhesion, and it is proposed that MMP-2 may also function to proteolyze cell surface components that mediate the attachment of the tumor cells to the extracellular matrix (Ray and Stetler-Stevenson, 1995).

TIMP-3 is a 24-kDa protein that binds to components of the extracellular matrix (Leco et al., 1994; Pavloff et al., 1992). Apte et al. have shown TIMP-3 to be an N-glycosylated protein that inhibits activated stromelysin 1, collagenase 1, MMP-2, and MMP-9 (Apte et al., 1995). Stromal fibroblasts have been shown to strongly express TIMP-3, further supporting the notion that the tumor stroma acts as a source of factors that influence invasion and thus human carcinoma growth and progression (Byrne et al., 1995). Mutations in TIMP-3 gene have been found in patients with Sorsbys' fundus dystrophy, a retinal degeneration characterized by abnormal deposits in Bruch's membrane and choroidal neovascularization (Fariss et al., 1997).

C. Migration

Central to the process of tumor metastasis is cell motility. Local invasion, intravasation, and extravasation of tumor cells and angiogenesis all require active cellular motility. Under normal physiological conditions, such as wound healing and the inflammatory response, cell motility is tightly controlled. However, in tumor cell motility aberrant regulation occurs, causing the migration of tumor cells to regions that constitute abnormal spread (Jiang et al., 1994; MacDonald and Steeg, 1993). Cell locomotion is a complex process that requires the coordinated extension of pseudopodia and membrane ruffling, formation of attachments to the extracellular matrix at the leading edge of the cell, and release of cell attachments at the rear of the cell and forward motion of the cell body (Guirguis et al., 1987; Jiang, 1995; Stossel, 1993).

Polymerization of filamentous (F)-actin of the cell cytoskeletal network is an important step in forming the leading edge pseudopodia. These finger-like protrusions are more often referred to as invadopodia, as they are the first structures of the cell to invade through the basement membranes. The constant polymerization and depolymerization of the actin filaments allows for the

remodeling and extension of the invadapodia (Zigmond, 1996). Attachment of these structures to the extracellular matrix is mediated primarily through the integrin family of cell surface receptors (Hynes, 1992). Other cell adhesion molecules have been shown to be important in these interactions as well. The attachment of the cell at the leading edge, the assembly and contraction of the cytoskeleton, and the coordinated release of previous contacts at the trailing edge result in cell translocation. Tumor cells have been found to respond in a motile fashion to a variety of agents, including host-derived motility and growth factors, extracellular matrix components, and tumor-secreted factors (Kantor and Zetter, 1996; Levine et al., 1995).

Several forms of cell motility have been defined based on the nature of the motile response and the source of stimulation: chemotaxis, chemokinesis, and haptotaxis. Chemotaxis is the directional migration of a cell in response to a positive gradient of a soluble factor, such as fragments of extracellular matrix molecules or growth factors. These chemoattractants both stimulate and orient the migration of the cells, the latter being caused by a polarization of the tumor cell so that it homes to the source of chemoattractant. Tumor cell chemotaxis has been shown to play an important role in local invasion and site-specific metastasis (Cerra and Nathanson, 1989; Ozaki et al., 1971; Terranova et al., 1986). Migration of cells in the direction of the chemotactic gradient increases as the concentration of the chemoattractant increases; however, at high concentrations motility may be inhibited. This occurs as a result of the cell moving up a concentration gradient and when the chemoattractant concentration is increased, cell surface receptors may saturate and the resulting homogenous stimulation may no longer elicit a motile response. This biphasic response is characteristic of neutrophil migration to FMLP and NIH3T3 cells to PDGF (Terranova et al., 1986).

Chemokinesis is stimulation of randomly directed motility and results in the migration of cells in a random manner away from their original site. This occurs in the absence of a concentration gradient. Chemokinetic factors such as hepatocyte growth factor/scatter factor (HGF/SF) (Weidner et al., 1993), autocrine motility factor (AMF) (Liotta et al., 1986), and the similarly named but distinct tumor autocrine motility factor (AMF) (Siletti et al., 1991) stimulate cellular motility in cells with the appropriate receptors and cause the cells to spread in a radial manner from the tumor.

Haptotaxis is the directed motility of a cell in response to a positive concentration gradient of immobilized attractant (Brandley and Schnaar, 1989). *In vivo*, tumor cells are exposed to a number of immobilized and soluble attractants in the form of extracellular matrix and growth factors, both bound and free. Activity of tumor-associated proteases results in the release and activation of extracellular matrix sequestered cytokines and growth factors providing a chemotactic gradient and chemokinetic stimulus. Furthermore, degradation of the extracellular matrix by the proteases results in the creation of a positive haptotactic gradient as well as serving as a source of extracellular matrix fragments that further provide an additional positive chemotactic gradient. Therefore, tumor cell motility results from chemotactic, chemokinetic, and haptotactic stimuli emanating from the local environment and autocrine secretion.

The capacity of tumor cells to migrate in response extracellular matrix proteins has been found to correlate positively with the *in vivo* invasive and metastatic capacity of tumor cell lines (Chelberg et al., 1990; Lester and McCarthy, 1992; McCarthy et al., 1986; Yabkowitz et al., 1993). Extracellular matrix proteins both intact and degraded that have been shown to act as motility factors for tumor cells include vitronectin (Basara

et al., 1985), fibronectin, laminin (Aresu et al., 1991), type I collagen (Faassen et al., 1992), type IV collagen (Aznavoorian et al., 1990), and thrombospondin (Yabkowitz et al., 1993), to name a few. Distinct domains in type IV collagen, fibronectin, and thrombospondin have been shown to stimulate the motility of tumor cells, whereas other domains are responsible for promoting adhesion.

Several of these extracellular matrix molecules mediate tumor cell motility via activation of the integrin heterodimeric cell surface receptors. Studies have shown that treatment of cells with anti-β1 integrin monoclonal antibodies inhibited migration and in vitro invasion capacity of human and murine tumor cells (Fujita et al., 1992; Yamada et al., 1990). Upregulation of α2β1, α3β1, and α6β1 was shown to correlate with metastatic potential and cell migration in human melanomas (Danen et al., 1993; Danen et al., 1993). Stable transfection of MCF-7 human breast cancer cells with mouse α7 integrin subunit yielded efficient adhesion and migration in response to laminin-1 and mixed laminin-2 and 4 substrates (Yao et al., 1996). The α4β1 integrin has been shown to be important in the adherence, cell spreading, and migration of CHO cells on fibronectin and VCAM-1-coated plates (Wu et al., 1995). When pancreatic cells that did not normally express the $\alpha_v\beta3$ integrin were transfected with β3 integrin cDNA, transfectants were shown to acquire a migratory capacity on vitronectin when compared with the non-transfected parental cells (Leavesley et al., 1992). Recently, Stefansson et al. has shown that cell migration can be inhibited by PAI-1 by blocking the $\alpha_v\beta3$ integrin interaction with vitronectin (Stefansson and Lawrence, 1996). In addition to its interaction with vitronectin, $\alpha_v\beta3$ has also been shown to bind MMP-2 (Brooks et al., 1996). The localized activity of this protease may be important in the degradation of matrix, and thus the creation of chemotactic gradients, increasing cell motility.

Another protein that associates with $\alpha_v\beta3$ has been identified recently as a receptor for the cell binding domain (CBD) of thrombospondin-1, a region important in its role in cell motility. The 50-kDa integrin-associated protein (IAP/CD47) has five putative hydrophobic helical membrane-spanning domains (Brooks et al., 1996; Lindberg et al., 1993; Lindberg et al., 1994), and an N-terminal immunoglobulin variable type extracellular domain that is important in the binding of thrombospondin (Lindberg et al., 1996). Functional blocking antibodies to IAP prevented migration to the CBD peptide of thrombospondin-1 or the intact thrombospondin-1 molecule establishing IAP as a receptor for the carboxy-terminal domain of thrombospondin (Gao et al., 1996; Gao et al., 1996). This establishes that the chemotaxis may be a direct biological consequence of CBD peptide binding to IAP. Furthermore, IAP-$\alpha_v\beta3$ complexes can regulate the function of other integrins through signal crosstalk (Blystone et al., 1994).

Other cell surface receptors, such as CD44, are involved in the process of cell motility. Recently, it was shown that the migration of highly aggressive melanoma cells on hyaluronate was fivefold greater when compared with low metastatic cells on hyaluronate, and the expression of CD44 isotype responsible for binding to hyaluronic acid (CD44s) was six times greater in the highly metastatic cells. Blocking antibodies to the CD44s isotype blocked haptotactic migration of the cells on hyaluronate showing the functional significance of the molecule; antibodies to the other CD44 isotypes had no effect. Additionally, synthesis of CD44 was increased three- to fivefold, indicating a reduction in CD44 half-life and up-regulation of turnover (Goebeler et al., 1996). Furthermore, the phosphorylation status of the CD44 receptor has been cited as important in the motility of tumor cells on this substrate. Peck et al. observed that cells expressing phosphorylation mu-

tants of CD44s did not migrate as well on hyaluronate-coated substrates as those cells expressing the wild-type CD44s (Peck and Isacke, 1996). Another receptor of hyaluronan that is also important in tumor cell motility is the receptor for hyaluronan-mediated motility (RHAMM). RHAMM is required for cell locomotion of a number of cell types, including *ras*-transformed fibrosarcoma cells and breast carcinoma cells (Hall and Turley, 1995). Hyaluronan and RHAMM interactions promote cell locomotion via a protein tyrosine kinase signal transduction pathway that targets focal adhesions. The tyrosine kinase pp60 (c-*src*) is associated with RHAMM and is required for RHAMM-mediated cell motility (Hall et al., 1996). It is possible that a RHAMM/*src* pathway induces focal adhesions to signal the cytoskeletal changes required for elevated cell motility seen in tumor progression, invasion, and metastasis (Entwistle et al., 1996).

In addition to the signaling initiated by ligand interaction, a number of growth factors, cytokines, and motility factors, through either paracrine or autocrine pathways, can activate the cellular migration machinery. These factors may cross-talk with adhesion components to elicit regulation. Autocrine stimulation is defined as secretion and response by cells that produce the factor. Paracrine stimulation refers to the response of a cell to a factor produced by an adjacent cell. Initially, many factors were identified as uniquely paracrine or autocrine; however, it is becoming apparent that many of these factors may serve in both ways.

Liotta and colleagues purified a 60-kDa tumor cell autocrine motility factor (AMF) from the conditioned media of the A2058 human melanoma cell line (Liotta et al., 1986). Stimulation of tumor cell motility by AMF is both chemokinetic and chemotactic in nature. It was also observed to lead to the production of pseudopodia, which were enriched in receptors toward laminin and fibronectin (Guirguis et al., 1987). It has also been shown that the presence of high levels of AMF in the urine of patients with bladder cancer correlated with increased tumor progression (Guirguis et al., 1988). Although AMF is defined as autocrine in nature, it has been proposed that nonproducing tumor cells in close proximity to those producing the factor might also be activated.

Recently, Stracke and colleagues have isolated, purified, and cloned a potent autocrine motility factor from the conditioned media of a human melanoma cell line. Autotaxin (ATX) has a molecular size of 125 kDa and is a basic glycoprotein (Murata et al., 1994; Stracke et al., 1992). It is active in the high picomolar to low nanomolar concentration range, stimulating both chemotactic and chemokinetic responses (Liotta et al., 1994; Stracke et al., 1991). ATX has marked homology to the PC-1 protein of activated B cells and plasma cells. In their extracellular domains, both ATX and PC-1 have pyrophosphatase, type I phosphodiesterase, and kinase activity and are expressed on their respective external cell surfaces as ectokinases (Stracke et al., 1991). Both can be cleaved; ATX is cleaved near its putative transmembrane domain (Murata et al., 1994). ATX may stimulate motility directly by binding a cell surface receptor or indirectly through one of its enzymatic products. The ability of ATX to stimulate tumor cell motility has been linked to the phosphodiesterase catalytic site. Mutation of a key threonine phosphorylation site of ATX both blocks activity and also abrogates its motility-stimulating activity (Clair et al., 1997).

Nabi and colleagues isolated a protein named gp78 that they described as a receptor for AMF (Nabi et al., 1990). Although it is not known if AMF or ATX can bind to gp78, Silletti and colleagues have isolated an autocrine motility factor that may (Siletti et al., 1991; Watanabe et al., 1991). This gp78 ligand is a 55-kDa tumor cell-secreted cytokine that, via its binding to its receptor, a 78-kDa cell surface glycoprotein, stimulates

tumor cell motility. Stimulation of metastatic murine melanoma B16a cells with this autocrine motility factor caused an increase in their spreading and adhesion on fibronectin. Additionally, the factor was also shown to stimulate integrin-mediated B16a cell adhesion, spreading, and invasion (Timar et al., 1996). Recently, this autocrine motility factor has been identified as neuroleukin/phosphohexose isomerase that has been implicated previously as having a role in cell motility and as a cancer progression marker (Watanabe et al., 1996). The expression of gp78 in human gastric cancer has been shown to correlate with disease progression (Hirono et al., 1996). In esophageal squamous cell carcinoma, patients who were shown to express high levels of gp78 had a decreased survival when compared with those who did not express the receptor (Maruyama et al., 1995). Expression of the AMF receptor in colorectal cancer has been shown to be a predictor of disease recurrence (Nakamori et al., 1994).

Hepatocyte growth factor, also known as scatter factor (HGF/SF), is both an autocrine and a paracrine motility factor that stimulates the motility of epithelial cells and endothelial cells (Stoker, 1989; Stoker and Gherardi, 1989; Stoker et al., 1987). HGF/SF is synthesized as a pre-propeptide of 728 amino acid residues with a 29 amino acid signal sequence. Active HGF/SF is formed by extracellular proteolytic cleavage that is mediated by HGF activator (Miyazawa et al., 1993). The protease uPA has also been reported as a candidate for HGF activation (Naldini et al., 1992). HGF/SF is known to be bound to the extracellular matrix and has been observed in the extracellular matrix of breast cancers, where it may act to promote tumor cell invasion and angiogenesis. Binding of HGF/SF to thrombospondin-1, fibronectin, and heparan sulfate proteoglycan has been observed (Lamszus et al., 1996). Active HGF/SF induces the scatter or chemokinetic locomotion of epithelial colonies, resulting in an invasive phenotype *in vivo* (Hartmann et al., 1992). The profound effects of HGF/SF on cell growth, morphology, and motility in a number of normal and tumor cell types has been well documented (Bhargava et al., 1993; Moriyama et al., 1996; Nusrat et al., 1994). In addition, high levels of HGF/SF in samples of breast tumors has been shown to correlate with shorter relapse-free and overall survival time (Yamashita et al., 1994).

HGF/SF is the preferred ligand for the c-*met* protooncogene product, a 190-kDa transmembrane tyrosine kinase-containing receptor that is widely expressed in normal epithelial tissues (Naldini et al., 1992). Overexpression of this receptor has been observed in a number of malignant tissues when compared with their normal counterparts (Jiang et al., 1993). HGF/SF and its receptor are also overexpressed in breast carcinoma when compared with benign breast tissue (Jin et al., 1996). The transfection of a mutated met oncogene into the human osteosarcoma cell line, HOS, induced an invasive phenotype *in vitro* and tumorigenic and metastatic ability *in vivo*. In another experimental setting, the transfection of murine met into NIH 3T3 cells already producing endogenous HGF/SF caused the cells to become highly tumorigenic and metastastic by completing the autocrine loop (Jin et al., 1996; Rong et al., 1992; Rong et al., 1994). These results indicate that HGF/SF and its receptor c-met can play an important role during tumor progression by stimulating the growth and motility of cancer cells.

The insulin-like growth factors (IGFs) have been shown to stimulate chemotactic responses in tumor cells. IGF-I has been shown to stimulate motility in a melanoma cell line and activates the cells through a type I IGF receptor (Stracke et al., 1988). Both IGF-I and II stimulate migration of melanoma cells in Boyden chamber assays (Kohn et al., 1990). In similar experiments, IGF-II has been shown to stimulate the motility of human rhabdomyosarcoma cells through a type II IGF/mannose-6-phosphate

receptor (Minniti et al., 1992). The mitogenic effect of IGF-II in these cells was shown to be through the type I IGF receptor, showing that a growth factor can have selective effects.

Epidermal growth factor (EGF) is known to be a potent mitogen for many tumor cells as well as normal cells. In addition to its ability to stimulate proliferation, it has also been shown to induce a motile response in a variety of tumor cells, including primary gliomas (Engebraaten et al., 1993), squamous carcinoma cells (Shibata et al., 1996), and renal carcinoma cells (Price et al., 1996). Mueller et al. demonstrated that antibodies toward the EGF receptor suppressed melanoma metastasis in *scid* mice (Naramura et al., 1993). EGF receptor has been shown to mediate the *in vivo* invasiveness of DU-145 human prostate carcinoma cells (Turner et al., 1996). Extension of lamellipodia in metastatic mammary adenocarcinoma cells was induced after EGF exposure by an actin-dependent mechanism (Segall et al., 1996). Activation of the EGF receptor in FG human pancreatic carcinoma cells led to their *de novo* motility on vitronectin. Without EGF, the cells only adhered to vitronectin and would not migrate. The EGF-stimulated motility in these cells could be blocked by the specific tyrosine kinase inhibitor, tyrphostin 25. Protein kinase C has also been shown to be involved in the pathway. Blockade of PKC with calphostin C also prevented the migration of FG cells (Klemke et al., 1994). Chen et al. have shown that phospholipase C-γ is required for EGF-induced motility in NR6 fibroblasts, and that this is mediated via hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP_2) and its subsequent release and mobilization of the actin modifying protein gelosin (Chen et al., 1996).

TGF-β_1 has been shown to be a powerful chemoattractant that promotes both chemotaxis and chemokinesis in a number of cell types (Wright et al., 1993). This growth factor can cause both pseudopodia and membrane ruffling in stimulated cells. TGF-β_1 has also been shown to regulate fibronectin production, and it has been shown by blocking studies that fibronectin is required for TGF-β_1 promoted locomotion (Stoker and Gherardi, 1991). Additionally, TGF-β_1 has been demonstrated to promote hyaluronan production that has also been linked to cell locomotion (Ellis and Schor, 1996; Turley et al., 1991). The ability of TGF-β_1 to increase protease production in transformed cells may also influence motility of tumor cells through protease release and activation of other growth factors, and extracellular matrix molecules that may further act as attractants or regulate cell surface receptors (Agarwal et al., 1994; Samuel et al., 1992; Welch et al., 1990).

Other growth factors such as platelet-derived growth factor (PDGF) (Pedersen et al., 1994), FGF-2 (Pienta et al., 1991), and TNF-α (Rosen et al., 1991) have also been shown to stimulate motility in a number of tumor cells and endothelial cells. Both tumor- and host-derived cytokines and growth factors can play an important role in the migration of endothelial cells, an important process for the formation of new blood vessels. Vascular endothelial growth factor (VEGF), FGF-2, and platelet-derived endothelial cell growth factor (PD-ECGF) all induce endothelial cell migration. VEGF is both chemokinetic and chemotactic in its action, whereas FGF-2 is only chemokinetic, and PD-ECGF was shown to be chemotactic (Sato and Rifkin, 1988; Simorre-Pinatel et al., 1994; Yoshida et al., 1996).

Regulation of gene expression of growth factors and cytokines by tumor cells and host cells undoubtedly plays an important role in tumor cell motility and invasion. However, regulation of these factors can also occur outside the cell by extracellular cellular matrix proteins and proteolytic enzymes. It is becoming apparent that many growth factors and cytokines bind to the com-

ponents of the extracellular matrix and can be released and activated by proteases (Taipale and Keski-Oja, 1997). Thus, tumor cells can further regulate their motility by protease production and localization of this activity at the invasive front allow for the release of chemoattractants in a directional location.

Other factors that are not classed as cytokines or growth factors have also been observed to stimulate tumor cell motility. The migration-stimulating factor (MSF), a 190-kDa secreted protein, is produced by human fetal fibroblasts. It has been shown to induce motility of adult fibroblasts in three-dimensional collagen gels (Schor et al., 1988). MSF has been detected in the serum of breast cancer patients before and after the resection of their tumors; however, no MSF could be detected in control sera. As circulating MSF persists after tumor resection, it has been hypothesized that a systemic effect of MSF may contribute to tumor formation and/or metastatic dissemination (Schor and Schor, 1990). It has been shown that this protein acts by stimulating hyaluronic acid secretion (Schor et al., 1989). Partial sequence analysis revealed a homology with the gelatin-binding domain of fibronectin (Schor et al., 1993); however, the relationship between MSF and motility requires further clarification.

The Mts-1 gene was initially identified as a result of its overexpression in a comparison made between highly metastatic murine breast carcinoma cells and their low metastatic parental cells (Ebralidze et al., 1989). Mts-1 is a member of the S100 family of Ca^{2+} binding proteins. Overexpression of a mts-1 antisense construct in mouse metastatic mammary carcinoma led to a reduction in metastatic potential (Grigorian et al., 1993). Overexpression of the mts-1 gene has been correlated with an increase in cell motility (Takenaga et al., 1994). Ford et al. demonstrated that mts-1 was responsible for motility but had no effect on the invasion of mouse mammary adenocarcinoma cell line in *in vitro* invasion motility assays and *in vivo* metastasis assays (Ford et al., 1995). The expression level of this gene correlated with cell motility in various murine normal and transformed cells (Takenaga et al., 1994). Mts-1 interacts with nonmuscle myosin II and therefore may mediate its effect on motility at the cytoskeletal level (Ford and Zain, 1995).

In addition to the cytokines and growth factors that stimulate tumor cell motility, several genes and their products have been shown to act as suppressors of tumor cell motility. TIMPs, natural inhibitors of the members of the metalloproteinase family, may indirectly inhibit motility by inhibiting the degradation of the extracellular matrix. However, in several studies the TIMPs have also been shown to be directly involved in inhibiting migration. Ray et al. demonstrated that by altering the expression of TIMP-2 by genetic manipulation and altering the balance between the inhibitor and proteases, not only was extracellular matrix degradation reduced but also cell attachment increased and motility decreased in a human melanoma cell line (Ray and Stetler-Stevenson, 1994). TIMP-1 and TIMP-2 have also been shown to inhibit the migration of endothelial cells (Johnson et al., 1994; Murphy et al., 1993). TIMP-3 is a potent inhibitor of both FGF-2-induced chemokinesis and VEGF-stimulated chemotaxis of endothelial cells. Therefore, the TIMPs may not only inhibit tumor cell invasion by inhibition of proteases involved in invasion but may also modulate cell attachment to the extracellular matrix altering cell motility.

The motility related protein-1 (MRP-1/CD9) is a transmembrane protein that not only is expressed on hemopoietic cells but is now known to be present on most solid tumor cells. Transfection and expression of MRP-1/CD9 in the human lung adenocarcinoma cell line MAC10 and the mouse melanoma cell line BL6, revealed that its expression suppressed tumor cell motility, growth,

and the metastatic potential of these cells, indicating that the protein may be a receptor for inhibiting ligands (Ikeyama et al., 1993; Miyake et al., 1991). Investigations with mouse melanoma BL6 cells and the BALB/c-nu/nu mouse system has showed the metastatic potential of transformants expressing MRP-1/CD9 was lower than that of their parental cells (Ikeyama et al., 1993). Analysis of breast cancer tumor specimens has indicated that MRP-1/CD9 is diminished as the stage of the breast cancer increased (Miyake et al., 1995). Recently, it has been shown that its low expression is associated with poor prognosis in breast cancer patients and may identify node-negative patients who are high risk for early disease recurrence (Miyake et al., 1996). In non-small cell lung cancer, low expression may be associated with poor prognosis (Higashiyama et al., 1995).

The small G-protein family of genes has been shown to be important in cell spreading, migration, and invasion. A new gene, Tiam-1, was isolated for its ability to alter T lymphoma invasion. Transfection of truncated Tiam-1 into noninvasive T lymphoma cells resulted in induction of invasive behavior. The predicted sequence encodes Dbl and pleckstrin homologue domains, known to be important in intracellular signaling and shared with other GDP-GTP exchangers utilized by other small G-proteins (Collard et al., 1996). Tiam-1 was shown to be a GDP-dissociation stimulator for Rho-like GTP-ases *in vitro*. Similar results have been observed in fibroblasts and may also implicate the Rac pathway.

IV. ANGIOGENESIS

Angiogenesis, the development of new blood vessels, is an important part of physiological processes such as wound healing, placentation, embryogenesis and development, and non-physiological disorders, including cancer (Folkman, 1971; Folkman and Shing, 1992). Early mouse experiments established that angiogenesis is absolutely required for tumor expansion by demonstrating that the transition from *in situ* carcinoma to invasive cancer was preceded by neovascularization. Tumor expansion was shown to be severely limited by nutrient requirements and by waste removal from the tumor into the surrounding medium, processes that depend on vascularization (Folkman et al., 1974). Experimental evidence suggested that in the absence of vascular support, tumors can remain dormant for long periods of time (Gimbrone et al., 1972) and eventually may become necrotic (Brem et al., 1978) or apoptotic (Holmgren et al., 1995; Parangi et al., 1996). The recent development of angiogenesis inhibitors confirmed the importance of neovascularization for tumor growth. Vascularization of tumors also has been found to promote metastatic spread. Liotta et al. demonstrated that the establishment of lung metastasis was related to the number of cells shed into the local circulation was first demonstrated in a transplantable mouse fibrosarcoma model (Liotta et al., 1974; Liotta et al., 1976). Subsequently, the clinical association of tumor vascularity with tumor aggressiveness was demonstrated. A vascular base in melanoma patients was correlated with development of metastatic disease (Srivastava et al., 1988). Increased incidence of metastatic disease was shown to be predicted by microvessel density for patients with breast cancer, prostate cancer, gastrointestinal tumors, and gliomas (Maria et al., 1993; Weidner et al., 1993). Increased tumor vessel density in advanced stage ovarian cancer was a predictor of decreased overall survival (Hollingsworth et al., 1995).

The vascular endothelium is a highly sophisticated, differentiated organ that regulates vascular tone in both conduit and smaller resistance vessels and plays a role in

homeostasis, cellular proliferation, inflammation, and immunity (Gottlieb et al., 1991). The vasculature is lined by endothelial cells, cells that are among the most quiescent cells in the body, proliferating slowly if at all under normal adult conditions. Tumors may release or secrete substances that stimulate endothelial cells to form new capillaries, increasing their proliferative potential more than 10,000-fold (Folkman, 1992). Tumor neovascularization is not delimited in time and space as is normal angiogenesis (Dvorak, 1986). The persistent vascularization of malignancy leads to a wide range of structural and functional abnormalities in vessel formation such as numerous trifurcations, arterio-venous shunts, and poorly developed basement membranes that may be discontinuous resulting in an increased vascular permeability (Galves, 1983; Jain, 1990).

Angiogenesis is a multistep process that has been shown to involve the same steps of invasion as malignant invasion (Kohn and Liotta, 1995). First, stimulatory agents activate migration and proliferation of endothelial cells. Then, local basement membrane dissolution occurs by proteolytic enzymes secreted by endothelial cells, perhaps augmented by stromal and tumor secreted proteases (Matrisian and Bowden, 1990). Interstitial collagenase (MMP-1), MMP-2, urokinase and TIMP-1 and -2 have been shown to be secreted by endothelial cells *in vitro* and are necessary for vessel formation *in vivo* (Johnson et al., 1994). The switch of endothelial cells from quiescence to rapid growth is regulated by angiogenic and angiostatic signals, including, among others, cytokines, fibrin, ECM molecules, and integrins (Brooks et al., 1994; Dvorak, 1986; Folkman and Klagsbrun, 1987; Ingber and Folkman, 1989). Neovessel formation is therefore the response to a shift in the net balance between angiogenic stimuli and inhibitors.

In vivo and *in vitro* model systems have been developed for the study of angiogenesis. Primary culture of large and small vessel endothelial cells provides a mechanism for study of angiostimulatory and inhibitory agents. *In vivo* models have been developed in order to identify factors that may be effective against tumors. The cornea is an avascular tissue but is adjacent to the highly vascularized limbus of the eye. Factors capable of stimulating angiogenesis may act as chemoattractants and angiogenesis inducers for capillaries to grow from the limbus toward the factor on the cornea. In the chick chorioallantoic membrane, assay angioregulatory factors are placed on the chorioallantoic membrane and progression of neovascularization can then be augmented or inhibited by exogenously added factors (Ausprunk et al., 1978).

Growth factors with marked effects on endothelial cells include fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), tumor necrosis factor α (TNF-α), platelet-derived growth factor (PDGF), interleukin-8 (IL-8), and hepatocyte growth factor/scatter factor (HGF/SF), among others (Folkman, 1996). These factors may be secreted by the endothelial cells, host cells, inflammatory cells, or tumor cells in an autocrine or paracrine fashion. Some of these molecules act directly on endothelial cells, whereas others recruit and activate other cells such as local inflammatory cells that may produce or activate angiogenic factors.

Basic fibroblast growth factor (bFGF, FGF-2) was the first angiostimulatory agent discovered and with acidic FGF (aFGF, FGF-1) belongs to a large family of growth factors. These factors are 14- to 16-kDa secreted proteins that are characterized by high-affinity binding to heparin within the ECM (Folkman et al., 1988). The FGFs are released from the ECM for presentation to FGF receptors through proteolytic digestion of the matrix (Miao et al., 1996). FGF-1 and FGF-2 stimulate endothelial cell growth, pro-

tease secretion, and motility of endothelial cells *in vitro* (Sato and Rifkin, 1988). FGF-2 also stimulates expression of $\alpha_v\beta3$ integrins on developing blood vessels and is believed to play a role in migration and proliferation during angiogenesis. Tumor regression is detected after treatment with anti-$\alpha_v\beta3$ (Brooks et al., 1994). FGFs mediate their activity through cell surface receptors that are widely expressed tyrosine kinases capable of coupling with downstream signal transduction cascades to mediate endothelial cell and tumor cell growth and invasion (Christofori, 1996).

FGF-2 has been shown to synergize with other growth factor such as TGF-β (Gajdusek et al., 1993) and VEGF to induce angiogenesis (Pepper et al., 1992). VEGF is a secreted 45-kDa protein that was originally believed to be a specific mitogen for vascular endothelial cells but more recently has been shown to stimulate a variety of responses in tumor cells (Ferrara et al., 1992). Molecular cloning has revealed that the VEGF family is comprised of four isoforms generated by alternatively splicing (Ferrara and Henzel, 1989). It appears to be a crucial mediator of tumor neovascularization *in vivo* and *in vitro*, as inhibition of VEGF-induced angiogenesis has been demonstrated to block tumor growth (Kim et al., 1993). Intraperitoneal injection of anti-VEGF monoclonal antibodies in nude mice injected with glioblastoma multiforme, human rhabdomyosarcoma, or leiomyosarcoma cells inhibited the tumor growth and exhibited a reduction in vascular density. The effect of the antibodies was specific to tumor vasculature as no decrease of tumor growth was observed. VEGF has been shown to induce plasminogen activator, plasminogen activator inhibitor, and interstitial collagenase, which themselves increase vascular permeability (Mandrioto et al., 1995; Unemori et al., 1992). Recently, two related members of the VEGF family have been identified, VEGF-B and VEGF-C, that also effect angiogenesis and endothelial cell growth (Joukov et al., 1996; Olofsson et al., 1996). Two high-affinity receptors to the VEGFs have been identified and characterized, kdr/flk-1 and flt-1. These receptors are transmembrane proteins that contain intracellular tyrosine kinase domains and are highly homologous to platelet-derived growth factor receptor (DeVries et al., 1992; Terman et al., 1992). Signaling through these receptors initiates with autophosphorylation and tyrosine phophorylation of numerous cytoplasmic proteins (Guo et al., 1995). Inhibition of flk-1 kinase activity has proven to be a potential therapeutic target for tumor growth inhibition (Strawn et al., 1996).

The angiogenic switch proposed by Folkman suggests that the activation of neovascular growth is controlled by the balance of inductive and inhibitory signals of angiogenesis (Hanahan and Folkman, 1996). High levels of inhibitor or low levels of inducer keep the angiogenic switch in check or in the off state. For example, an angiogenic inhibitor thrombospondin, TSP-1, is expressed at high levels in normal rodent and human cells and at lower levels in tumor cells (Good et al., 1990). Alternatively, blocking the activity of angiostimulatory agents such as VEGF with antibodies blocks neovascularization. These and other experiments have led to the suggestion that angiogenesis is switched on by decreasing the production of inhibitors (Folkman, 1996). To date, 11 endogenously expressed inhibitors of angiogenesis have been identified (Folkman, 1995; O'Reilly et al., 1997), including thrombospondin, platelet factor-4, angiostatin, 16-kDa prolactin fragment, tissue inhibitor of metalloproteinase -1 and -2, TGF-β, interferons α, placental proliferin-related proteins, interleukin-12, and endostatin.

The five thrombospondin family members are heterotrimeric secreted, calcium-binding glycoproteins (Lawler et al., 1995; Oldberg et al., 1992). TSP-1 has been shown

to modulate a variety of endothelial and tumor cell functions, such as proliferation, adhesion, and migration. Furthermore, TSP-1 and fragments contained within TSP-1 have been identified as effective inhibitors of angiogenesis (Good et al., 1990; Tolsma et al., 1993). The inhibitory activity of TSP-1 has been shown to be regulated by wild-type p53 tumor suppressor protein in fibroblasts and mammary epithelial cells (Volpert et al., 1995). Loss of the tumor suppressor activity and subsequent reduction in TSP-1 expression can lead to the activation of the angiogenic switch. TSP has four unique cell-binding domains. A RGD sequence in the calcium-binding region mediates interactions with $\alpha_v\beta3$ integrin receptor (Varner et al., 1995). TSP-1 contains a binding and activation site for TGF-β (Schultz-Cherry et al., 1995). It has been proposed that the anti-angiogenic potency of TSP-1 relates to its ability to inhibit protease enzymatic activity and to cross-talk to other signaling pathways (Roberts, 1996).

It has been hypothesized that there are natural angio-inhibitors stored as cryptic components of larger molecules. A potent inhibitor of angiogenesis, angiostatin, a 38-kDa protein, was isolated and characterized (O'Reilly et al., 1994). Angiostatin is an internal fragment of plasminogen, from amino acid 79 to 440. Angiostatin can be generated by proteolytic cleavage of plasminogen *in vitro*. Angiostatin is composed of four unique kringle domains that are triple-loop, disulfide-linked structures. Angiostatin activity requires appropriate protein folding and arrangement of the four units (Cao et al., 1996). It was shown in *in vitro* experiments to specifically inhibit endothelial cell proliferation and to inhibit neovascularization *in vivo* (O'Reilly et al., 1994). In these experiments, treatment of tumor-bearing animals with angiostatin yielded tumor reduction due to loss of vascularization. Further, it has been shown that after treatment with angiostatin, there is increased vascular apoptosis. An anti-angiogenic fragment of collagen type XVIII, called endostatin, has been identified recently (O'Reilly et al., 1997).

Platelet factor-4 is an angiogenesis inhibitor when given at high concentrations (Koch et al., 1996; Maione et al., 1990). However, fragments from platelet factor-4 derived from proteolytic cleavage have been shown to be 30 to 50 times more efficient than the parent molecule at inhibiting proliferation (Gupta et al., 1995). A 16-kDa fragment from human prolactin has been shown to inhibit endothelial cell proliferation and block angiogenesis *in vivo* (Clapp et al., 1993). Experiments have suggested that this fragment functions by inhibiting activation of mitogen-activated protein kinase that is activated by the angiogenic stimulators VEGF and FGF-2 in the endothelial cells. Interleukin-12 (IL-12) was previously identified as a potent tumor inhibitor based on its ability to inhibit metastasis of murine tumors (Brunda et al., 1993; Nastala et al., 1994). It is a species-specific inhibitor of angiogenesis that has been shown to cause inhibition of angiogenesis in a mouse cornea model system but not in a chick chorioallantoic membrane assay system (Voest et al., 1995). The identification of the components of the angiogenic switch has the potential to aim clinical research to drive an alteration in the balance of angiogenic inducers and inhibitors and hence block angiogenesis tumor growth.

V. THERAPEUTIC TARGETS FOR CANCER PROGRESSION

A. Antiangiogenesis Therapies

TNP-470 (AGM-1470), a semisynthetic analogue of fumagillin, a natural product iso-

lated from *Aspergillus fumigatus*, has been found to have an inhibitory effect on endothelial cell proliferation and migration (Ingber et al., 1990; Kusaka et al., 1994). TNP-470 inhibited the growth of human umbilical vein endothelial cells in a biphasic manner, both cytostatic and cytotoxic, with an arrest in the Go/G1 phase of the cell cycle. Yamaoka and colleagues found potent inhibition of prostate cancer and breast cancer growth by TNP-470 both *in vitro* and in animal models, suggesting that inhibition of tumor cell growth *in vivo* may not only be due to anti-angiogenic activity but by direct action on the cancer cell (Yamaoka et al., 1993). Administration of TNP-470 has also been effective in ovarian cancer, endometrial cancer, and choriocarcinoma xenografts (Taki et al., 1994; Yanase et al., 1993; Yazaki et al., 1995). Phase I and II clinical trials are ongoing with this agent in patients with solid tumors and in HIV-related Kaposi's sarcoma.

Thalidomide has been shown to have anti-angiogenic activity, and it is now in phase I and II clinical trials (D'Amato et al., 1994). Thalidomide, initially developed in the 1950s as a sedative and antiemetic for pregnant women, was found to be a potent teratogen causing limb dysgenesis. An association between maternal thalidomide usage and limb defects was first described by McBride and Lenz (Lenz, 1962; McBride, 1961). The teratogenic effects of the drug were experimentally reproduced many years later in rabbits; however, the mechanism of teratogenicity was still unresolved (Stephens, 1988). Recently, D'Amato et al. demonstrated that orally administered thalidomide inhibited FGF-2-induced angiogenesis in the rabbit cornea model, leading to ultrastructural changes similar to those seen in the deformed limb bud vasculature of thalidomide-exposed embryos (D'Amato et al., 1994). Hepatic metabolism is required to yield an active metabolite. Thalidomide has been shown to cause regression in AIDS-related Karposis sarcoma (Soler et al., 1996). Phase II clinical trials of thalidomide have been initiated in breast cancer, glioblastoma multiforme, and prostate cancer.

Interleukin-12 (IL-12) is a heterodimeric cytokine that plays a pivotal role in directing cell-mediated immunity and previously has been shown to have potent antitumor activity (Brunda et al., 1993; Nastala et al., 1994). In preclinical mouse models, intraperitoneal administration of IL-12 has been shown to inhibit experimental pulmonary metastasis and subcutaneous growth of B16F10 melanoma cells, resulting in an increased survival. Established experimental hepatic metastases, subcutaneous M5076 reticulum cell sarcoma, and Renca renal cell adenocarcinoma tumors were effectively growth inhibited with IL-12. Systemic IL-12 treatment of mice bearing subcutaneous tumors results in tumor growth inhibition, prolonged survival, and in some models tumor regression (Brunda et al., 1996). In addition, administration of IL-12 with pulse IL-2 was shown to eradicate murine renal carcinoma (Wigginton et al., 1996). Recently, Voest and colleagues have shown IL-12 to be a potent inhibitor of neovascularization, not by direct inhibition of endothelial cell proliferation, but mediated through induction of IFN-γ (Voest et al., 1995). IL-12 has been demonstrated to inhibit angiogenesis induced by human tumor cell lines *in vivo* in an IFN-γ-dependent fashion. As expected, IFN-γ alone also inhibited tumor cell-induced angiogenic activity (Majewski et al., 1996). IFN-γ is known to induce the production of inducible protein-10 (IP-10), which is known to have antiangiogenic activity. Thus, the activity of IL-12 may be due to generation of IP-10 (Luster et al., 1985; Sgadari et al., 1996). Previously, IP-10 has been shown to inhibit metalloproteinase production, which may also explain its inhibition of angiogenesis and of tumor cell inva-

sion (Shapiro et al., 1990). Gene therapy using IL-12 has been promising in the laboratory and is planned to begin for patients (Tahara et al., 1996).

B. Matrix Metalloproteinase Inhibitors

Regulation of the TIMP/MMP balance is critical to localized inhibition of matrix breakdown. A key action of TIMP-2 is to maintain the homeostasis of local proteolysis, its site of action becomes an important therapeutic target for the development of synthetic inhibitors of MMPs. BB94 (Batimastat) is a low-molecular-weight synthetic hydroxamate compound that has been shown to inhibit activity of most MMPs (Davies et al., 1993). Its effective concentrations range from 3 n*M* for interstitial collagenase (MMP-1) to 20 n*M* for stromelysin (MMP-3). BB-94 inactivates the proteolytic site of MMPs by binding to the catalytically active zinc atom in the holoenzyme, thus mimicking the physiologic function of TIMPs. Treatment of an ovarian carcinoma xenograft in nude mice with BB-94 caused resolution of ascites, reduction of net tumor burden, and a 5- to 6-fold increase in survival (Davies et al., 1993). BB-94 was also shown to reduce ascites and formation of peritoneal deposits in a human colorectal cancer mouse model (Watson et al., 1996). Recently, it was demonstrated to be effective in the control of lymphatic and hematogenous metastasis of a rat mammary carcinoma (Eccles et al., 1996). Antiinvasive and antiangiogenic activity has also been observed (Davies et al., 1993; Taraboletti et al., 1995; Wang et al., 1994). A phase I/II clinical trial of BB94 administered intraperitoneally to ovarian cancer patients found the drug to be well tolerated, with no significant acute toxicity (Brown, 1994). In 9 of 15 patients, ascites did not reaccumulate for at least 60 d after treatment. An oral analogue, marmistat and BB-94 are in phase I and II clinical trials.

C. Signal Transduction Therapy

The loss of balance in the cellular communication process may allow for dysregulation leading to tumorigenicity, invasion, and metastases (Kohn and Liotta, 1995; Liotta et al., 1991). Information is transferred from the extracellular environment into a cell or between cells through the process of transmembrane signal transduction. New therapeutic efforts in cancer prevention and treatment are being focused at the level of signaling pathways or selective modulatory proteins (Cole and Kohn, 1994; Gibbs, 1991; Powis, 1994). Investigations into the signaling pathways underlying metastasis have suggested that protein kinase activity, calcium homeostasis, and ras activation are important signals and therefore may be key regulatory sites for therapeutic intervention.

Several natural products have been found that inhibit protein tyrosine kinase activity and may possess antiproliferative or antiinvasive properties. These include genistein, herbimycin, and lavendustin A (Nussbaumer et al., 1994). Genistein is a naturally occurring isoflavinoid that has been demonstrated to inhibit endothelial cell proliferation and *in vitro* angiogenesis. Genistein is known to be an inhibitor of tyrosine phosphorylation and has been shown to inhibit ATP-induced calcium influx in PC12 pheochromocytoma cells (Kozawa et al., 1995). Calcium influx has been shown to be important in the process of angiogenesis, and therefore genistein may mediate its anti-angiogenic effects via this action (Kohn et al., 1995). The tyrphostins, a group of synthetic compounds designed to block phosphorylation of tyrosine residues, have been shown to be potent

inhibitors of cell proliferation *in vitro* (Levitzki and Gilon, 1991). Substitutions in the structure of the tyrphostins confer specificity to different receptor tyrosine kinases. The tyrphostin PD15305 is a specific inhibitor of the EGF receptor tyrosine kinase that selectively blocks EGF-mediated cellular events, including mitogenesis, early gene expression, and oncogenic transformation in both fibroblasts and human epidermoid carcinoma cells (Fry et al., 1994).

Protein kinases C (PKCs) form a family of serine/threonine kinases that mediate phosphorylation events involved in the pathway of many growth factors, matrix components, and neurotransmitters. Phorbol esters, initially described as tumor promoters, were found to stimulate a subset of PKCs in place of diacylglycerol, an endogenous activator of PKC (Castagna et al., 1982; Hata et al., 1993). PKC activity has been linked to metastasis through studies of PKC blockers that inhibited cancer cell adhesion, growth factor receptor phosphorylation, and tumor growth (Dumont et al., 1992; Liu et al., 1992; Stanwell et al., 1994). These findings suggest that PKC may be a potent target for therapeutic intervention. Staurosporine, an antibiotic isolated from the *Streptomyces* species, has general protein kinase inhibitory activity but has been studied primarily for its inhibitory effects on PKC. It has been shown to inhibit invasion and metastases of bladder carcinoma models (Schwartz et al., 1990) and to inhibit adhesion with possible modulation of MMP-9 production in human promyelocytic cell line, HL-60 (Reis et al., 1994). UCN-01, an analogue of staurosporine, is now in clinical trial. Salfingol, an optical isomer of dihydrosphingosine, is a specific inhibitor of PKC. In preclinical animal studies, Salfingol was nontoxic at doses that yielded serum levels sufficient to inhibit PKC enzyme activity (Kedderis et al., 1995). It was shown to inhibit gastric cancer cell invasion and to potentiate apoptosis in C-treated gastric cancer cells (Schwartz et al., 1995; Schwartz et al., 1993). Salfingol is a cytostatic agent *in vivo*; however, the combination of Salfingol with conventional chemotherapeutic agents such as doxorubicin and cisplatin resulted in potentiation of the anti-tumor effects. Salfingol has recently entered phase I clinical trials alone and in combination with doxorubicin. This agent has been well tolerated, and potentially effective serum concentrations of Salfingol have been achieved. Two minor responses, one each in pancreatic carcinoma and sarcoma, and resolution of ascites in a patient with pancreatic cancer have been observed.

The bryostatins, macrocyclic lactones derived from the marine bryozian *Burgula neritina* (sea mosses), interfere with PKC activity by replacing diacylglycerol in the normal activation process of PKC, causing a net down-regulation of PKC. The bryostatins have been shown to inhibit phorbol ester-induced tumor promotion both *in vitro* and *in vivo* in models of ovarian sarcoma, melanoma, and lymphocytic leukemia (Schuchter et al., 1993). In a phase I clinical trial, bryostatin was administered as a weekly 24-h intravenous infusion for 8 weeks and was performed in patients with advanced malignancy. Myalgia was found to be the dose-limiting toxicity and was cumulative and dose related. Response was observed in four patients, including two partial responses of 4-month duration and two minor responses. The two partial responses were observed in patients with ovarian carcinoma and low-grade non-Hodgkins lymphoma (Jayson et al., 1995).

Intracellular calcium homeostasis has also been found to be a common regulator of the major categories of transmembrane signal transduction and the process of invasion, metastasis, and angiogenesis (Chapron et al., 1989; Cole and Kohn, 1994; Kohn et al., 1995; Savarese et al., 1992; Tanaguchi et al., 1993). The integral roles that intracellular calcium homeostasis plays makes it an

interesting target for chemotherapeutic intervention. In a screen for agents that would both inhibit migration *in vitro* and inhibit motility-requiring signaling pathways, a novel inhibitor of calcium mobilization, CAI (carboxyamido-triazole), was identified (Kohn and Liotta, 1990). CAI was shown to inhibit calcium influx through nonvoltage-gated calcium channels, and it has also been shown to inhibit other calcium influx-dependent downstream signaling pathways such as selected tyrosine kinase activities and release of arachidonic acid from phospholipase A2 (Felder et al., 1991; Gusovsky et al., 1993; Kohn et al., 1994). CAI activity appears to be specific for influx of calcium from the extracellular environment.

A structure-function relationship study significantly linked inhibition of calcium influx and calcium-mediated signaling to inhibition of tumor cell growth and metastasis. CAI has been shown to inhibit proliferation of a wide array of human cancer cell types and endothelial cells *in vitro* (Kohn et al., 1995; Kohn et al., 1994; Kohn et al., 1992). CAI treatment has been shown to down-regulate expression and activity of MMP-2 (Kohn et al., 1994). A marked inhibitory activity against neovascularization was observed with CAI *in vitro* and *in vivo*. Xenograft studies revealed reduction of both tumor growth and dissemination of human ovarian cancer and melanoma xenografts oral administration of CAI. The plasma concentrations attained in the animals were 1 to 10 μM, which was effective in the inhibition of calcium influx and calcium-mediated signaling pathways *in vitro*. No significant toxicity was demonstrated in the animal cohorts that received daily or every other day CAI. Phase I clinical trials of CAI have been completed and phase II studies are opening (Berlin et al., 1997; Kohn et al., 1997; Kohn et al., 1996). CAI was cytostatic with disease stabilization in almost half of the patients treated and minor or partial remission in two patients. Oral daily administration yielded plasma concentrations in the 1 to 10 μM concentration range. Predominant toxicity was formulation-dependent mild-moderate nausea and vomiting and sensory peripheral neuropathy. Phase II studies of CAI are under development.

VI. CONCLUSION

The cellular and molecular biology revolution that has occurred over the last 2 decades has allowed for an in-depth investigation into the multiple steps of tumor progression. This great increase in knowledge of the importance of adhesion, motility, proteolysis, and angiogenesis has not only served our intellectual curiosity but, more importantly, identified novel targets for the treatment and prevention of cancer initiation, progression, and metastasis. Many agents, identified as inhibitory toward various steps of the metastatic cascade in the laboratory, have translated with success to the clinical environment. The early initiation of angiogenesis and invasion suggest that these agents may be useful in the chemoprevention of cancer in high-risk patients and for the prevention of relapse. Their utility in combination with existing cytotoxic drugs may increase the efficacy of these agents and improve disease-free and overall survival.

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