NOTE TO USERS

CELL-CELL AND CELL-MATRIX INTERACTIONS INVOLVED IN CANCER INVASION

by

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DEDICATION

This dissertation is dedicated to those people whose lives have been affected by cancer. This includes both of my grandfathers who passed away from lung cancer before I was born and the rest of my family who had to cope with the loss of these two men that I did not have the opportunity to know. It is my hope that cancer research taking place today will result in the development of better diagnostic indicators and improved treatments to ease the pain of both cancer patients and their families.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF TABLES

ABSTRACT

In order for a cancer to metastasize, it must first invade through the basement membrane that surrounds it, invade blood vessels and travel through the bloodstream to a new location where it extravasates the vessel and begins growing at the new site. The mechanisms by which a cancer becomes able to invade and metastasize are currently under intense study. Interactions of the cell with its environment via cell-cell contacts, extracellular matrix (ECM) interactions, and circulating proteins are thought to play a major role in signaling for these invasive processes to occur. Upregulation of proteolytic enzymes, such as the matrix metalloproteases, is suspected of being involved in the metastatic process. Cell-cell and cell-matrix contacts via integrins and cadherins are necessary for upregulation of the matrix metalloprotease matrilysin in oral squamous cell carcinoma. In an effort to identify the factors involved in upregulation of matrilysin expression detected in a co-culture of oral squamous cell carcinoma (SCC) cells and fibroblast cells, a coculture model designed to represent the actual tumor environment, we show that inhibition of β1 integrin, E-cadherin, and N-cadherin with blocking antibodies thoroughly decreases the induction of matrilysin in the co-culture model. This demonstrates that interactions between cancer cells and normal cells surrounding them may allow for invasion and metastasis. The protein 90K may also play a role in the invasive process of prostate cancer. It functions as an immune modulator upregulating cytokines that induce MMPs and we show that it can induce matrilysin expression in prostate cancer cells. It also functions in cell aggregation, which can help cells survive during metastasis. For this reason, expression of 90K in prostate cancer, which we

examined, may be indicative of aggressive disease, making 90K a potentially useful tumor marker. Cell-matrix contacts are also important for the transmembrane matrix metalloprotease MT1-MMP cleavage of laminin-10. We demonstrate that recombinant MT1-MMP is able to cleave human laminin-10 into four distinct products. This allows for prostate cancer cell migration on laminin-10 coated substrates, which can be inhibited with the addition of MT1-MMP antisense oligonucleotides. Ln-10 cleavage also occurs *in vivo* in human prostate tissue, indicating that this cell-matrix interaction has *in vivo* relevance in human prostate cancer.

I. INTRODUCTION

Epidemiology and Natural History of Oral Squamous Cell Carcinoma

Oral squamous cell carcinoma (SCC) is the sixth most common malignancy in the world, and is a major cause of cancer morbidity worldwide. Globally, over 500,000 new cases of oral cancers are diagnosed each year, and three quarters of these new cases are from developing countries (1). In the U.S., 28,000 new cases of oral cancer are expected in 2004, and 7,230 deaths due to oral cavity cancers are expected (2). The five-year survival rate for oral cancers is 57% and this drops dramatically to 26% if local lymph nodes are involved (2). The most common cause of oral SCC is tobacco use, either smokeless tobacco or smoke tobacco (3). Tobacco contains over 2,500 different compounds and 300 of these compounds are considered carcinogenic, including nitrosamines, polycyclic aromatic hydrocarbons, α-particle emitting ^{210}Po (Polonium), trace metals, carbon monoxide, hydrogen cyanide, and phenols (4-6). In Western countries, where smoke tobacco use is more prevalent, most oral SCCs arise on the tongue or the floor of the mouth (7). Not all tobacco users are SCC patients, which suggests that genetic differences such as differences in drug metabolizing and detoxifying enzymes may predispose individuals towards oral cancer. In addition to tobacco and genetic susceptibility, other factors involved in oral SCC include alcohol, diet, and viruses such as Human Papilloma Virus and Epstein-Barr Virus (8).

As with most cancers, early diagnosis is a major factor with regards to outcome of the disease. Symptoms of the disease include oral bleeding, presence of an ulcer, loose teeth, ill-fitting dentures, throat irritation or pain, drooling, and respiratory difficulties.

Unfortunately, this disease remains fairly asymptomatic until it reaches an advanced stage and as a consequence, by the time a diagnosis is made, extensive disease is often present. Oral SCCs present early as leukoplakias and erythroplakias. Leukoplakia is characterized by a white colorization of the mucous membranes, a thickened surface keratin layer, and chronic inflammatory cells in the underlying connective layer. Only about 3% of leukoplakias become malignant, however, the longer the lesion is present the greater the chance that it may become malignant. Leukoplakia on the base of the mouth and on the ventral surface of the tongue has the greatest propensity for malignant transformation. Erythroplakia is a red velvety patch with an irregular outline that can be clearly differentiated from the surrounding normal tissue. Erythroplakias are usually areas of extreme epithelial dysplasia or carcinoma *in situ*, and as such, are seventeen times more likely to become malignant than leukoplakias. In many cases, oral SCCs arise *de novo* from clinically normal mucosa. These cancers tend to be even more aggressive with a poorer prognosis than those which arise from a tobacco-induced lesion (3). Screening techniques currently in use involve physical examination of an oral lesion, along with a biopsy of that lesion. Also important for screening are examination of the teeth and oral mucosa, and palpitation of the neck. Computed tomography (CT), Positron emission tomography (PET) and magnetic resonance imaging (MRI) are also useful tools for screening and diagnosis of oral tumors, but their expense makes their usefulness as screening tools prohibitive.

Oral SCC is characterized as exophytic (growing outward from an epithelial surface), ulcerative (extending into the underlying lamina), or both (9). Exophytic

lesions are less common, slower growing and less invasive than ulcerative lesions. Ulcerative lesions are more common and appear as red or grayish ulcers that bleed easily. Treatment of SCC involves surgery, radiotherapy, chemotherapy, or a combination of these. For early, localized disease, surgery alone is considered to provide the best outcome. Advanced-stage disease, on the other hand, requires combined therapy to lower the risk of metastatic disease (10). Based on the location of the cancer, side effects of treatments include adverse effects on chewing, swallowing, speaking, sight, smell, and hearing. As a result, treatment usually involves an interdisciplinary coordination of dental oncologists, reconstructive surgeons, speech pathologists, physical therapists, and maxillofacial prosthodontists. Even with extensive treatment, local recurrence and appearance of distant metastases are a major problem with oral SCC. Therefore, prognoses for patients with oral SCC is considered to be related to the status of the regional lymph nodes, with a common metastatic occurrence being delayed neck metastases (DNM), also called occult nodal metastases (11). Other sites of metastasis include the lungs and the liver. Therefore, identification of oral tumor biomarkers that can differentiate cancer from normal epithelium and biomarkers that can establish prognosis are of utmost importance (12, 13), as there are currently no reliable markers that can predict the likelihood of having an SCC metastasis at the time of diagnosis (14).

Epidemiology and Natural History of Prostate Cancer

Prostate adenocarcinoma is the second leading cause of cancer deaths in males and is currently the most commonly diagnosed neoplasm in men in the United States (15,

16). Five-year survival statistics for prostate cancer have improved since the 1970s, due in part to more accurate diagnostic indicators and more efficient therapies for low-grade disease (17). However, epidemiologists predict that by the year 2020 there will be a dramatic increase in both the incidence of and death rate from prostate cancer due to the increasing population of men considered to be at risk for developing prostate cancer. Thus, improvements in prevention, early diagnosis, and treatment are needed (18).

The major risk factors for prostate cancer include age, race, diet, and heredity. Prostate cancer risk increases more rapidly with advancing age than any other cancer. Men under the age of 50 are at relatively low risk for developing the disease. From the age of 55 onward, the risk for developing prostate cancer increases dramatically at about 1 percent per year of age, resulting in an overall incidence of 1,000 cases per 100,000 men aged 85 and older. It has been estimated that a man has about a 10% chance of developing clinically detectable prostate cancer during his lifetime (19). African-American men have an incidence rate twice that of white Americans and a mortality rate that is 120% higher than white Americans (20-22). This results in their risk of prostate cancer to be significantly higher than white Americans, while Asian populations have the lowest risk.

A Western diet, a diet that includes high intake of animal fat and low levels of vegetables, has been associated with an increased risk of developing prostate cancer. In countries such as Japan and China, where a non-Western diet, high in vegetable consumption and low in meat consumption, is employed, there is historically a low incidence of prostate cancer. However, incidence in these countries is on the rise,

correlating with a loss of the traditional Asian diet and the acceptance of a Western lifestyle and diet (23). Also, age-adjusted prostate cancer incidence and mortality rates have risen along with per capita consumption of fat in studies in the U.S. among both Caucasians and African-Americans (24). Other studies suggest that dietary fat may be a promotional factor rather than a causative factor in the development of prostate cancer (25).

Studies of prostate cancer amongst close relatives have suggested that there are high-penetrance susceptibility genes that can predispose men to develop prostate cancer. This susceptibility accounts for 5-10% of all prostate cancer cases in men and is normally associated with early onset disease (26, 27). Two familial susceptibility loci have already been mapped, one to the X chromosome, and the other to a region on chromosome 1q (28, 29), and the genes responsible for this susceptibility are currently under investigation.

Early diagnosis is a problem because many men with prostate cancer are asymptomatic although some common symptoms of prostate cancer include weight loss, bone pain, lethargy, and bladder outflow problems. Problems such as bladder outflow problems and other pelvic pain symptoms are usually characteristic of local extension of the tumor and can also be attributable to benign prostatic hyperplasia (BPH) (30). Symptoms of metastatic disease include bone pain, specifically common in the lower back and the neck of the femur due to bone metastases. The most common sites of prostate cancer metastasis are to the local lymph nodes (31) and bone tissues of the pelvis and lower spinal column (32). Due to the potential lack of symptoms in early, localized

prostate cancer, screening high-risk populations is extremely important for early detection. The American Cancer Society and the American Urological Association recommend annual prostate cancer screening using both the Digital Rectal Examination (DRE) and Prostate-Specific Antigen (PSA) serum tests for men starting at age 50 (33). The DRE is used to diagnose both BPH and macroscopic prostate cancer but it is ineffective in diagnosing small, early-stage prostate cancer. However, the DRE is useful for prostate cancer detection because most prostate cancers develop in the peripheral zone of the prostate. Although problems with the DRE include low sensitivity and subjective results, 25% of prostate cancers can still be detected by DRE in men with normal PSA levels (34). PSA is normally secreted into the lumen of prostatic ducts and is found in high concentrations in seminal fluid (35). PSA can also leak from the ductal system into the prostatic stroma and into the bloodstream where it can be measured (36). Problems with the PSA test are a fairly large number of false positives and false negatives (37) indicating that advances in screening for and detection of prostate cancer are necessary.

Currently, the only curative therapy for prostate cancer is surgical resection of prostate confined disease and there are no curative treatments for disseminated cancer. Hopefully, understanding molecular events of prostate cancer progression will lead to the development of novel therapies for the disease. A prostate cancer's ability to develop an invasive and metastatic phenotype is arguably the most important determinant in the clinical relevance of prostate cancer; and therefore, the study of factors involved in the development of this phenotype is of primary importance. There are at least two different phenotypes of prostate cancer. One is a clinically aggressive cancer that can become life-

threatening relatively fast, while the other is a slow-growing, "latent" form, which may never present itself clinically (38).

Prostate cancer is thought to arise through a precursor lesion, prostatic intraepithelial neoplasia (PIN) (39). However, early signs of PIN lesions have been found in men over the age 20 who have died of other causes, indicating that not all PIN lesions will progress to cancer. Early PIN lesions are not often associated with cancer, but high grade PIN lesions are frequently found in areas of the prostate with evidence of cancer (40). High grade PIN appear as distended glands with thickend atypical epithelium and areas of basal cell loss that exhibit loss of the β4 integrin and its ligand laminin-5 (41, 42).

The investigational methods currently in use do not allow clinicians to differentiate between aggressive and quiescent prostate tumors, leading to potential over- and under- treatment of this disease (43). For this reason, identification and understanding of molecular alterations during prostate cancer progression is essential to distinguish between cases that will progress rapidly to advanced metastatic cancer and those with little likelihood of progressing. This will hopefully lead to more accurate prognoses and appropriate treatments. In addition, identification of biomarkers will be useful for recognizing individuals with a higher risk for prostate cancer and will allow for earlier detection and better treatment of advanced disease. In particular, there is a growing need for biomarkers that access the tumor microenvironment as predictors of metastases (44).

The Metastatic Spread of Cancer

Metastasis is the growth of a secondary tumor foci at a site that is separate from the primary tumor lesion (45). The major cause of cancer morbidity is not due to a primary tumor. Rather, it is the metastatic lesion that is the major cause of death. It is much more complicated to effectively treat and remove a tumor that has spread to distant sites in the body compared to treatment and removal of a primary lesion. Because of this, patients with metastatic lesions have a much higher mortality than patients treated for a primary tumor (46, 47). The steps in the metastatic process include vascularization of the primary tumor, local invasion of cancer cells within the primary tumor, intravasation into the circulatory system (blood or lymphatic), transport and survival within the circulatory system, extravasation from the vessel, invasion of the tissue at the distant site, and establishment of new tumor growth (Figure 1) (48).

Cell motility is crucial for metastasis. Thus, alterations that can increase tumor cell motility are likely to result in increased metastatic capacity. The mechanisms by which tumor cells metastasize are mediated, in part, by matrix metalloprotease-mediated proteolysis of the basement membrane (BM). Detachment of cells from the primary tumor site involves alteration of several different types of cell adhesion molecules (49, 50). There are two ways in which cell adhesion functions in migration. CD44, the major receptor for hyaluronan, and other sugar receptors initiate temporary and flexible adhesion at sites of cellular extensions. Then, more stable adhesion is mediated by integrins, which produces an actin-based force that is able to pull the cell forward (51).

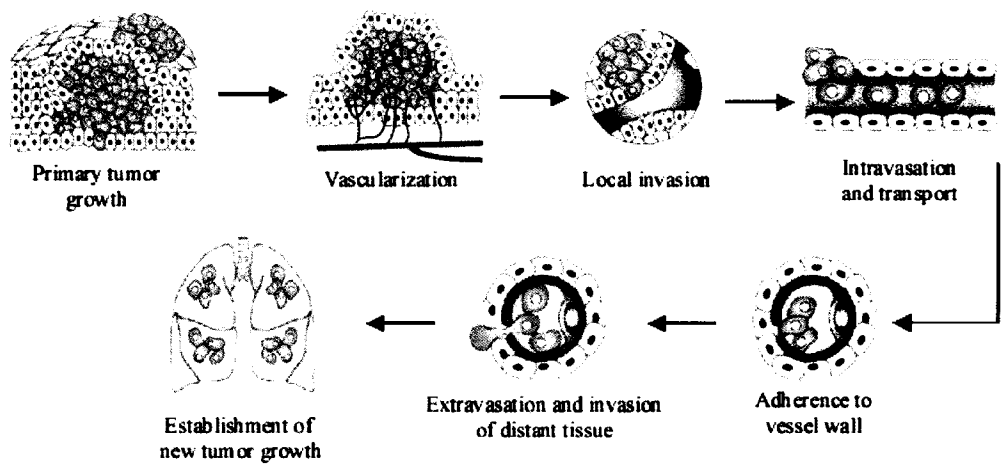

Figure 1. The process of metastasis.
Modified from (48).

The next step in metastasis is usually to gain access to the blood or lymphatic circulation. A tumor cell is able to intravasate a vessel through a regulated process, which begins with attachment to the vessel wall. Once attached to the vessel wall, proteases and adhesion molecules facilitate the overall process of intravasation (and extravasation) (52, 53). The tumor cells form extensions, called invadopodia, which contain high levels of integrins and metalloproteases such as MMP-2 and MT1-MMP with low levels of TIMP-2 (53). These invadopodia mediate focal degradation of the BM via proteolysis and extend through the endothelial cell junctions. This induces endothelial cell retraction and facilitates passage through the BM via cell migration. Finally, the cell escapes into the lumen of the vessel. Once the tumor cell has entered the bloodstream it desiminated, it finally extravasates into a distant secondary environment. Here, it must survive and proliferate in order to give rise to a metastatic lesion. Oral SCC tends to metastasize via the lymphatic system (54). Prostate cancer most often initially metastasizes via migration along the peripheral nerves, however they can also metastasize via the lymphatic and blood vessels (55, 56).

During invasion, tumor cells are capable of modulating their environment by secreting different ECM proteins and thus forming a BM different than that found in normal structures. In addition, tumors can secrete proteolytic enzymes that digest BM proteins, again modifying the BM surrounding the cancer (57, 58). Invading cells can then interact with the new BM to promote migration (59, 60). Invasive carcinoma is also associated with changes in cell adhesion receptors. In particular, loss of E-cadherin, gain of N-cadherin, and alterations in integrins are known to occur (59, 61, 62).

Extracellular Matrix

Cells are continuously sending and receiving information, either by cell-cell contacts, signals from the surrounding extracellular matrix (ECM), or from circulating soluble hormones and growth factors. The ECM is a complex network of proteins surrounding cells, serving as a structural element in tissues. The ECM provides cells with information about their environment and thus influences tissue development. The basal lamina (BL) is a component of basement membranes (BM), and are thin, specialized forms of ECM that surround muscle, fat, and peripheral nerve cells, and underlay all epithelial and endothelial cells. BLs are responsible for tissue compartmentalization, acting as a barrier for cells. The main components of BLs include laminins, collagen IVs, nidogens, and the proteoglycans agrin and perlecan. In addition, collagen XV, collagen VII, and collagen XVIII are also found in BLs (63, 64). The BL consists of two networks that can self-assemble independently of each other, but are then connected together by nidogen. One of the networks is formed by laminin, which assemble through N-terminal interactions of the three short arms (64) and is thought to give dynamic flexibility to the BL and give signals that lead to cell polarization. The second network consists of different type IV collagens and is thought to give mechanical stability to the BL (64-66). The formation and organization of the BL is depicted in Figure 2. The adhesion of cells to BLs influences tissue formation in many ways. In addition to stabilizing tissue, basement membranes regulate cell behavior through different signaling cascades, which can start differentiation, movements, proliferation,

growth, and cell survival. It is known that disturbances or mutations in BL proteins can lead to diseases such as muscular dystrophies, epidermolysis bullosa, or cancer. The BL components are well conserved throughout evolution, indicating the importance of correct BL proteins for the development of multicellular life.

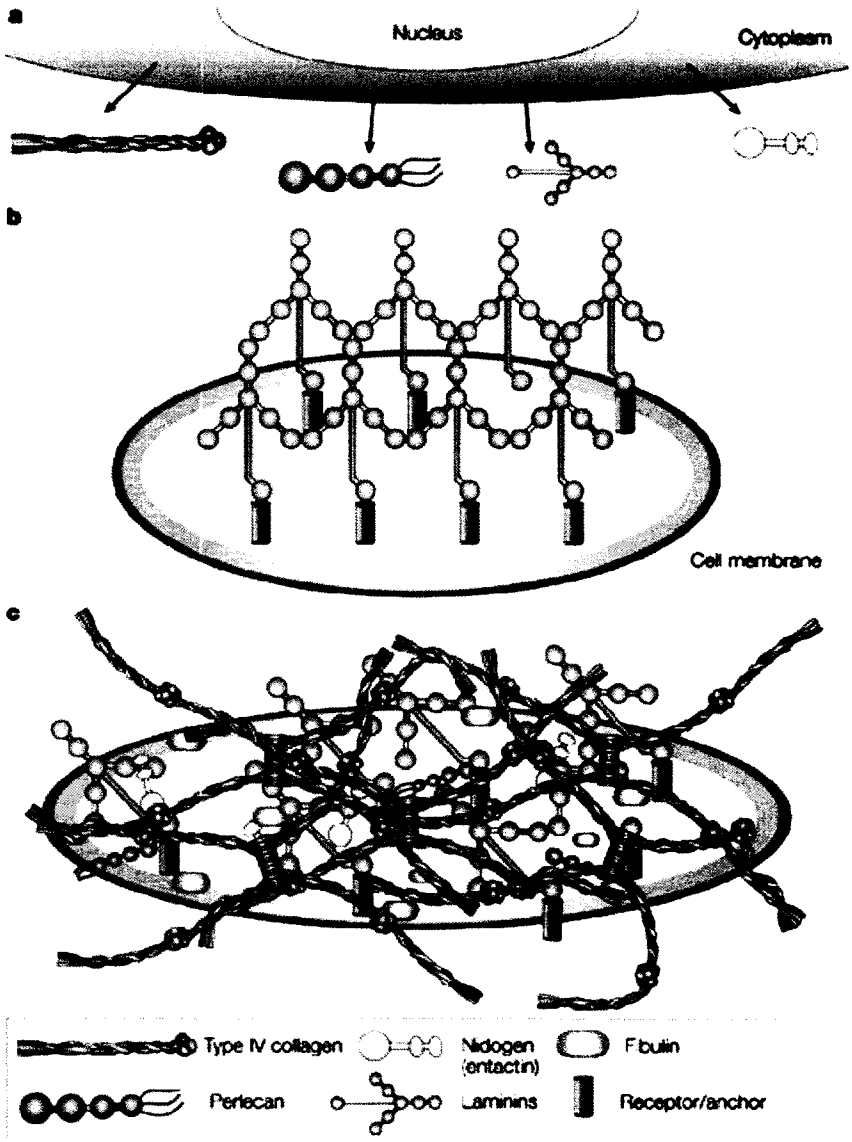

Figure 2. Illustration of basement membrane (BM) formation.

a) Secretion of BL components by the cell. b) Polymerization of laminin network. c) Association of laminin network through interactions with other BM components. Modified from (67).

Laminins

Laminins are large (400-600 kDa) cross-shaped heterotrimers that are components of the extracellular matrix. All laminin chains are separate gene products. A few of the individual polypeptide chains can be alternatively spliced (68) and some undergo extracellular proteolytic processing (68-70). Laminin trimers are assembled intracellularly, through interactions of domain I of the C-terminal part of the chains and then secreted (71). Interestingly, the three chains that make up a laminin are not secreted separately or as dimers, with the exception of the α chain, which suggests that although all chains have a signal sequence for secretion, the α chain is the chain that drives secretion of the trimer (72). Dimers of βγ are formed initially, but not secreted, followed by incorporation of the α chain, after which the trimer is secreted. All laminins have the same basic domain structure, as demonstrated for Ln-10 in Figure 3. The α chains have five homologous laminin globular (LG) domains, named LG1-5, at the C-terminal end. Cell surface receptors such as dystroglycan and integrins bind to the LG domains (73-75). Domains I and II make up the triple alpha-helical coiled-coil structure forming the rod-like part of the long arm. Domains IIIa, IIIb, and V in all three short arms of the laminin molecule consist of three to eight laminin EGF-like (LE) repeats. These regions are rod-like and separate the globular domains IVa, IVb, and VI (76).

The globular domain VI, also called the LN module, terminates the short arms and is the most conserved part of the laminins. Self-assembly of laminin isoforms into a network occurs when the three short arms interact via the N-terminus of their LN modules. This self-assembly mechanism indicates that only laminins with three full-

length chains can form a laminin network, including Ln-1, -2, -4, and -10/11 (66, 77).

Currently, there are fifteen different laminins described in tissues, with five α, three β, and three γ chains identified, indicating a theoretical possibility for up to forty-five different trimers, indicating that it is likely that new chain combinations have yet to be discovered. However, certain chains are never seen paired with each other, so the actual number of combinations is probably much smaller (78, 79). Each laminin is thought to transmit a unique set of signals to a cell, as knock-outs of each laminin chain in mice exhibit unique phenotypes (80).

Many laminins, including laminins-2, -4, -5, -6, -7, -10, and –11 undergo proteolytic cleavage within the LG domains or the short arms. Cleavage in the LG domains modulates binding of cell surface receptors. Cleavage in the short arm of Ln-5 affects cell migration, possibly through the release of a soluble EGF-like fragment of the short arm (81). The assembled heterotrimer undergoes extracellular proteolytic processing to produce the mature protein for some laminins, including Ln-5. The α3 subunit is processed from the 200 kDa precursor to a 165 kDa chain by plasmin (82) and the γ2 subunit is then processed from 140 kDa to 100 kDa by MMP-2 (82). This mature Ln-5 is then able to support adhesion and migration. Human Ln-5 β3 chain can also be proteolytically cleaved further by MT1-MMP. This cleavage allows for increased cellular migration and invasion on human Ln-5 (83). In addition, the rat γ2 chain of Ln-5 is cleaved by MT1-MMP and MMP-2, though this cleavage has not been directly observed in human Ln-5 (84, 85). Cleavage in the α chain has most often been described in the LG domains, and this cleavage usually allows for increased integrin binding (82)

and thus more stringent adhesion.

Laminin-10

In adults, the α5 chain is the major α chain of most BMs, where it forms laminin-10 (Ln-10) and laminin-11 (Ln-11) trimers (68, 86, 87). Ln-10 contains the α5, β1, and γ1 chains to make up its heterotrimer. The α5 chain is also shared with Ln-11 (α5β2γ1). The α5 chain is widely expressed in tissues including skin, placenta, heart, skeletal muscle, kidney, lung, pancreas, and prostate (86, 88, 89). Ln-10/11 are secreted by many human cancer cell lines including lung, choriocarcinomas, renal carcinomas, and colon carcinomas (90).

The importance of Ln-10 has been demonstrated by transgenic mice with the α5 chain knocked out. These mice die late in embryogenesis and exhibit defects in many tissues. Limbs do not form separate digits (syndactyly), the brain is enlarged, misshapen, and not covered by skin in 60% of the mice, and the placenta is malformed. Defects are also seen in the lungs, heart, intestines, and kidneys (91, 92).

Ln-10 is a highly adhesive protein, comparable to Ln-5 in mediating cell attachment and spreading and also mediates cell proliferation and migration (93, 94). Adhesion to Ln-10 is mediated by multiple integrin receptors including α2β1, α3β1, α6β1, and α6β4 (95, 96). The morphology of cells grown on Ln-10 is different than that of cells grown on Ln-5. Cells on Ln-10 have an enlarged morphology, with multiple thin processes, indicative of a migratory phenotype, while cells on Ln-5 have a cobblestone-like morphology (93).

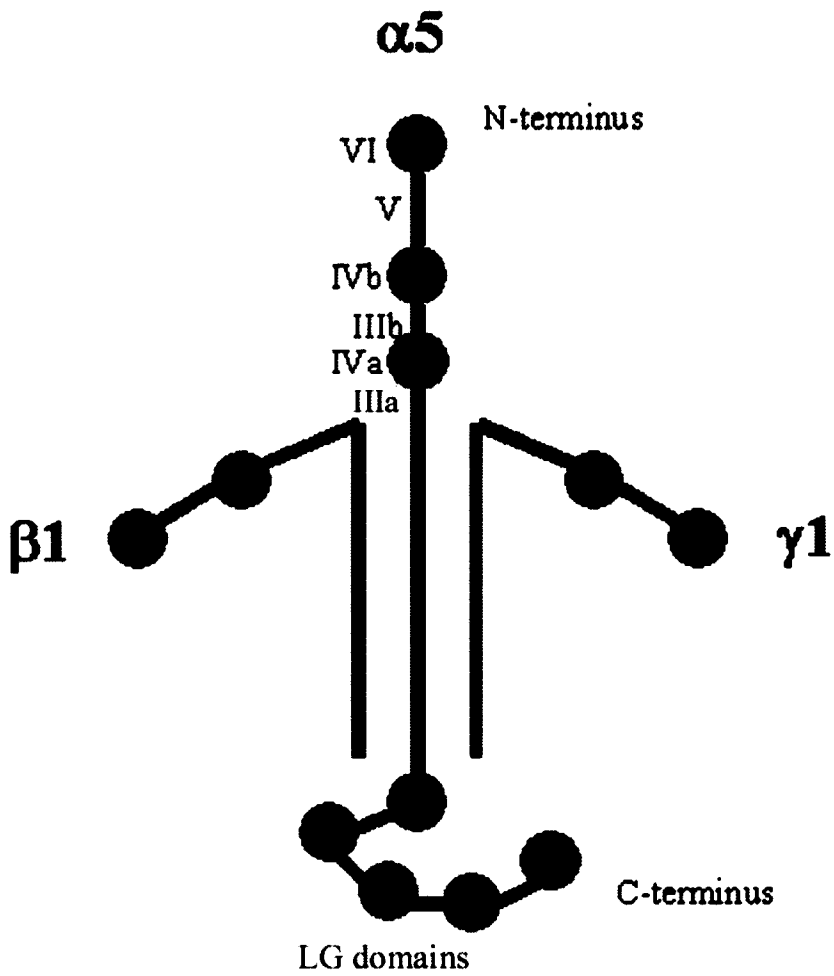

Figure 3. Structural representation of Laminin-10.

Laminin-10 is composed of the α5, β1, and γ1 chains. Domains are indicated by roman numbers.

Matrix Metalloproteases and the mechanism of metastatic invasion

The matrix metalloproteases (MMPs) are a family of over twenty enzymes that require zinc-binding for activity and are involved in degrading the extracellular matrix. MMPs possess broad spectrum proteolytic activity for a wide variety of ECM components including collagens I through IV, laminin, fibronectin, gelatin, elastin, and proteoglycans (97). MMPs are subdivided based on substrate specificity and structural characteristics into the collagenases (MMPs –1, -8, -13), gelatinases (MMPs-2 and –9) stromelysins (MMPs-3, -7, 10, and –11), and membrane anchored metalloproteases (MT-1, -2, -3, -4, and –5 MMP). Selected MMPs and their substrates are outlines in Table 1. MMPs are associated with both normal and pathological conditions involving matrix degradation and remodeling (98). MMPs are highly expressed in the endometrium during menstruation and in the involuting breast, uterus, and prostate (99). MMPs are also essential for connective tissue development and wound healing (100, 101). Tissue destruction caused by MMPs is noted in different diseases including periodontis, rheumatoid arthritis, and macular degeneration. MMPs also facilitate the invasion of primary cancers and the metastasis of tumor cells to distant sites, such as lymph nodes and bone (102-104). MMP-directed matrix degradation can liberate actual peptides or growth factors sequestered by the ECM. Degradation also may reveal ECM cryptic sites called matrikines, which are able to initiate signaling by binding to and activating cytokine or growth factor receptors. These factors can influence tumor cell invasion by affecting integrin regulation or MMP expression, activation, and activity (105, 106).

MMP	Size (latent/active)	Reported Substrates*
Matrilysin (MMP-7)	28,000/19,000	Collagen IV, elastin, fibronectin, gelatin, laminin, pro-MMP-1, -2, and –9
Collagenase-1 (MMP-1)	55,000/45,000	Aggrecan, collagens, gelatin, pro-MMP-2 and -9
Collagenase-3 (MMP-13)	60,000/48,000	Collagens I, II, III, and IV, gelatin
Stromelysin-1 (MMP-3)	57,000/45,000	Collagens III and IV, fibronectin, gelatin, pro-MMP-1, -7, -9, and -13, proteoglycans,
Stromelysin-2 (MMP-10)	57,000/44,000	Collagens III, IV, V, fibronectin, gelatin, pro-MMP-1
Stromelysin-3 (MMP-11)	51,000/44,000	Fibronectin(weakly), laminin
Gelatinase A (MMP-2)	72,000/66,000	Collagens, elastin, fibronectin, gelatin, laminin, pro-MMP-9 and -13
Gelatinase B (MMP-9)	92,000/86,000	Collagens IV, V, and VII, fibronectin, gelatin
MT1-MMP (MMP-14)	66,000/60,000	Collagens I, II, and III, elastin, entactin, fibronectin, gelatin, laminin, pro-MMP-2 and -13, proteoglycans, vitronectin
MT2-MMP (MMP-15)	72,000/?	Fibronectin, gelatin, pro-MMP-2, tenascin
MT3-MMP (MMP-16)	64,000/52,000	Collagen III, fibronectin, pro-MMP-2
*Not an inclusive list		

Table 1. Selected Tumor Associated Matrix Metalloproteases and Their Substrates. From (107), with modifications.

MMP expression is primarily regulated at the transcriptional level by growth factors, cytokines, and contact to ECM (108). MMPs are secreted as inactive zymogens that require removal of the amino-terminal prodomain to become catalytically active (109). Following activation, MMPs can be inhibited by the tissue inhibitors of metalloproteases (TIMPs) that bind to the highly conserved zinc binding site (110). The TIMP family includes four members, TIMP-1, -2, -3, and –4. Different TIMPs are expressed in different tissues, and each has an affinity only for certain MMPs (111). Therefore, consequences or overexpression of MMPs in cancer are dependent upon the level of TIMP expression. All MMPs characterized to date contain a signal sequence, which allows for secretion of the inactive protein, a prodomain, which must be cleaved off for the enzyme to be catalytically active, and a catalytic domain containing a zinc-binding site. Most MMPs, with the exception of MMP-7 and MMP-23, contain a hemopexin-like domain that determines substrate specificity. This domain mediates interactions with the TIMPs. The membrane-type metalloproteases (MT-MMPs) contain a transmembrane domain that directs membrane localization. These structural characteristics are demonstrated in Figure 4.

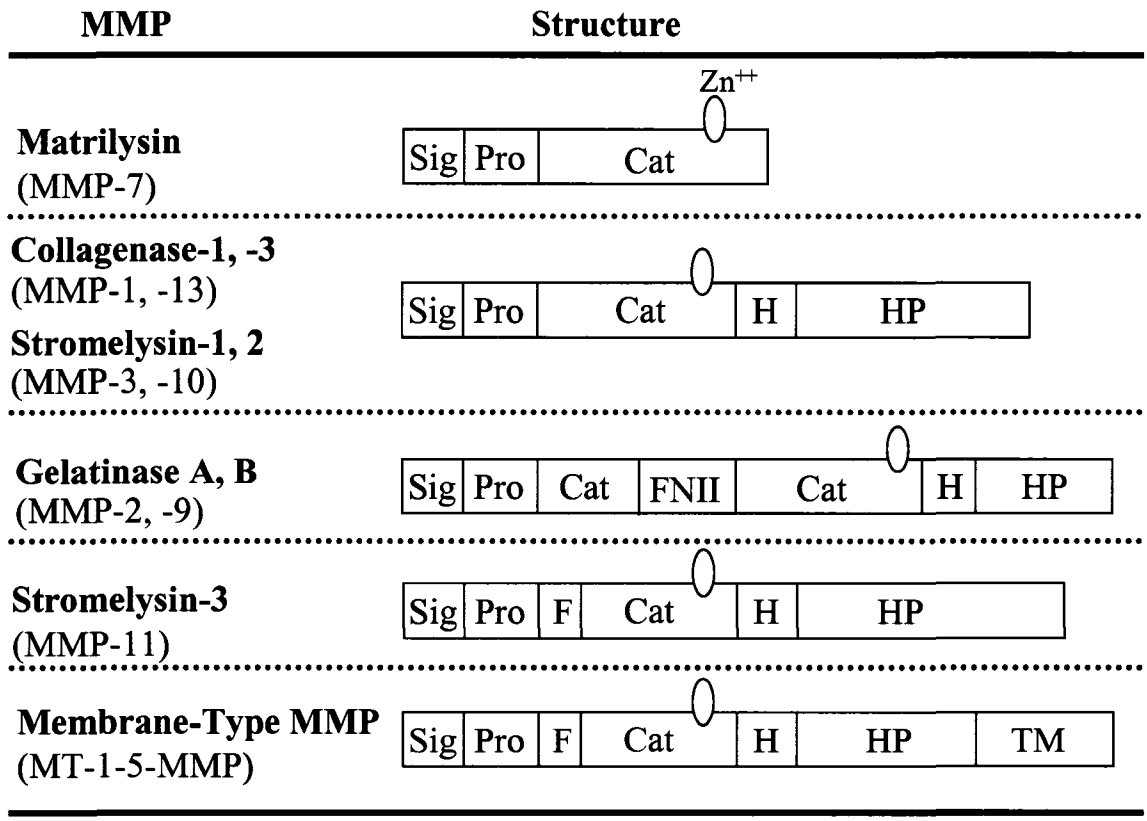

Figure 4. Structure of tumor cell associated matrix metalloproteases.

Subgroups of MMPs are illustrated according to the characteristics of each domain. Sig = signal peptide, Pro = pro-domain, F = furin cleavage sequence, Cat = catalytic domain containing the active zinc, FNII = fibronectin type II repeats, H = hinge region, HP = hemopexin-like domain, TM = transmembrane and cytoplasmic domain. Modified from (107).

Matrix metalloprotease overexpression has been shown to be common in many cancer types including breast (112), squamous cell carcinomas (102), lung carcinomas (113), and prostate carcinomas (114). Generally, MMPs appear to be involved in many of the steps in invasion and metastasis of cancer and understanding the role of these proteases in specific cancers will provide important information for designing preventative and therapeutic treatments for cancer.

Matrilysin

The smallest member of the MMP family, matrilysin (MMP-7, PUMP-1), a member of the stromelysin family that lacks the hemopexin domain, has been shown to degrade many ECM proteins including proteoglycans, fibronectin, entactin, laminin, gelatin, and elastin (115). Entactin, the protein that links type IV collagen to laminin, is the preferred substrate of matrilysin (115). Matrilysin is also able to cleave and activate other MMPs such as progelatinase B (116) and procollagenase (117) which in turn degrade other ECM components. Matrilysin is expressed in cells during normal processes such as wound healing (115), bone differentiation (118), and in the cycling endometrium (119). However, overexpression of matrilysin has been associated with malignant tumors including breast (112), prostate (120), stomach (121), skin (122), colon (123), esophageal, gastric (124), head and neck, and lung carcinomas (125). Because matrilysin is a secreted protease, local secretion makes its expression potentially useful for diagnosis. This is true for both oral SCC, where matrilysin secretion into saliva may indicate aggressive disease, and for prostate cancer, were matrilysin secretion into serum

or prostatic fluid may indicate aggressive disease.

Matrilysin's effects on tumor cells have been studied by altering matrilysin expression in different cancer cell lines. Inhibition of matrilysin expression in the colon cancer cell line BM314 decreased their *in vitro* invasiveness (126). Blocking matrilysin expression with antisense oligonucleotides in the colon cancer cell line WiDr injected into nude mice led to decreased liver metastases compared to control cells (127). In addition, overexpression of matrilysin in the prostate cell line DU-145 increased their *in vivo* invasiveness (128).

Matrilysin in Oral SCC

Many MMPs have been demonstrated to be upregulated in oral SCCs, including MMPs –1, -2, -3, -7, -9, -10, -12, -13, -14 and –26 (129-131). A less aggressive oral cancer variant, verrucous carcinoma, did not display the same MMP expression profile, including no detectable MMP-7, -13, -2, or -9, further implicating MMPs, including matrilysin, as markers for aggressive oral SCC (131). The expression of secreted MMPs in tissue sections from patients with oral SCC is associated with increased local invasion or with incidence of lymph node metastases (129, 130). While matrilysin expression and effects on oral SCC prognosis have not been examined in detail, the staining patterns of matrilysin in oral SCC correlate with matrilysin expression in other tumors. Since expression of matrilysin tends to be associated with aggressive disease in other tumors, it is thought that this similar pattern of expression in oral SCC is also indicative of aggressive disease for this cancer type (132). Matrilysin expression in oral SCC was

located at the invasive front of cancer, further implicating this MMP as being associated with an aggressive phenotype (131).

Membrane-Type-1 Matrix Metalloprotease

MT1-MMP was the first membrane associated MMP to be discovered, and was described as the cellular receptor and activator of MMP-2 (133). Since its discovery, MT1-MMP has also be described as a proteolytic enzyme capable of degrading extracellular matrix (ECM) molecules such as collagens, fibronectin, entactin, gelatin, tenascin, vitronectin, and laminin (134-136). Because it is anchored to the cell membrane, MT1-MMP is thought to play a role in pericellular proteolysis, unlike secreted MMPs, which may act on substrates at a distant location from the cell that secretes them. MT1-MMP is expressed in cancers including breast, gastric, lung, ovarian, colon, pancreatic, bladder, urothelial, thyroid, cervical, and hepatocellular (137).

MT1-MMP has been demonstrated to be involved in tumor cell migration in many ways. First, MT1-MMP localizes at the invading front of migrating cells. This localization allows for degradation of the ECM barrier and therefore facilitates invasion. MT1-MMP localizes at the invading front via its interactions with the actin cytoskeleton. This is not a direct interaction though the cytoplasmic tail of MT1-MMP, however. MT1-MMP associates with CD44 on the cell surface, which has a cytoplasmic domain that anchors to actin (138). Also, overexpression of MT1-MMP in noninvasive cells is enough to stimulate cell migration and activate extracellular signal-regulated kinase

(ERK) (139). Finally, MT1-MMP can cleave human Ln-5, and this cleavage allows for increased prostate cancer cell invasion and migration (83).

MT1-MMP has a furin consensus site located near its prodomain that allows for activation by the transmembrane serine protease furin, found in the trans-golgi network (140). Activated MT1-MMP on the membrane binds TIMP-2 in its catalytic domain and this complex acts as a receptor for pro-MMP-2, which binds to TIMP-2 through its catalytic domain. A second MT1-MMP dimerizes with the hemopexin domain of the first MT1-MMP molecule and cleaves the pro-domain from pro-MMP-2, releasing the active MMP-2. Once expressed on the cell's surface, MT1-MMP is inactivated by either TIMP binding (TIMPs –2, -3, or –4) or by proteolytic degradation by either another MT1-MMP molecule or by MMP-2. The inactive MT1-MMP is then internalized via its cytoplasmic tail (141, 142).

In addition to ECM molecules, MT1-MMP can cleave cell adhesion molecules such as CD44 (143), pro αv integrin (144), and a tissue transglutaminase that binds fibronectin (145). It is thought that MT1-MMP mediated cleavage of cell surface receptors may aid in invasion by altering adhesion or by generating novel fragments that can act as transcription factors to promote migration (51).

Matrilysin and MT1-MMP in Prostate Cancer

Many members of the MMP family have been demonstrated to be expressed in normal prostate tissue and in prostate cancers including: MMPs-2, 7, and -9 (146). Prostate cancers express high levels of MMPs, with a concurrent decrease in TIMP levels

(147). Matrilysin expression has been demonstrated to be focal in small groups of prostate cancer cells. Matrilysin expression was also detected in normal prostate tissue, but only in areas with inflammation (120). Expression of matrilysin in patients with prostate cancer has been correlated with vascular and lymphatic invasion (148). More recently, MT1-MMP has been demonstrated to be expressed in the cytoplasm of secretory cells in high grade PIN lesions (149) and overexpressed in some prostate tumors. It was not found to be expressed in the secretory cells of normal prostate although basal cells did show some expression. Therefore, the importance of MT1-MMP in prostate cancer progression has yet to be fully explored but its expression profile indicates that it is most likely a very important factor in the progression of this disease.

Integrins

The integrins are a family of cell surface proteins that connect cells to the surrounding ECM, and these interactions initiate cell signaling that mediates a wide variety of biological effects such as cell growth, death, differentiation, and movement (150). Integrins are heterodimeric integral membrane glycoproteins composed of one α and one β chain. Integrin binding specificity for different ECM proteins is determined by which $\alpha\beta$ combination is adopted (151). Many cells express multiple integrins, each capable of interacting with specific extracellular proteins. The $\alpha\beta$ pairing specifies the ligand binding abilities of the integrin. Differences in adhesiveness are dependent not only on the affinity to the ligand, but also on cell surface clustering and cytoskeletal associations. Binding to the ligands also requires divalent cations such as Mg^{2+} and Ca^{2+}

(152, 153). Most integrins bind more than one ECM protein and one ligand can recognize several integrins.

So far, 18 α and 8 β chains have been described, many of which exist in multiple isoforms generated by alternative mRNA splicing. At least 24 different combinations of α and β subunits have been discovered (154). Integrins contain a large extracellular domain, consisting of approximately 1,000 residues in the α chain and approximately 750 residues in the β chain. They also contain a transmembrane domain and a short cytoplasmic domain, which consists of 50 residues or less. The exception is β4, which contains greater than 1,000 residues in the cytoplasmic domain (150, 151). The extracellular domains of integrins bind to the ECM or to counter receptors on other cells whereas the cytoplasmic domains bind to cytoskeletal elements, forming a bridge between the intracellular and extracellular space (150). By connecting the extracellular environment with the intracellular structures, integrins may directly or indirectly activate cytoskeletal signaling networks. Because they form a bridge between the actin cytoskeleton and the ECM, integrins allow cells to exert force onto the surrounding environment (155) which is necessary for cells to be motile (156).

The cell adhesive properties of integrins have been described in detail, but it is also known that integrins are also involved in signaling for cell growth and motility via "inside-out" and "outside-in" signaling. "Inside-out" signaling refers to a process by which intracellular signaling mechanisms regulate the ligand-binding activities of integrins on the surface of the cell. This signaling can alter either the avidity or affinity of an integrin for its ligand. Changes in avidity result from redistribution of integrins,

which causes clustering of integrins in focal complexes at sites of ligand binding. Changes in affinity result from an intracellular signal that can cause a conformational change in the integrin, rendering it active so that it can bind its ligand (157). Integrins also become activated upon binding to their ligand. The α subunit cytoplasmic domain inhibits function of the β cytoplasmic domain, which is the domain involved in signaling. When the ligand binds, a conformational change occurs, relieving the inhibition of the β chain, allowing signaling to occur in the "outside-in" signaling mechanism (158).

Cadherins

Cadherins are transmembrane glycoproteins that interact in a calcium-dependent manner to form homotypic adhesions with neighboring cells. They play an important role in cell differentiation and migration and in maintaining tissue integrity (159). Cadherins consist of three protein domains, an extracellular amino terminus, a transmembrane domain, and an intracellular carboxy terminus. Cadherins bind to catenins intracellularly via their cytoplasmic domain (160) and this binding is necessary for their cell-adhesive properties (161, 162). In turn, catenins anchor the cadherins to the actin cytoskeleton (163), another example of a transmembrane protein forming a bridge between the intracellular and extracellular spaces. Functional adhesion by cadherins requires a coordinated homophilic binding of the extracellular domains on two cells and the binding of the cadherin cytoplasmic domain with the actin cytoskeleton (164). Homophilic binding of identical cadherins is preferred, and experiments have

demonstrated that self-sorting of a mixed cell population is dependent upon the cadherins expressed on the cells (165).

As with integrins, cadherins are capable of transducing signals to affect cellular processes. Cadherin signaling occurs upon cadherin mediated cell-cell receptor recognition or in conjunction with receptor tyrosine kinases such as fibroblast growth factor receptor (166). E-cadherin (epithelial cadherin) is the main cell-cell adhesion molecule in epithelial cells and is essential for the adhesion of glandular epithelium to each other and for cell polarity (167). Decreased E-cadherin expression is an important factor in the regulation of carcinoma invasiveness and metastasis (168) and downregulation or loss of E-cadherin expression is common in various tumors including stomach, colon, head and neck, bladder, breast, and prostate (169-174). N-cadherin (neural cadherin) expression is reported to induce a motile and scattered phenotype in breast carcinoma (175) and squamous cell carcinoma (176). Expression of N-cadherin by a cell has been shown to be able to overcome E-cadherin mediated cell-cell adhesion in its ability to promote cell migration. It is therefore thought that a "cadherin switch" occurs in the progression of cancer from a noninvasive to an invasive phenotype (177). It is thought that expression of N-cadherin by tumor cells allows for interactions with new cells with which an E-cadherin-only expression cell would not be capable of interacting. N-cadherin may also mediate pro-migratory signals by inducing activation of MAP kinase pathway (178).

90K

90K is a secreted cell adhesive glycopotein that was originally identified as a tumor-associated antigen in breast cancer and was named 90K after its molecular mass (179). Expression of this protein has been demonstrated to be associated with a poor prognosis in various cancers and it is therefore considered a tumor marker for these cancers. 90K is expressed and secreted by different cell types including hematapoetic and epithelial cells (180, 181). The functions of 90K are not well-defined at this point, but it has the ability to upregulate various cytokines, including IL-1, IL-2, and IL-6 (181, 182). Therefore it is involved in the host response to tumors and to infections such as HIV, Hepatitis B, Hepatitis C, and other autoimmune diseases (183-186). It is found in the cytoplasm and in the ECM in different tissues where it binds collagens, fibronectin and entactin (180, 187-189). It is known to promote cell adhesion and spreading by binding β1 integrins (189, 190). 90K was originally known as galectin-3 binding protein or mac-2 binding protein due to its first identified ligand, galectin-3 (formerly mac-2), a carbohydrate-binding protein (179-181, 191). However, since its ligands are now known to also include galectins -1, and –7 (192, 193) in addition to its ECM ligands, it is now called 90K. 90K can be detected in the μg/ml range in serum and other biological fluids of different cancers (179, 180). High levels of 90K in the serum of breast cancer patients were associated with poor survival and metastatic spread to the liver (194). Also, it is expressed in about 30% of stage I non-small cell lung cancers, as detected by immunohistochemisty, and its expression was associated with poor prognosis (195). Therefore, Mac-2 BP is considered a tumor marker for these cancers.

Statement of the Problem

Clearly, a role for MMPs in cancer progression exists. How the function of MMPs exists in context with the cellular environment with regards to expression and function is not clearly understood, however. In this report, I will discuss how the extracellular environment is important for MMP expression in oral SCC and prostate cancer, and how cancers are able to specifically alter their BMs by cleaving specific proteins, effectively breaking down the barriers that keep them in place. The composition of the ECM together with the receptors expressed on the cell surface decides whether a cell will survive, proliferate, or exit the cell cycle and differentiate. Invasiveness of cells occurs due to a coordinated regulation of cell-cell interactions, cell-matrix interactions, cytoskeletal dynamics, and pericellular proteolysis degrading matrix barriers, including the basement membrane. Communication amongst various cell adhesion molecules such as integrins and cadherins is necessary for this coordination. For example, E-cadherin activity allows for cells to break away from the primary tumor mass (196). This is followed by increased involvement of intergrins in cell-substrate interactions during increased motility. Laminin-binding integrins are involved in this process in tumor cells (145). These diverse requirements for metastasis are met as a consequence of interactions between the different classes of adhesion receptors.

The first goal of these studies was to elucidate the mechanism by which matrilysin is expressed in oral SCC after interactions with stromal cells. Fibroblast cells were cocultured with SCC-25 cells to mimic the environment of a tumor, complete with

cell adhesion molecule and ECM protein interactions. In this environment, increased matrilysin expression by SCC-25 cells was observed. In relation to the stromal environment and metastasis, a second goal of this study was to determine the effects of 90K expression on prognosis in prostate cancer, with specific emphasis on upregulation of matrilysin. We therefore examined the expression of 90K in prostate cell lines and prostate tissue. In addition, 90K upregulates expressions of various cytokines, which we had previously demonstrated to upregulate matrilysin in prostate cancer cells. This led to the hypothesis that 90K would mediate matrilysin expression in prostate cancer cells. Finally, the third goal was to examine the mechanism by which prostate tumors are able to invade with the hypothesis that MT1-MMP expressed by prostate cancer cells cleave Ln-10, which allows the cells to invade through their basement membrane.

II. MATERIALS AND METHODS

Cell Culture

The human oral squamous cell carcinoma line, SCC-25, was obtained from American Type Tissue Culture Collection (Rockville, MD). Human Foreskin Fibroblasts (HFF cells) were obtained as previously described (197). SCC-25 and HFF cells were maintained in Ham's F:12/Dulbecco's Modified Eagles Medium (DMEM) purchased from Gibco BRL (Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) from Gemini-Bio-Products (Calabasas, CA), 0.1% hydrocortisone, and penicillin (100 units/ml)/streptomycin (100 μg/ml). Cells were grown in a humidified incubator at 37°C and 5% CO_2. Co-cultures were seeded at a 1:1 ratio. For all experiments (unless otherwise noted), SCC-25 and HFF cells were seeded in full medium and allowed to attach for 24 hours before proceeding further. To generate SCC-25 conditioned medium (CM) and HFF CM the cells were seeded at a density of $3x10^5$ cells/well in 6-well plates and were then incubated for 24 hours in serum free medium (SFM) supplemented with 0.1% hydrocortisone and penicillin (100 units/ml)/streptomycin (100 μg/ml). The medium was then clarified by centrifugation. Tissue culture plasticware was purchased from Costar (Cambridge, Mass.), cell inserts from Falcon (Franklin Lakes, NJ), and EGTA from Sigma (St. Louis, MO). For calcium free medium experiments, calcium free DMEM was purchased from Gibco BRL (Grand Island, NY). The human prostate cancer cell lines PC3, PC3-N (variant of PC3), TSU-Pr1, JCA-1, LNCaP, PPC-1, DU-145, and MCF-7 cells were maintained in Dulbecco's Modified Eagle medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (JRH

Biosciences, Lenexa, KS), penicillin (100 U/ml, Invitrogen, Carlsbad, CA), streptomycin (100 μg/ml, Invitrogen, Carlsbad, CA), and 0.2 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA). All cells were maintained at 37 °C in 5% CO_2 and passaged with trypsin/EDTA (Invitrogen Life Technologies, Carlsbad, CA) when confluent.

Antibodies and Reagents

Salt free, preservative free anti-human-β-1 integrin antibody, 1μg/μl, (clone P4C10) and A-CAM antibody, an anti-N-cadherin antibody, 3μg/μl, (clone GC-4) were obtained from Sigma (St. Louis, MO). Mouse IgG, 3.4μg/μl, was also purchased from Sigma-Aldrich (St. Louis, MO) and used as the control antibody in blocking experiments. A polyclonal anti-pan-cadherin antibody was produced as described previously (62). Purified anti-E-cadherin antibody, 1μg/μl, (clone 67A4) and recombinant human MT1-MMP catalytic domain, polyclonal MT1-MMP antibody (AB815) were obtained from Chemicon (Temecula, CA). Rabbit polyclonal antibodies (Ab470) raised against the synthetic peptide RECPYAIREGNEK derived from the protein sequence of MT1-MMP was obtained from Dr. Stetler-Stevenson, NCI, Bethesda, Maryland. Laminin-10/11 α5 chain antibody, 15H5, was purified as described previously (93) and 4C7 was a gift from Dr. Eva Engvall (The Burnham Institute, La Jolla, CA). 90K antibody clone SP-2 was purchased from Bender MedSystems, and 90K antibodies 1H9 and 4D1 were prepared as described previously (198). Il-6 neutralizing antibody was obtained from Calbiochem (La Jolla, CA).

Peptides

HYD1 peptide, which blocks cell binding through the β1 integrins, and HYD1-S peptide (the scrambled form of HYD1) were obtained as described previously (199).

Removal of cells from matrix

Cells were removed from their matrix so that the matrix could be used to examine cell-matrix interactions following a previously established protocol, with modifications (200). One cell type was seeded for 24-48 hours, allowing it to establish matrix. The cells were then removed by replacing the growth medium with 5mM EDTA in SFM for 30 minutes at 37°C. Cells were washed twice in 1X PBS. Then 40 mM NH_4OH was added for 20 minutes, completely removing the cells. This method removed the cells without disrupting the cell matrix. The matrix was washed twice with 1X PBS then with double distilled H_2O. At this point, the matrix was either frozen for future use or the other cell type was seeded on the matrix in SFM.

ELISA for promatrilysin

An antibody sandwich assay for promatrilysin was developed previously (201). The capture antibody (10D2, a mouse monoclonal antibody produced in the laboratory of Dr. Raymond Nagle using purified promatrilysin from Dr. Mark Navre, Syntex, Palo Alto, CA) was coated onto 96-well micotiter plates (Costar, Cambridge, Mass.). Because this antibody is specific for human promatrilysin, the ELISA will not detect active matrilysin. The detection antibodies included Rb2, from Santa Cruz Biotechnology (Santa Cruz,

CA), a rabbit polyclonal antibody to human matrilysin, followed by a horseradish-peroxidase-conjugated goat anti-rabbit antibody (Pierce, Rockford, IL) used to detect bound Rb2. Horseradish peroxidase activity was quantitated using a hydrogen peroxide/*o*-phenyldiamine (Sigma, St. Louis, MO) colorimetric system. Purified promatrilysin was used to generate a standard curve with each assay. The assay was linear in the range of 0.2 to 12.5ng/ml. If necessary, samples were diluted until the readings were within the linear range and then the results were multiplied by the dilution factor.

Western analysis

Cell lysates were prepared and separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroporetically transferred to nitrocellulose, as described previously (62). Cells were washed twice with phosphate buffered saline containing 1mM phenylmethylsulfonyl fluoride (PMSF). Cells were scraped in PBS + PMSF, transferred to a microfuge tube and centrifuged. The pellet was lysed with 2X SDS sample buffer (0.25 M Tris-HCl, pH 6.8, 10% SDS, 25% glycerol) and sonicated. Protein concentrations were measured using the DC BioRad assay procedure (Hercules, CA) with bovine serum albumin (BSA) as a standard. Each lane was loaded with 30 μg of cellular protein. Antigens were detected using the pan-cadherin antibody, followed by peroxidase-conjugated goat anti-rabbit. Protein bands were identified by chemiluminescence exposed on X-OMAT AR film (Kodak, Rochester, NY). Protein samples run under non-reducing conditions were mixed with a non-

reducing sample buffer (25 % 4X Tris-Cl/SDS, pH6.8, 20% glycerol, 4% SDS, 0.001% bromophenol blue) before loading on the SDS-PAGE gel.

Real-Time-PCR

Real-time-PCR was performed on the PE Biosystem GeneAmp 5700 sequence detection system (PE Applied Biosystems, Foster City, CA) using Assay-on-Demand expression products consisting of a 20X mix of unlabeled PCR primers and TaqMan Universal PCR Master Mix No AmpErase UNG buffer (Applied Biosystems, Foster City, CA) and MultiScribe and Rnase Inhibitor Mix (Applied Biosystems, Foster City, CA). These assays are designed for the detection and quantitation of specific human genetic sequences in RNA samples converted to cDNA. The specific primers used for PCR were obtained from Applied Biosystems Assays-on-Demand Gene Expression Producers (taqMan R MGD probes, FAM TM dye-labeled) 90K (Hs00174774) and GAPDH (Hs99999905). Reverse transcription and amplification were carried out using one-step RT-PCR. The RT reaction was performed for 30 minutes at 48°C. The samples were then incubated for 10 minutes at 95°C to inactivate the reverse transcriptase (RT) and activate the AmpliTaq Gold (Applied Biosystems Foster City, CA). The PCR reaction was completed after 40 cycles for 15 seconds at 95°C followed by 1 minute at 60°C. A no-template control was included to establish baseline fluorescence. The increase in intensity of fluorescence of the reporter dye (ΔRn) for 90K was plotted against the cycle number. The point at which the ΔRn values cross a set threshold is defined at the cycle threshold (C_t) that is calculated by the sequence detection software (Applied Biosystems,

Foster City, CA). At the end of each extension phase, fluorescence was observed at C_t. All samples were normalized to human GAPDH expression. The relative expression of 90K in each sample was determined using the comparative C_t method of relative quantitation of gene expression as per the manufacturer's protocol (Applied Biosystems User Bulletin 2). This method uses the average C_t from both the 90K and GAPDH samples and calculates the ΔC_t, or the normalized 90K C_t relative to the corresponding GAPDH C_t.

Prostate Tumor Tissue Array Construction and Immunohistochemistry

A tissue microarray containing four morphologically representative areas of tissue from 299 patients was obtained from the Cooperative Prostate Cancer Tissue Resource (CPCTR) (National Cancer Institute, Bethesda, MD)(202). Sections were incubated with 90K primary antibody (SP-2) in PBS for 30 minutes and washed with PBS. This was followed with the LSAB (labeled streptavidin-biotin) peroxidase kit (Dako, Carpinteria, CA). Sections were colored with diaminobenzidine incubation for 10 minutes at room temperature, washed in tap water, counterstained with hematoxylin and mounted in permanent coverslipping medium.

Coverslip Immunohistochemistry

Prostate cancer cell lines were grown on coverslips and fixed in methanol/acetone. Sections were incubated with 90K antibody, SP-2 as described for prostate tissue. Coverslips were also incubated with DAPI to stain nuclei. Antibody detection was

performed by incubating slides with fluorescent labeled secondary antibodies (Alexa 485 and 565, Molecular Probes, Eugene, OR). Slides were analyzed on a Zeiss LSM 410 UV (Carl Zeiss, Oberkochen, Germany) dual laser confocal microscope using the argon/krypton ion laser operating at 488 and 568 nm.

Immunohistochemistry

For detection of Ln-10, frozen prostate tissue sections (3 μm) were placed on positively charged glass slides and were fixed in acetone for 5 minutes and incubated with primary antibody in PBS for 30 min at room temperature. Antibody detection was performed by incubating slides with fluorescent labeled secondary antibodies (Alexa 485 and 565, Molecular Probes, Eugene, OR). For detection of MT1-MMP, slides were fixed in 2% formaldehyde for 7 minutes, 50 mM NH_4Cl for 5 minutes, and 0.2% Triton-X100 for 3 minutes. Slides were analyzed on a Zeiss LSM 410 UV (Carl Zeiss, Oberkochen, Germany) dual laser confocal microscope using the argon/krypton ion laser operating at 488 and 568 nm. Tissue sections were also stained with hematoxylin and eosin (H&E) to identify tissue structures.

Purification of Human Ln-10 from A549 Serum-free Conditioned Medium (CM)

Human Ln-10 was purified as described previously (93). Briefly, the human lung carcinoma cell line A549 was grown in 175 cm^2 culture flasks. After the cells reached confluence, the conditioned medium was harvested. Endogenous protease activity was minimized by the addition of 5 mM EDTA, 50 μM phenylmethysufonyl floride, and 50

μM *N*-ethylmaleimide. The A549 CM was passed through a 4C7-Sepharose CL-4B affinity column prepared by coupling the anti-Ln-10 α5 chain monoclonal antibody 4C7 to cyanogen-activated Sepharose CL-4B (Amersham Biosciences, Piscataway, NJ). Ln-10 was eluted from the affinity column with 0.1 M glycine (pH 2.7), and neutralized by addition of Tris-HCl (pH 8.0). The protein concentration was determined with Advanced Protein Assay Reagent (Cytoskeleton, Inc., Denver, CO).

Cleavage of Ln-10 by MT1-MMP

Purified Ln-10 was adsorbed and dried onto a 96-well plate and incubated with the recombinant catalytic domain of MT1-MMP (from 0.034 nmol to 2.1 nmol) for 6-18 hr at 37 °C in 50 mM Tris, pH 7.5, 0.005% Brij-35, 10 mM $CaCl_2$ as described previously for Ln-5 (83, 84). After incubation, each mixture was solubilized off the plate with sample buffer and electrophoresed on a 4-10% SDS-PAGE gradient under reducing conditions. Gels were analyzed by either silver or Coomassie Blue staining or by Western blot analysis as described previously (203) with mouse monoclonal antibody 15H5 to the α5 chain of Ln-10.

Mass Spectrometry Analysis of Cleaved Fragments

Cleaved Ln-10 samples were separated by SDS-PAGE. After staining with Biosafe Coomassie Brilliant Blue (BioRad, Hercules, CA), bands were excised and identified by mass spectrometry analysis as described previously (83) using the Proteomics Core facility of the Southwest Environmental Health Sciences Center at the University of

Arizona. Briefly, the protein bands were excised, cut into small pieces (1x1 mm) and subjected to in-gel digestion using trypsin or chymotrypsin. The extracted peptides after digestion were analysed by liquid chromatography-tandem MS using a quadropole ion trap Finnigan LCQ class mass spectrometer equipped with a Michrom (Auburn, CA) MAGIC 2002 high performance liquid chromatography and a nano-electrospray ionization source (University of Washington, Seattle, WA). The peptides were eluted from a pulled tip capillary column packed with Vydac (Hesperia, CA) C18 material. The gradient was from 0 to 65% solvent B (98% methanol/ 2% water/ 0.5% formic acid/ 0.01% triflouroacetic acid) over 60 minutes at a flow rate of 200-300 nl/min. Tandem mass spectrometry spectra of the peptides were analyzed with the SEQUEST program (Turbo Sequest) to assign peptide sequence to the spectra. SEQUEST analyses were performed against the publicly available nonredundant database.

Adhesion Assays

Ln-10 (1 ug) was adsorbed and dried onto a 96-well plate well and treated with MT1-MMP (2 ug/ml) for 18 hours at 37 °C. Wells were then blocked with 1% BSA in PBS for 30 minutes. DU-145 cells (0.5×10^5) in serum free medium (SFM) were then added to the wells and adhesion at 20 minute intervals was determined. Briefly, unattached cells were removed by aspiration and washing with PBS and attached cells were stained for 10 minutes with 5% crystal violet/20% methanol. Fixed cells were then washed with ddH_2O until no more dye was leached. After air drying, dye was eluted with 0.1 M citric acid

and absorbance was read on a plate reader at 570 nm. By quantifying the absorbance, the percentage of attached cells was calculated.

Cell Migration Assays

The linear migration assays were performed as described previously (204). In brief, Teflon-printed microscope slides (CSM, Inc. Phoenix, Arizona) subdivided into 10 wells, were precoated overnight with purified human Ln-10 (1μg/well) at 4°C. Five wells were used as control and the other 5 wells were treated with MT1-MMP (2μg/ml) for 16 hours in a humidified incubator at 37 °C and 5% CO_2. After incubation the excess liquid in the wells was removed, the wells were rinsed with PBS and covered with 70 μl of serum free medium. A cell sedimentation manifold was placed on the slide, and 1 μl of cell suspension (2,000 cells) was placed in each cylinder, and incubated at 37 °C for 4 h in 5% CO_2 allowing the cells to attach before removal of the manifold. After the manifold was removed, the initial sedimentation area was recorded using Axiocam camera scanner with CCD sensor, attached to an inverted microscope (Carl Zeiss, Göttingen, Germany). Cell migration area was quantified at each time point with an image analysis system (Axioplan 2, Carl Zeiss, Germany). The initial area of sedimentation was used as a migration reference point and the migration area was normalized to this initial area. Migration was measured in microns. Each experiment was performed at least three times in triplicate. Transmigration assays were preformed as described previously (205), with modifications. Briefly, 0.8 μm filter bottom cell-culture inserts (Corning-Costar, Acton, MA) were coated with 1 μg purified Ln-10 treated with either 2 μg/ml MT1-MMP or

PBS. DU-145 cells were then seeded inside the insert and transmigration through the insert was quantified by crystal violet-methanol staining of invaded cells. One group of DU-145 cells used in both the linear migration and transmigration assays were pretreated for 2 days with 10 μM human antisense oligonucleotide or scrambled antisense sequences against human MT1-MMP as described previously (84). These cells were then seeded in either the migration assay manifold or the cell culture inserts. The antisense oligonucleotides (10 μM) were added to the medium for the linear migration assays and to both the upper and lower chambers of the tissue culture plate for the transmigration assays.

Cell-Mediated Cleavage of Ln-10

Ln-10 (2 μg) was coated on a 6-well plate overnight at 4 °C. Cells were plated on the precoated wells ($0.5x10^6$ cells/well) for 48 hr. Conditioned medium (CM) was collected and proteins were precipitate with trichloroacetic acid. After washing the pellet, protein was resuspended in sample buffer for Western analysis. Cells were removed by addition of 1ml of 5 mM EDTA for 30 min. Wells were washed three times with PBS and collected cells were lysed with RIPA buffer. Sample buffer (50 μl) was added to the wells to solubilize the coated Ln-10. Samples were separated by SDS-PAGE and immunoblotted with 15H5 (matrix samples, CM, and cell lysates) and AB815 (cell lysates).

III. INTEGRIN AND CADHERIN MEDIATED INDUCTION OF THE MATRIX METALLOPROTEASE MATRILYSIN IN CO-CULTURES OF MALIGNANT ORAL SQUAMOUS CELL CARCINOMA CELLS AND DERMAL FIBROBLASTS

Introduction

Matrix metalloproteases (MMPs) are a family of enzymes that degrade components of the extracellular matrix (ECM). MMP degradation of the ECM occurs in normal and necessary cellular processes such as tissue morphogenesis, differentiation, and wound healing (115). However, expression of MMPs is also associated with pathological conditions such as rheumatoid arthritis, cystic fibrosis, Alzheimer's disease, and cancer (206). Three observations that suggest MMPs play an important role in cancer are: 1) the relative level of individual MMPs has been shown to increase with tumor progression (111), 2) noninvasive tumor cell lines transfected with genes encoding proteolytic enzymes have acquired an invasive phenotype (128, 207, 208), and 3) inhibitors of proteolytic enzymes have been shown to suppress tumor cell invasion (208-210).

Overexpression of MMPs causes cells to be released from processes that the ECM regulates such as proliferation and migration (128). Alterations in cell-cell contacts allow these malignant tumor cells to separate from primary tumor masses (61, 211, 212), attach to the basement membrane, and secrete matrix metalloproteases to degrade and penetrate the basement membrane allowing the tumors to metastasize to other parts of the body (213, 214). These pathological conditions have led to an increasing interest in discovering more information on the molecular mechanisms that regulate the expression of MMPs.

One particular MMP, matrilysin (PUMP-1, MMP-7), has been shown to degrade many ECM proteins including proteoglycans, fibronectin, entactin, laminin, gelatin, and elastin (115). Matrilysin is expressed in cells during normal processes such as wound healing (115), bone differentiation (118), and in the cycling endometrium (119). However, increased levels of matrilysin have also been found in invading malignant tumors including breast (112), prostate (120), stomach (121), colon (123), head and neck, and lung carcinomas (125). The oral squamous cell carcinoma line, SCC-25, was derived from a tongue squamous cell carcinoma typical of head and neck tumors potentially caused by tobacco use. After an oral squamous cell carcinoma develops, there is only a 35% to 50% survival rate after 5 years (215, 216) because these tongue carcinomas are highly metastatic. Therefore, we were interested in investigating the matrilysin expression patterns in this highly invasive cell line. The human foreskin fibroblast (HFF) cells are similar to the type of non-cancerous stromal cells that could be found surrounding a squamous cell tumor. Therefore, a co-culture of these two cell types is similar to the physiological environment in which the SCC-25 cells would normally be found.

We and others have demonstrated that matrilysin expression in malignant tumors can be mediated through either paracrine factors (201), cell-cell contact (217-222), or a combination of both (197). Cell adhesion involves both cell-cell interactions as well as cell-matrix interactions. Calcium dependent cell-cell adhesion is mediated by cadherins, a supergene family of cell surface receptors (223). Previously, E-cadherin interactions were shown to be important in the induction of matrilysin expression in II-4 squamous

cell carcinomas (221). E-cadherin has been described as a tumor suppressor gene shown to restrict cell movement by promoting tight cell-cell adhesion leading to the hypothesis that the regulation of matrilysin expression by E-cadherin can be an initial step in the metastatic cascade and that the matrilysin expression can then downregulate E-cadherin expression, allowing for metastasis. N-cadherin, which is expressed on other types of squamous cell carcinomas has also been shown to be expressed in invasive prostate cell lines and in prostate stromal fibroblasts (62). N-cadherin has been hypothesized to promote stable interactions between carcinoma and stroma cells that can facilitate cell survival and cell migration (224-226) and has been shown to be expressed in more invasive cell lines (62). Cell-matrix interactions are mediated in part by integrins, cell surface receptors that require cations to function and are responsible for adhesion to the cell matrix. β1 integrins are implicated in processes such as cancer progression, invasion, and metastasis (227, 228), and have been implicated in the induction of matrilysin expression in a co-culture of colon carcinoma cells with fibroblast cells (229).

We performed experiments to determine if the induction of promatrilysin expression by HFF cells co-cultured with SCC-25 cells is dependant on cell-cell contact, cell-matrix contact, a factor secreted by HFF cells inducing promatrilysin expression in the SCC-25 cells, or a factor secreted by the SCC-25 cells inducing promatrilysin expression in the HFF cells.

In this report, we find that SCC-25 cells do not produce large amounts of promatrilysin by themselves, but when co-cultured with HFF cells they express high levels (125ng/ml within 24 hours) of promatrilysin. Through experiments which blocked

the function of specific cell adhesion molecules, we demonstrate for the first time that the induction of promatrilysin expression in the co-culture of SCC-25 and fibroblast cells is due to a cooperative effect between cell-cell (cadherin) and cell-matrix (integrin) interactions, as current reports implicate cadherins only in same-cell systems and there are no reports of both integrins and cadherins mediating promatrilysin expression in a co-culture system. These results indicate that promatrilysin regulation in the SCC-25 cells is a complex process involving at least three different cell surface receptors, E-cadherins, N-cadherins, and β1 integrins, as blocking these three receptors together caused matrilysin expression to return to basal levels, while blocking each receptor individually only partially decreased matrilysin expression in the co-culture.

Results

Promatrilysin expression in co-cultures of SCC-25 and HFF cells is not mediated by a secreted factor alone.

HFF cells do not express promatrilysin by themselves and SCC-25 cells only express low levels of promatrilysin (average of 2 ng/ml within 24 hours). However, in a co-culture of $3x10^5$ HFF and $3x10^5$ SCC-25 cells, a significant level of expression of promatrilysin was observed. We tested to determine whether a soluble factor secreted by one cell type induced secretion of promatrilysin in the other cell type. To test for a soluble factor-mediated mechanism of induction, SCC-25 cells were incubated in SFM alone or in HFF CM; and HFF cells were incubated in SFM alone or in SCC-25 CM. A co-culture of SCC-25 and HFF cells was also incubated in SFM. After 24 hours, CM

samples were collected and the amount of promatrilysin protein secreted into the medium was quantitated using ELISA analysis. The co-culture medium contained 128ng/ml of promatrilysin, representing a 40-fold increase in secreted promatrilysin protein. However, no significant promatrilysin induction was observed in CM treated cell lines (Figure 5A).

Cell inserts were also used to search for a short half-life paracrine factor mechanism responsible for the induction of promatrilysin expression. Cell inserts allow the two cell lines to share medium without physically contacting each other by seeding one cell type in a tissue culture well and another cell type on a 3.0 μm filter-bottom insert, placed in the well. SCC-25 cells were plated in a well, with HFF cells in an insert. For consistency, SCC-25 cells were also plated in an insert, with HFF cells plated in a well. A co-culture was also plated in a well. After attachment, the medium containing serum was removed from both the wells and inserts and they were washed twice with 1X PBS to remove any traces of serum and SFM was added to the wells. The inserts were placed in the appropriate wells (HFF insert in SCC-25 well and vice versa). After a 24 hour incubation period, samples were collected and the amount of promatrilysin protein secreted into the medium was determined by ELISA. Promatrilysin induction was seen in the co-culture where physical contact between the cells did occur, with a 40-fold increase in promatrilysin expression. In the absence of physical contact between the cell lines no promatrilysin induction occurred (Figure 5B). Also, an experiment in which a co-culture was plated in a well with SCC-25 cells plated in an insert, matrilysin induction was not increased over the induction level in co-culture CM alone (data not shown).

These results, as well as the conditioned medium results, indicate that the major factor in promatrilysin induction is not a soluble factor secreted into the medium, suggesting that cellular contact, either cell-to-cell or cell-to-matrix contact, induces promatrilysin expression.

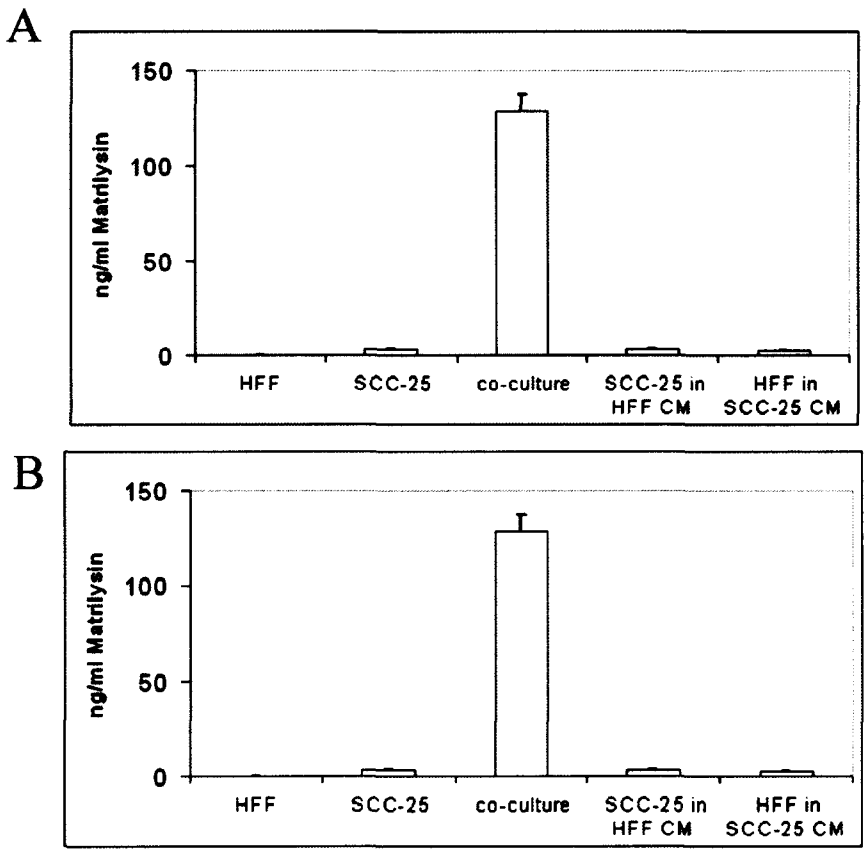

Figure 5. Matrilysin expression is not controlled by the release of paracrine factors.

A representative ELISA of promatrilysin protein in medium is shown. A) CM samples were collected and placed on cells of the opposite type or cells were incubated in serum free medium (SFM). After 24 hours, media samples were collected and analyzed for promatrilysin protein by ELISA. B) After seeding, cells were incubated for 24 hours with SFM grown either in a well or insert forming a transwell system. Media samples were collected and analyzed for matrilsyin protein by ELISA. In the absence of physical contact between the cells there is no induction of matrilysin expression. Results represent the means and 95% confidence intervals representative of at least three experiments run in triplicate.

Promatrilysin expression in the co-culture is calcium dependent.

Because cadherins and integrins rely on Ca^{2+} to function (230, 231), the addition of the cation chelator, EGTA, to a co-culture of SCC-25 and HFF cells will disrupt cadherin and integrin function. SCC-25 and HFF cells were plated together and after seeding the cells were washed and the full medium was replaced with either SFM or 1.5 mM EGTA in SFM. After a 24 hour incubation medium samples were collected and assayed for secreted promatrilysin protein by ELISA. The results (Figure 6A) show a significant inhibition of promatrilysin expression in the presence of EGTA. The co-culture in the absence of EGTA demonstrates a 100-fold increase of promatrilysin expression as compared to the co-culture in 1.5 mM EGTA. When EGTA was removed and SFM was added back to the cells, matrilysin expression increased linearly with time (data not shown). This suggests that calcium dependent cell-cell interactions are needed for the induction of promatrilysin expression.

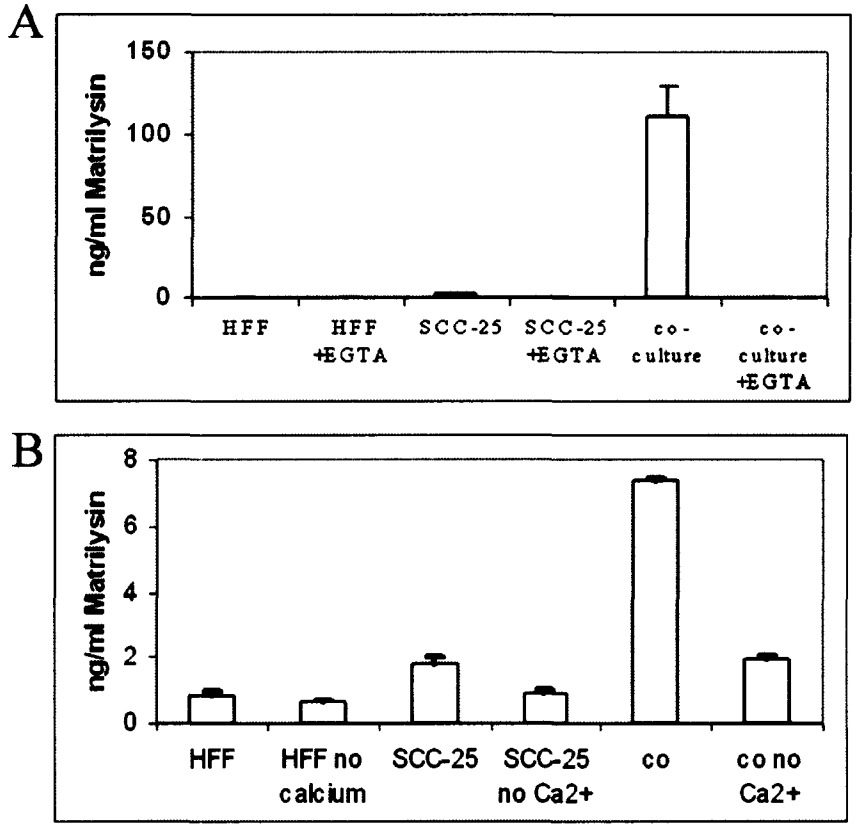

Figure 6. Calcium is required for the induction of promatrilysin expression in the co-culture.

A representative ELISA of secreted promatrilysin protein is shown. A) Cells were seeded in the presence or absence of 1.5mM EGTA, a calcium chelator, in serum free medium. In the presence of EGTA, promatrilysin expression is significantly reduced, indicating that calcium is required for the induction of promatrilysin. This suggests that cadherins are involved in the induction mechanism. B) Cells were seeded in either serum-free medium or in the presence of calcium free, serum free medium and incubated for 9 hours, causing the difference in total protein seen. In the absence of calcium, promatrilysin expression in the co-culture was significantly reduced. Results represent the means and 95% confidence intervals of at least three experiments run in triplicate.

Calcium free medium was used to determine whether the basal level of promatrilysin expression could be due to calcium dependent pathways other than cadherin and integrin signaling. Cells were grown in SFM or in calcium-free, serum-free medium for 9 hours, after which CM samples were collected and analyzed for promatrilysin expression by ELISA. The results (Figure 6B) show that in the presence of calcium, the SCC-25 cells express basal levels of promatrilysin and the co-culture expresses higher levels of promatrilysin. In the absence of calcium, the induction of promatrilysin expression under co-culture conditions was almost completely inhibited, suggesting that cell adhesion molecules are involved in the promatrilysin induction, justifying further experimentation on the involvement of cell adhesion molecules in this system.

Promatrilysin induction is not a result of cell-matrix contacts with inactive matrix proteins.

In order to determine if integrin mediated interactions between the SCC-25 cells and HFF matrix or interactions between the HFF cells and SCC-25 matrix are necessary for the induction of promatrilysin expression seen in the co-culture, SCC-25 cells were plated in SFM on HFF matrix and HFF cells were plated in SFM on SCC-25 matrix. After a 24 hour incubation time, medium samples were collected and analyzed for promatrilysin protein by ELISA. Neither the HFF cells plated on SCC-25 matrix or the SCC-25 cells plated on HFF matrix showed a significant increase in promatrilysin expression. The co-culture showed an increase in secreted promatrilysin protein as

compared to the SCC-25 cells plated on HFF matrix. The results (Figure 7) indicate that cell-matrix interactions in the absence of active matrix proteins secreted by cells do not mediate the induction of promatrilysin expression in the co-culture. SCC-25 cells plated on a co-culture matrix also showed no induction in matrilysin expression (data not shown).

HYD1 peptide inhibits promatrilysin expression in co-cultures of SCC-25 and HFF cells.

Having shown that inactive matrix proteins do not affect promatrilysin induction, we chose to further explore the potential role for cell-matrix interactions in the induction of promatrilysin expression by studying β1 integrins, which were found to be functional on the cell surface of SCC-25 cells by cell adhesion assays (data not shown). We used HYD1 peptide, a synthetic peptide reported to specifically block cell adhesion via β1 integrin (199) to test the role for β1 integrins on promatrilysin expression in the co-culture. HFF cells were allowed to attach overnight and the SCC-25 cells were then plated in the presence of either the HYD1 or the HYD1-S (scrambled) peptide. The HYD1 peptide inhibited attachment by about 40% at 3 hours (199) and did not affect the viability of the cells (data not shown). Among the co-culture samples, treatment with HYD1 significantly inhibited the induction of promatrilysin expression whereas no effect was seen with the HYD1-S peptide (Figure 8). Treatment with HYD1 did not inhibit promatrilysin expression due to the increased time required for adhesion of the cells, as a co-culture seeded 3 hours after the original co-culture (to account for adhesion time) demonstrated similar levels of promatrilysin as the original co-culture (data not shown).

These data show that β1 integrin is involved in promatrilysin induction but that β1 integrin is not fully responsible for the induction. Therefore, we explored potential cell-cell contact mechanisms of induction.

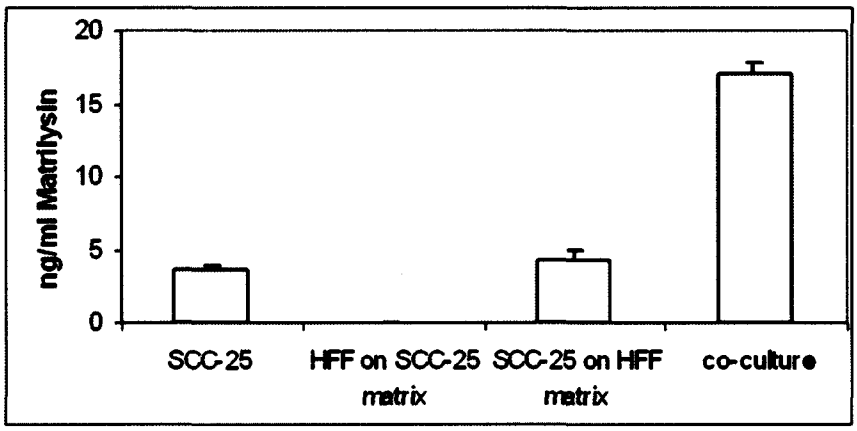

Figure 7. Cell-matrix mediated interactions do not induce matrilysin expression in either the HFF or the SCC-25 cells in the absence of activated fibronectin.

A representative ELISA of promatrilysin protein in medium is shown. HFF cells were plated on SCC-25 matrix in serum free medium and SCC-25 cells were plated on HFF matrix in serum free medium to look for a cell-matrix interaction mechanism of matrilysin induction. CM samples were collected 24 hours after plating in SFM, explaining the lower levels of induction seen. No induction was seen in either matrix-plated cell line compared to the large induction seen in the co-culture where cell-cell interactions can occur. Results represent the means and 95% confidence intervals representative of at least three experiments run in triplicate.

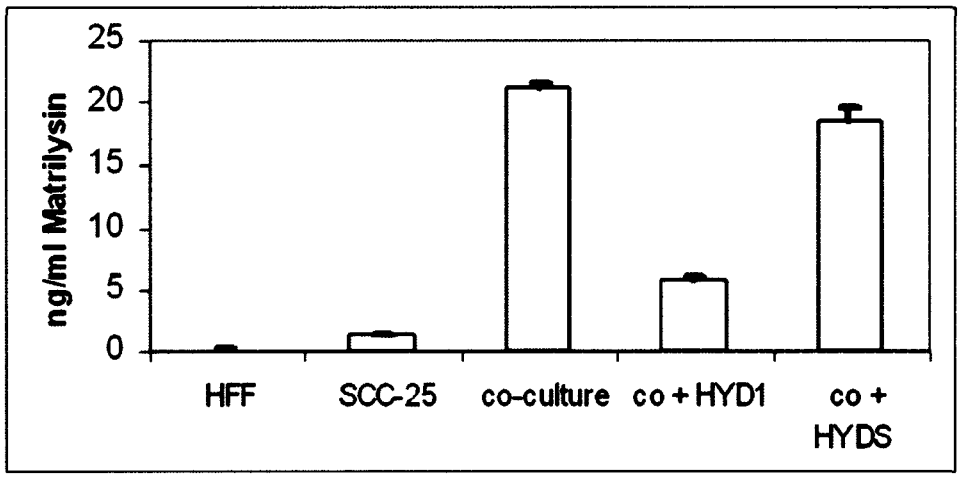

Figure 8. Loss of adhesion causes reduction in promatrilysin expression in the co-culture.

HFF cells were seeded overnight and SCC-25 cells were plated on the HFF cells in SFM with one of the following treatments: 40ug/ml HYD1, 40ug/ml HYD1-S, or SFM alone. Medium samples were collected after 24 hours and analyzed for promatrilysin by ELISA. In the presence of HYD1, the SCC-25 cells did not attach as readily, and promatrilysin expression was significantly reduced. This suggests that contact between the HFF and SCC-25 cells is required for promatrilysin expression. Results represent the means and 95% confidence intervals of at least three experiments run in triplicate.

Promatrilysin expression increases linearly with the degree of cell-cell contact in the co-culture, but not in the SCC-25 cells alone.

To determine if the induction of promatrilysin expression in the co-culture could be due to the increased degree of cell-cell contact , $3x10^5$ cells of each line were plated alone or in a co-culture in 1mL of medium in culture plates of various sizes. After seeding, the full medium was removed and cells were incubated in 1mL SFM. Under these conditions, the only variable was cell density, as the number of cells and the amount of medium were kept constant. Each culture was incubated for 24 hours after which CM was collected and analyzed by ELISA. The results (Figure 9) indicate that promatrilysin expression in the co-culture increases linearly as cell density increases whereas promatrilysin expression in the SCC-25 cells alone does not increase significantly as cell density increases. This implicates cell-cell contact in the induction of promatrilysin protein.

Western analysis reveals that only N-cadherins are expressed in both the HFF and SCC-25 cells.

Given the importance of Ca^{2+} to the function of cadherins, we determined which cadherins are expressed in the SCC-25 and HFF cells utilizing a pan-cadherin antibody that detects all classical cadherins (62). The results (Figure 10) indicate that N-cadherin is expressed in the SCC-25 cells, the HFF cells and in a co-culture of the two cell types. E-cadherin is expressed only in the SCC-25 cell line and in the co-culture of the two cell

lines. This pattern of E and N cadherin subtype expression was confirmed by northern analysis, performed as described previously (62) (data not shown). This suggests that both N and E cadherins could be potential mediators of promatrilysin expression in the co-culture of the SCC-25 and HFF cells.

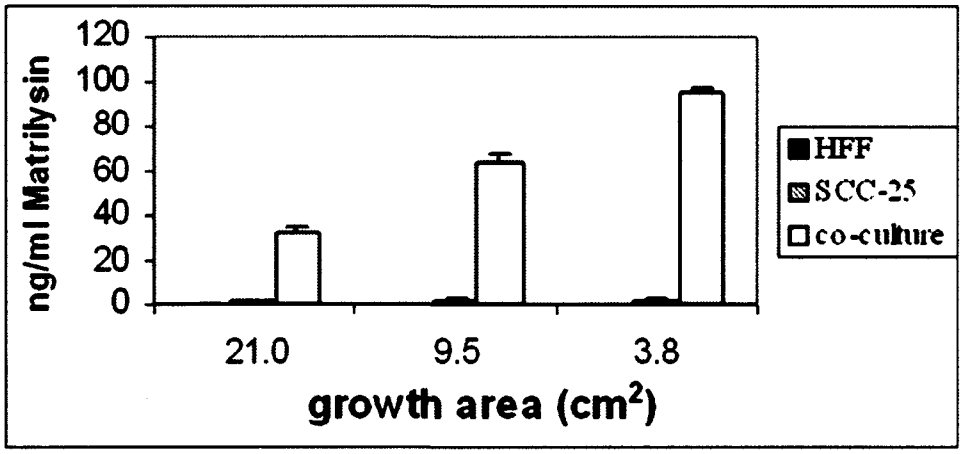

Figure 9. Cell density affects promatrilysin expression in the co-culture but not in the SCC-25 cells alone.

A representative ELISA of promatrilysin protein in medium is shown. The same number of cells ($3x10^5$) were plated in the same amount of media (1 ml) in tissue culture plates of varying sizes (growth areas of 9.5, 3.8, and 1.9 cm^2) and allowed to seed overnight, causing varying degrees of cell density. Cells were then incubated in 1 ml serum free medium for 24 hours, after which conditioned media samples were collected. As cell density increased in the co-culture, promatrilysin expression increased linearly. However, as cell density increased in the SCC-25cells alone, there was no significant increase in promatrilysin expression. Results represent the means and 95% confidence intervals representative of at least three experiments run in triplicate.

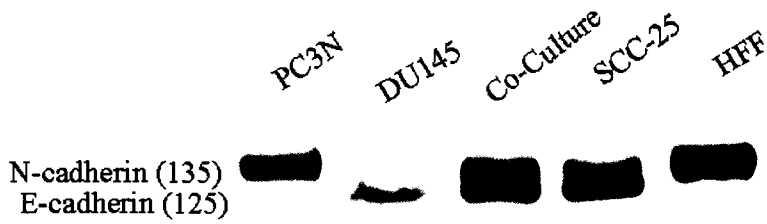

Figure 10. Cadherin expression in SCC-25 and HFF cells.

A representative Western of cadherin expression in SCC-25 and HFF cells is shown. Cells were lysed with 2XSDS solution and assayed for total protein. 30μg protein were run per lane and a pan cadherin primary antibody was used. PC3N, a prostate cell lysate known to express N cadherin and DU145, a prostate cell lysate known to express E cadherin (62) were run as controls. The HFF cells express N cadherin, the SCC-25 cells express N cadherin and E cadherin. The co-culture of the two cell types shows the expression of N and E cadherins.

N-cadherins, E-cadherins, and β1 integrins together are responsible for the induction of promatrilysin expression in the co-culture of SCC-25 and HFF cells.

In order to determine if N and E cadherins and β1 integrins were cooperatively involved in the induction mechanism, HFF and SCC-25 co-cultures were incubated in the presence of antibodies known to block either cadherin or integrin function. Neutralizing A-CAM antibody was used to block N-cadherin function in the cells, anti-E cadherin antibody was used to block E-cadherin function and β1 integrin blocking antibody was used to block β1 integrin function. HFF and SCC-25 cells were seeded in the presence of A-CAM, anti-E cadherin, and anti-β1 integrin antibodies at a 1:50 dilution. The antibodies had no effect on promatrilysin expression in SCC-25 cells alone (data not shown). A new antibody addition was made after 3 hours of incubation and again after a total of 6 hours incubation time to compensate for endocytosed antibody. In the presence of cell adhesion molecule antibodies, cell adhesion decreased by about 40% at 1 hour and eventually almost all of the cells attached (data not shown). CM samples were collected 3 hours after the last antibody addition (9 hours after plating) and were analyzed for promatrilysin protein by ELISA. Promatrilysin expression was decreased by 40% in the presence of the A-CAM antibody alone, 20% by the anti-E-cadherin alone, 30% by the anti-β1 integrin antibody alone, 45% with the A-CAM and E cadherin antibodies, 60% with anti-E-cadherin and anti-β1 integrin antibodies, 55% with A-CAM and anti-β1 integrin antibodies, and almost to basal levels with the anti N and E cadherin and the anti β1 integrin antibodies at a 1:50 dilution (Figure 11). The decreased promatrilysin expression seen in the presence of the antibodies is not due to the increased time

necessary for adhesion, as a co-culture plated after the antibody treated cells had adhered showed similar levels of promatrilysin expression as the original co-culture (data not shown). This indicates that N-cadherins, E-cadherins, and β1 integrins are involved in the induction of promatrilysin expression in the co-culture.

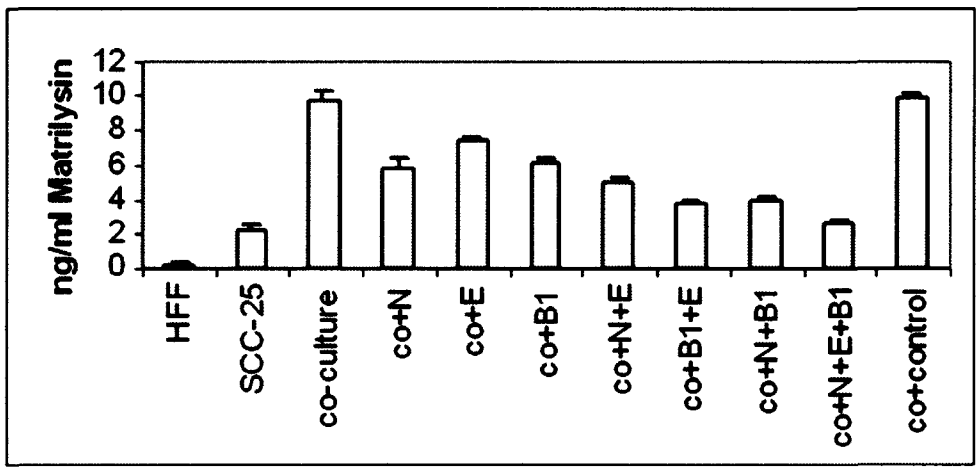

Figure 11. Cadherins and integrins together regulate promatrilysin expression in the co-culture.

A representative ELISA of secreted promatrilysin protein is shown. Cells were seeded in the presence of a 1:50 dilution of anti N-cadherin (84μg/ml) and E-cadherin (20μg/ml) and anti B-1 integrin (20μg/ml) antibodies. The control antibody was added at a concentration of 124μg/ml to account for the total concentration of antibody in the group with all three antibodies added. Cells were incubated for a total of 9 hours. In the presence of all three blocking antibodies, promatrilysin expression was inhibited to basal levels. Results represent the means and 95% confidence intervals of at least three experiments run in triplicate.

Discussion

It has been shown that MMPs including matrilysin can be regulated at the transcriptional level by growth factors and cytokines (201). However, our experiments led us to the hypothesis that cell-cell or cell-matrix interactions play a major role in the induction of promatrilysin in the co-culture of SCC-25 and HFF cells.

We have shown that calcium dependent cellular interactions are necessary for the induction of promatrilysin expression in our experiments involving EGTA and calcium free medium. In the absence of properly functioning cadherins, promatrilysin expression was significantly reduced. There was a complete inhibition of promatrilysin expression in the EGTA treated co-culture, compared to the non-treated co-culture. When grown in calcium free medium, promatrilysin in the co-culture was reduced. The calcium chelator and calcium free medium experiments which inhibited promatrilysin expression led us to look at cell-cell interactions in the induction of promatrilysin. The cell density experiment in which promatrilysin expression increased with increased amounts of cell-cell contact implicated a cell-cell contact mechanism of promatrilysin expression, and the anti-cadherin antibody experiments demonstrate that cadherins are involved in promatrilysin induction.

We removed attached cells from a dish, leaving the cell matrix to determine if cell-matrix interactions played a role in promatrilysin induction. With this technique we found no promatrilysin induction. However, this technique does not take into account active forms of cell matrix proteins, such as fibronectin, which require the cells that secrete matrix to be present for activation (232). There could be an interaction between

an integrin (such as β1 integrin) and active fibronectin in our system that is not seen when the HFF cells are removed from their matrix. For this reason, we continued our investigation into the possible role of integrins in the expression of promatrilysin by using a peptide that inhibits cell binding via the β1 integrin. Using the peptide, we found a significant reduction in promatrilysin expression. These findings agree with the experiment where we used an anti-β1-integrin antibody and were able to partially block promatrilysin expression.

It is not surprising that our results show that both cadherins and integrins are involved in the promatrilysin induction. There have been numerous proposals of cross-talk between integrins and cadherins, and cross-talk between these two cell adhesion molecules may explain the necessity of both these molecules in the induction of promatrilysin. Recently αv integrins and E cadherins were shown to cross-talk by down-regulating each other (233). One of the proposed mechanisms of this cross-talk is through β-catenin.

β-catenin signaling is known to be activated by cadherin interactions. Others have shown matrilysin expression to be dependent on the β-catenin pathway of activation in colon tumor cells (234, 235), and similar activation of matrilysin expression could occur in the SCC-25 cells. This would explain why blocking both N and E cadherins have an effect on promatrilysin expression. Also if β-catenin is indeed the regulator of integrin-cadherin crosstalk, this would explain the involvement of both cadherins and integrins in promatrilysin expression in the co-culture. Blocking β1 integrins as well as N and E cadherins may block the translocation of β-catenin to the nucleus thereby

blocking matrilysin expression.

Another possible explanation for the co-culture effect would be that the cell-cell interaction via cadherins could induce the HFF cells to secrete a growth factor that would, in turn, activate the expression of promatrilysin in the SCC-25 cells. Recently it has been hypothesized that after heterotypic cell contact a soluble factor is secreted into the intercellular space in a co-culture of mast cells and fibroblasts (236). Potentially, this could explain the significance of the cell contact in our co-culture system and could be the cause for promatrilysin induction. In our system, no induction was seen in the absence of cell-cell contact or using conditioned medium from the co-culture. Also, in experiments where a co-culture was incubated in a well with HFF or SCC-25 cells in an insert, promatrilysin expression was not increased above normal co-culture expression from the cells seeded in the well (data not shown). Therefore, if a paracrine factor is involved, it must be a result of cell-cell contact and must be released into the intercellular space, explaining the lack of induction in the co-culture conditioned medium experiments. However, preliminary experiments on this subject show that neither IL-6 nor IL-1β, cytokines known to induce the expression of promatrilysin expression in LnCaP prostate cells (201), significantly induce matrilysin expression in SCC-25 or HFF cells (data not shown).

A final explanation for the involvement of N and E cadherins and β1 integrins is heterotypic interactions between the cadherins and the integrins. The E-cadherins on the SCC-25 cells could form heterotypic interactions with the N-cadherins, which are predominately expressed on the HFF cells, inducing cadherin signaling. The β1 integrins

may form heterotypic interactions with the cadherins, though this has not been previously described for β1 integrins and N or E cadherins. However, heterophilic interactions have been described for αEβ7 integrin with E-cadherin (237-240) and E-cadherin is described as an alternative receptor for αEβ7 integrin (239). More likely, the integrins are involved in cross talk with the cadherins, possibly signaled through β-catenin as described by von Schlippe et al.(233), or an active fibronectin matrix interaction induces a similar signaling pathway as the cadherins.

It is interesting that cadherins and integrins play such a significant role in matrilysin expression in these cells. Cadherins mediate cell-cell interactions and integrins mediate cell-matrix interactions, and theoretically, these cell-cell and cell-matrix interactions would cause a tumor cell to remain immobile. However, we have previously proposed that different MMPs are expressed at discreet stages in tumor progression, and that the alterations in MMP expression are due to alterations in tumor cell-cell contact (221).

This information will be important in future experiments. Since the squamous cell carcinomas are such invasive tumors, and since matrilysin is one of the mechanisms of this invasiveness, knowing the cause of matrilysin expression and therefore, the cause of metastasis is significant. Further studies can be done to determine the precise signaling pathway activated upon cell-cell and cell-matrix contact that is responsible for matrilysin induction in this system.

IV. 90K INDUCES PROMATRILYSIN AND IS EXPRESSED IN PROSTATE CANCER

Introduction

Prostate cancer is currently the most commonly diagnosed neoplasm in men in the United States, having surpassed lung cancer incidence in 1990, and is the second leading cause of cancer deaths after lung cancer (2). The high mortality rate for prostate cancer can be attributed to the fact that prostate cancer is often diagnosed at an advanced stage as there are currently not many prognostic or diagnostic indicators for the disease. Prostate cancer will continue to be a national health problem as the population ages, as prostate cancer incidence is strongly associated with increasing age (241). Also, there is a wide variation in individual outcomes for prostate cancer due to the fact that some prostate cancers are much more aggressive and likely to cause metastatic disease than others. Unfortunately, there are currently no standard methods, which could be applied to biopsy material, for determining which prostate cancers are more likely to metastasize. Therefore, research into prevention, early diagnosis, and treatment are needed. Research to identify better prognostic indicators can be used to determine whether a man has aggressive prostate cancer that needs to be treated more aggressively.

In light of this, the identification of tumor markers for prostate cancer progression is of utmost importance. Some possible biological markers have been reported and are in use to detect possible evidence of disease, such as prostate specific antigen (PSA) (242), alkaline phosphatase, and serum chromogranin A, but none of these are without problems in the evaluation of prostate cancer. A major problem with these biomarkers is a high number of false positives (243).

90K (aka Mac-2 Binding protein, galectin-3 binding protein) was originally identified as a tumor-associated antigen in breast cancer. It is a large oligomeric glycoprotein composed of 90 kDa subunits and is a ligand for galectin-3 (formerly Mac-2), a carbohydrate-binding protein (179-181, 191) that is present in the ECM of several tissues (189) and in extracellular fluids such as serum and milk (180). 90K expression is present in the ug/ml range in serum and other biological fluids from different cancer patients (179, 180). Serum levels ≥ 11 ug/ml were associated with poor survival and metastatic spread to the liver in patients with operable breast cancer (194). Also, it is expressed in about 30% of stage I non-small cell lung cancers and its expression was associated with a significantly worse outcome (195). Therefore, 90K is considered a tumor marker for these cancers.

The functions of 90K are not well-defined yet; however, it has been suggested to be involved in the host response to tumors and to infection. This finding is consistent with the fact that 90K induces the expression of various cytokines, including IL-1, IL-2, and IL-6 (181, 182). It is a member of a protein superfamily defined by a special motif characteristic of the scavenger receptor cysteine- rich (SRCR) domain (181), a domain implicated in the immune response of higher organisms (244). 90K promotes homotypic cell-cell contacts and cell adhesion through its ability to bind collagens IV, V and VI, fibronectin, and nidogen in the ECM (180, 187-189) and through its ability to bind β1 integrins (189).

Matrix metalloproteases (MMPs) are a family of zinc-dependent enzymes that degrade components of the extracellular matrix (ECM). Cytokines and growth factors

are known to regulate the expression of MMPs. Through degradation of the ECM, MMPs also facilitate the invasion of primary tumors and the metastasis of tumor cells to distant sites, such as lymph nodes and bone (28-30). One particular MMP, matrilysin (MMP-7), has been demonstrated to degrade many ECM proteins including proteoglycans, entactin, laminin, gelatin, and elastin. We have previously shown that matrilysin expression can be induced by a variety of factors, including cellular signals (245) and cytokines (201, 246). Of interest in relation to 90K, we have shown that matrilysin expression can be induced in the prostate cancer cell line, LNCaP, via IL-1β induced IL-6 expression and signaling through STAT-3 (246). Since 90K is known to induce expression of IL-6, we chose to look at 90K induced expression of promatrilysin in LNCaP cells. We found that 90K does induce promatrilysin in LNCaP cells and we suggest that this is a novel mechanism of 90K-associated poor prognosis in various cancers.

While 90K expression studies have been performed on various other cancers, studies on its expression in prostate cancer have been lacking. In this study, we examined by immunohistochemistry the expression pattern of 90K in 299 patients who underwent biopsy to detect prostate cancer in order to assess the prognostic value of 90K for prostate cancer. We found that while expression of 90K was mostly lost in PIN (prostatic intraepithelial neoplasia) the protein was re-expressed in about 40% of the tumors analyzed. Due to the lack of prognostic data from the tissues examined, it is impossible to determine at this time whether the tumors observed to express 90K were more invasive than those that did not. However, we demonstrated that 90K can induce

promatrilysin expression in prostate cancer cells and therefore we suggest that 90K may be a relevant tumor marker for prostate cancer progression.

Results

Expression of 90K in prostate cells

We examined the expression pattern of 90K in a series of prostate cancer cell lines by Western blot analysis. Lysates extracted from each line demonstrated (Figure 12) that PC3 cells had the highest amount of 90K expression; PC3N, LNCaP, JCA-1 and TSU-Pr1 also had detectable levels of 90K , although in LNCaP cells, in addition to a potentially differentially glycosylated 90 kDa band, 67 kDa and 32 kDa species were also observed, which may correspond to 67 kDa and 27 kDa cleavage products of 90K previously identified (180). Although protein was detected in JCA-1 cells, levels were less than those seen in other positive cell lines. Among the cell lines screened, only PPC-1 and DU-145 were negative for 90K expression. The same results were seen with immunohistochemistry of cell lines grown on coverslips (data not shown).

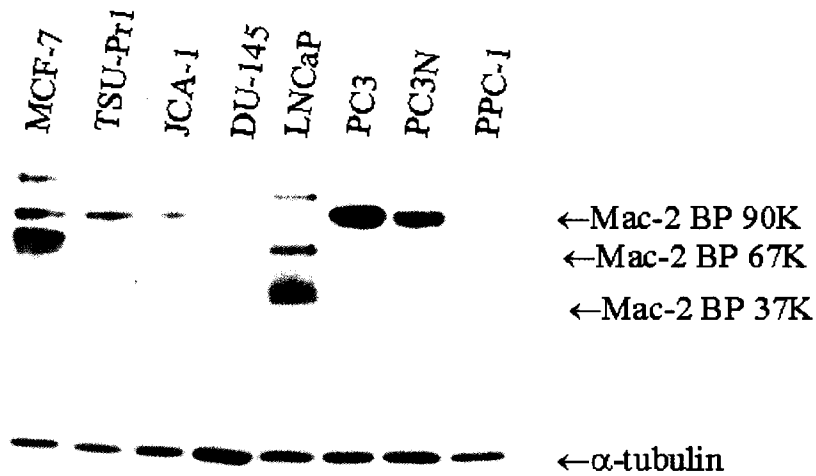

Figure 12. 90K expression in prostate cancer cell lines.

Western blot of whole cell lysate of prostate cancer cell line indicated. MCF-7 breast cancer cell lines were used as a positive control for 90K expression. Full-length 90K is seen at 90 kDa and as cleaved 90K at 67 kDa and 32 kDa. Alpha-tubulin expression is also shown for all cell lines as a loading control. Results are representative of three experiments.

We next examined the expression pattern of 90K message in each of the prostate cell lines by Quantitative Real-Time PCR. Fold-expression over GAPDH (Figure 13) reveals that MCF-7, TSU-Pr1, JCA-1, LNCaP, PC3, and PC3N cells express detectable levels of 90K message while DU-145 and PPC-1 cells have significantly less. These data correlate with the protein expression data in the cell lines.

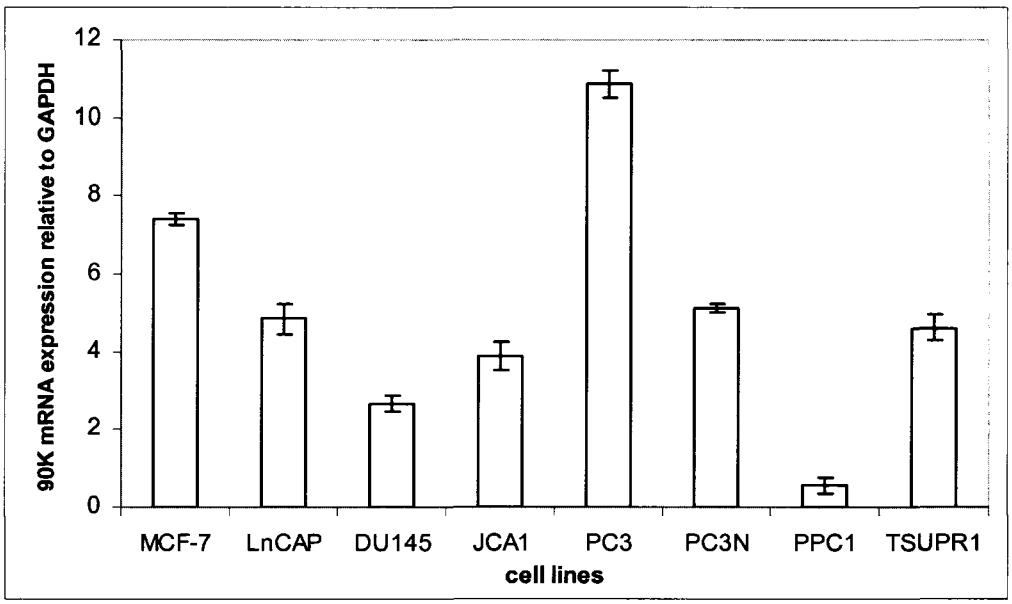

Figure 13. 90K message levels in prostate cancer cell lines.

mRNA isolated from prostate cancer cells was analyzed for 90K message expression by real time RT-PCR. Expression levels are representative as fold over GAPDH levels for all cell lines studied.

Expression of 90K in prostate tissue

To examine 90K distribution in human prostate tissue, we performed immunohistochemical analysis on a prostate tissue microarray obtained from the CPCTR (Figure 14). We examined expression in a total of 884 prostate biopsies, from a total of 300 patients on a prostate tissue array. Of these patients, 286 had evidence of prostate cancer and 119 of these cancerous biopsies did not exhibit any normal glands. Normal ducts only were observed in 7 patients. In normal glandular tissue, 31/174 patients demonstrated positive staining for 90K, whereas only 2/14 PIN lesions were positive. Interestingly, in prostate cancer, 111/286 patients demonstrated positive staining (Table 2). Since PIN is considered a precursor for prostate cancer and most PIN lesions were negative, the high percentage of cancers exhibiting 90K staining suggest that 90K is re-expressed in some prostate cancers. Staining occurred mainly in epithelial cells with some evidence of positive staining of intra luminal secretions (Figure 15). Staining of neoplastic glands did not correlate with Gleason grade although the staining pattern suggests that patients with a higher Gleason sum score (greater than 7) are more likely to exhibit 90K expression (Table 3). Atrophic glandular cells in all specimens stained negatively. Staining patterns were heterogeneous, with different ducts or glands demonstrating cytoplasmic staining, nuclear staining, focal staining, or secretion staining (Figure 15). The majority of cancers positive for 90K demonstrated nuclear staining with a greater intensity than cytoplasmic staining (54/111) with an average of 58.5% of nuclei exhibiting expression in each gland (Tables 4-5). The majority of normal glands positive for 90K expression demonstrated nuclear staining, though only 33.9% of nuclei in each

normal gland were positive. The exact function of the nuclear localization of 90K is unknown, but has been described previously in tissue from patients with lung cancer (195).

Figure 14. Prostate tissue array.

Overview of prostate tumor tissue array after staining with Mac-2 BP antibody to demonstrate the layout of tissue samples on the microscope slide.

	Positive Cases/ Total Cases	(%)
Normal	31/174	17.8%
PIN	2/14	14.3%
Cancer	111/286	38.4%

Table 2. Mac-2 BP staining in prostate tissue

Gleason Sum Score	Positive Cases/Total Cases	% Positive
≥ 7	84/197	42.6%
< 7	27/89	30.3 %

Table 3. 90K expression in carcinoma vs. Gleason Sum score.

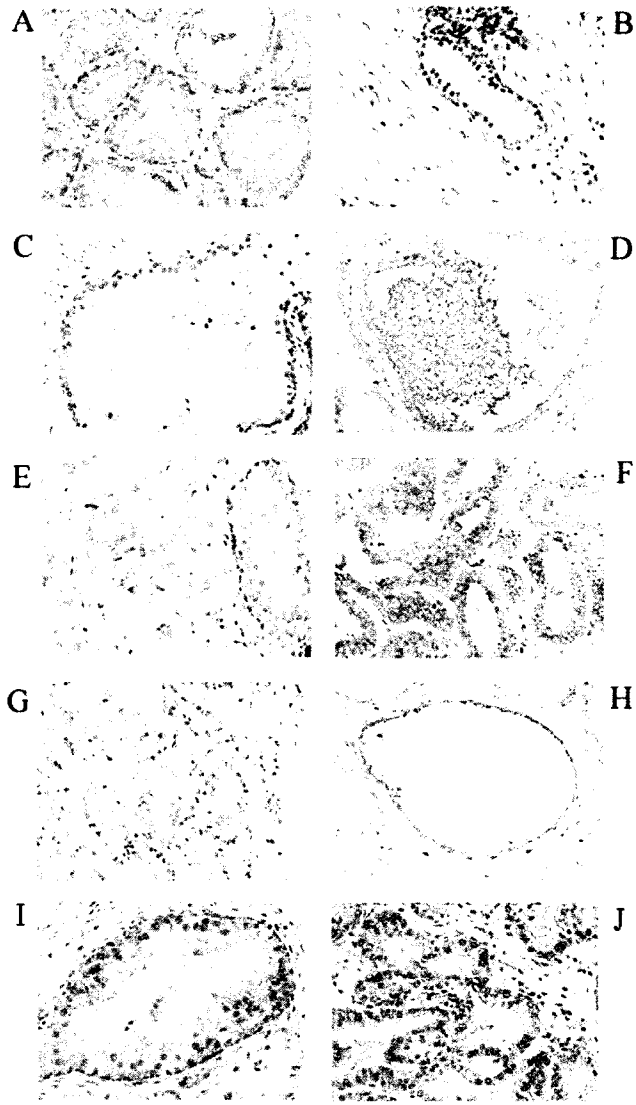

Figure 15. 90K expression in human prostate tissue.

Samples were subjected to immunohistochemistry using a monoclonal antibody to 90K. Pictures were taken at a magnification of x20. Panels are: A) normal glands positive (3+), B) a normal duct that is weakly positive (1+), C) a normal duct that is negative, D) a normal duct containing positive secretions, E) a PIN lesion (P) that is negative and a cancer (C) with weak staining (1+), F) a cancer that is positive (3+), G) a cancer that is negative, H) an atrophic duct that is negative, I) a normal gland with nuclear staining, and J) cancer with nuclear staining.

	Normal	%	PIN	%	Cancer	%
Cytoplasm	3	9.60%	2	100%	37	33.3%
Nucleus	27	87.1%	0	0%	54	48.6%
Equal intensity	1	3.23%	0	0%	20	18.0%

Table 4. Location of Highest Intensity Staining for 90K.

	Average % of nuclei positive per gland
Normal	33.9 %
PIN	0 %
Cancer	58.5 %

Table 5. Average percent of gland exhibiting nuclear staining of 90K.

Induction of Promatrilysin by 90K

To test whether 90K could induce promatrilysin expression in a prostate cancer cell line, we chose to use a cell line previously demonstrated to be capable of expressing IL-6 when induced with IL-1β, which is the LNCaP cell line (246). Recombinant 90K was added to serum-starved LNCaP cells at varying concentrations, and induction of promatrilysin was seen at concentrations ranging from 0.5 to 5 ng/ml (Figure 16A). For time course assays, we chose 1ng/ml and treated the cells for 24-72 hours. Some induction of promatrilysin is seen at 24 hrs, but strong induction is seen at 48 hrs, with a slight decrease in expression at 72 hrs (Figure 16B). To verify that this induction is a result of 90K treatment, we used blocking antibodies to 90K (SP-2, IH9 and 4D1) and treated LNCaP cells with both 90K and the blocking antibodies for 48hr. Results (Figure 16C) demonstrate that blocking 90K with the blocking antibodies reduces promatrilysin expression. It is not known whether these blocking antibodies block the function of 90K or if they simply immuno-deplete 90K in the medium. Either of these mechanisms would be effective in blocking 90Ks effects on the LNCaP cells. Also, to verify that induction is through IL-6 signaling, we used an IL-6 neutralizing antibody. Cells were pretreated for 1 hour with neutralizing antibody, after which cells were also treated with 90K. Results show (Figure 16C) that neutralizing IL-6 also reduces promatrilysin expression in the presence of recombinant 90K.

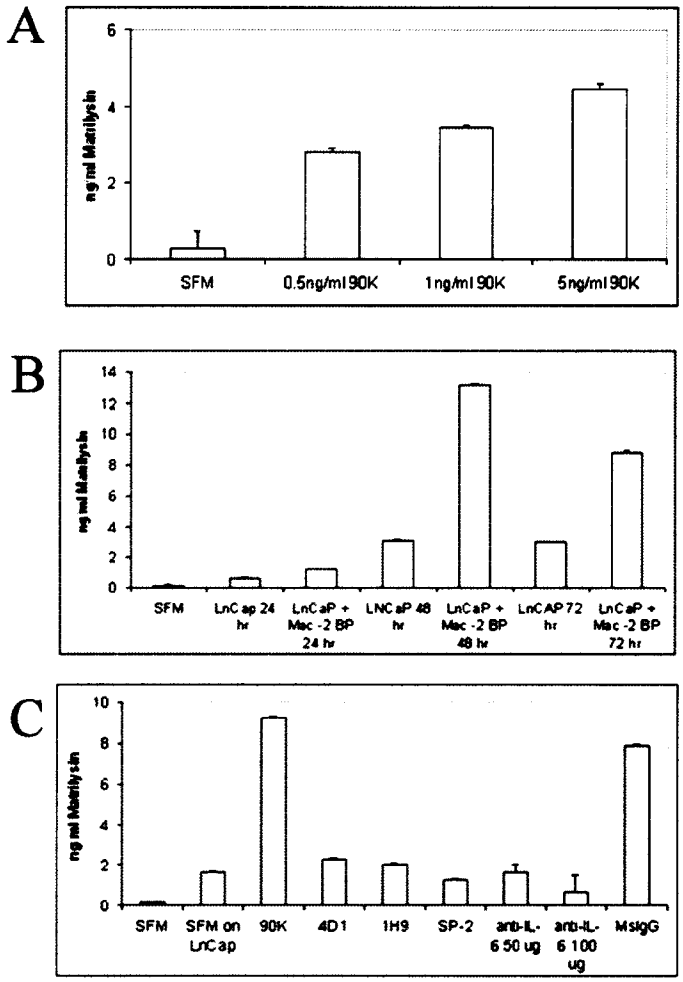

Figure 16. 90K induced expression of promatrilysin.

A. ELISA for promatrilysin in LNCaP cells with 90K treatment at indicated concentration for 24 hrs. B. ELISA for pro-MMP-7 in LNCaP cells over 72 hrs of treatment with recombinant 90K. C. Expression of promatrilysin with 90K treatment in the presence of blocking antibodies to 90K (4D1, 1H9, SP-2) or an IL-6 neutralizing antibody (IL-6). Mouse IgG (MsIgG) was used as a control. Results are representative of three experiments performed in triplicate. Error bars represent standard deviation of representative experiment.

Discussion

These observations for 90K expression in prostate cancer are consistent with previous reports demonstrating positive staining in malignant cells of breast, lung, ovarian, melanoma, hepatocellular carcinoma and lymphoma (181, 194, 195, 247-250). These reports indicate that 90K expression may have a biologic role in tumor progression and may be useful prognostic indicators in these neoplasms. In lung cancer, expression of 90K was significantly associated with the establishment of distant metastases, with no correlation between 90K expression and tumor size, type, or grade (195). This was also true for node-positive breast cancer patients (179). Here, we have shown that 90K is expressed in 40% of prostate cancers. This suggests that 90K expression may be indicative of aggressiveness in prostate cancer as well, since in our study, the patients with the highest 90K expression were the patients who exhibited prostate cancer progression. Interestingly, we demonstrated 90K expression in a small fraction of normal prostate glands, which is contradictory to expression in normal tissues in other published reports (179). However, these data correlate with a separate report which studied 90K expression in normal tissues and found high levels of 90K message in these normal tissues, which included tissues from breast, lung, colon, ovary, small intestine, stomach, spleen, and bladder (181).

How 90K expression intervenes in tumor progression and metastasis remains to be elucidated. Normally, 90K has stimulatory activity on natural killer cells and lymphokine-activated killer cells (181), mediated by 90K induction of cytokine expression (181, 182). For this reason, 90K is thought to be associated with tumor

suppression, yet its expression profile in various cancers seems to contradict this theory. Possibly, the reason for this discrepancy is a result of cytokine induction by 90K. 90K upregulates IL-1, IL-2, and IL-6, which in turn, can upregulate other proteins associated with tumor cell invasion, such as matrix metalloproteases. In fact, IL-1 induces MMPs-1, -2, -3, -7, and –9 (201, 251), IL-2 induces MMP-2 and MMP-9 (252), and IL-6 induces MMPs-1, -2, -7, and –9 (253-255) in prostate cancer cells. Matrilysin expression by IL-1β occurs through NFκB mediated induction of IL-6 and IL-6 signaling through STAT-3 to induce promatrilysin (246). Here, we demonstrate for the first time that 90K is capable of inducing promatrilysin expression in the prostate cell line LNCaP. We suggest that the mechanism behind the induction of promatrilysin by 90K is through 90K-induced expression of IL-6 and IL-6 induces promatrilysin expression as previously described in LNCaP cells (246). This has important implications for cancer progression, as matrilysin is known to be involved in tumor invasion and metastasis (112, 120, 123, 124). In addition to this direct mechanism of promatrilysin induction, infiltrating inflammatory cells may be induced to express cytokines such as IL-1β or IL-6 by 90K (181, 189) and these cytokines may then upregulate promatrilysin in prostate cancer. This mechanism explains the dramatic expression of matrilysin in inflamed ducts in the prostate as described previously (120).

It is important to note that in most instances, 90K expression in other cancers was determined by circulating levels of 90K in the serum. We chose to examine 90K expression using immunohistochemistry rather than serum levels of 90K in order to verify the localization of 90K at the prostate. Localized expression of 90K would be the

most significant for initial effects on prostate cancer invasion, especially considering its MMP-7 inductive properties. However, we feel that examination of 90K expression in the serum of patients with prostate cancer and correlation with expression in the prostate is still an important study that should be done. Systemic 90K could be important for later metastatic steps making both localized 90K and systemic 90K potentially useful indicators of prognosis.

90K may also be involved in cell survival during metastasis through its cell-adhesive capabilities. 90K mediates adhesion of cells into homotypic aggregates more likely to survive the metastatic process than a single cell through interactions with galectin-1 and galectin-3 (195). Other considerations for the role of 90K in cancer metastasis include the upregulation of I-CAM-1 and V-CAM-1, which may allow tumor cells to adhere to the vasculature. Finally, 90K has been found deposited in the ECM, interacting with collagens and fibronectin, mediating cell adhesion through β1 integrins (189, 190). Through these properties, it has been proposed that cancer cells expressing high levels of 90K release the protein locally at high concentrations, creating a microenvironment that favors their survival in the bloodstream (through cell aggregation), their adhesion to endothelia (through I-CAM-1 and V-CAM-1) and to ECM, allowing for the establishment of new tumor colonies (195).

In this study, we report that 90K is expressed by a wide variety of prostate cancer cell lines and that its expression is detected in approximately 40% of human prostate cancers. Although patient outcome data on the tissues examined for 90K expression are not comprehensive at this time, these results demonstrate the importance of studying its

expression profile in prostate cancer in more detail. We cannot conclude that 90K expression is associated with prostate cancer progression, but its ability to induce promatrilysin expression suggests that 90K expression may be a useful tumor marker for prostate cancer. It will be important to further elucidate the role of 90K in prostate cancer progression to determine whether 90K will be useful as an early prognostic indicator of aggressive disease.

V. MEMBRANE-TYPE-1 MATRIX METALLOPROTEASE (MT1-MMP) CLEAVES LAMININ-10 AND PROMOTES PROSTATE CANCER CELL INVASION

Introduction

Remodeling of the extracellular matrix (ECM) through proteolysis of ECM proteins is an important step in the metastatic progression of cancer, allowing for invasion of neoplastic cells through the basal lamina (BL) and into the stroma (256). Proteolysis creates paths for migration, releases signaling molecules such as growth factors bound in the ECM, and generates biologically active ECM fragments (257-261). Matrix metalloproteases are a family of zinc-dependent enzymes that degrade components of the ECM and have been implicated in the pathological remodeling of ECM in tumor invasion and metastasis (262-264). There are currently twenty-six members of the MMP family, most of which are secreted as inactive zymogens that must be activated extracellularly through proteolytic removal of a pro-domain. The membrane-type matrix metalloproteases, of which there are currently six members, are not secreted MMPs. Rather, they contain a transmembrane domain that anchors them into the cell membrane and they are activated intracellularly through removal of their pro-domain by furin-type convertases. MT1-MMP, a member of the transmembrane metalloproteases, was first described as a 66 kDa activator of pro-MMP-2 (Gelatinase A) (133, 265) but has also been found to possess proteolytic activity for ECM proteins including gelatin, fibronectin, K-elastin, vitronectin, collagens, and laminin-5 (65, 83, 84, 133, 134, 266, 267). MT1-MMP is

expressed in a wide variety of human tissues under both normal and pathological conditions, though its expression is enhanced in tumor tissues (268). MT1-MMP has been demonstrated to be upregulated in the progression of prostate cancer and its expression is correlated with an increase in the invasiveness of tumor cells (265, 269-272), indicating that it may have a role in the invasion and metastasis of this cancer.

Laminins are extracellular matrix glycoprotein components of all BLs. They play essential roles in tissues such as providing the major structure of BLs (65, 66), attaching cells to the extracellular matrix via interactions with cell surface components (273), and interacting with cellular receptors such as integrins to induce intracellular signaling (274, 275). Each laminin consists of three distinct chains (α,β,γ) arranged in a cruciform structure. So far, five α, three β, and three γ chains have been identified, giving rise to 15 different heterotrimers (80, 276). All laminin chains share structural similarity, each consisting of small globular domains, EGF-like repeats, and an α helical coiled-coil in the long arm (277). Laminins are important for normal development and laminin deficiencies are known to cause severe developmental defects. For example, laminin α5 chain knockout mice are not viable, demonstrating multiple morphological abnormalities in several tissues (91). Ln-5 mutations lead to a neonatal lethal skin blistering disease, Herlitz's variant junctional epidermolysis bullosa (JEB), characterized by disruption of the dermal-epidermal junction (278).

Being the major components of BL, laminins are structural barriers that separate connective tissue from epithelium and must be penetrated by tumor cells during invasion and metastasis. This occurs via proteolytic degradation of the BLs and mobilization of

tumor cells through the degraded BL. Some laminins have been previously shown to be proteolytically processed (68-70), which may aid in this mobilization. The α1 chain of Ln-1 has been shown to be cleaved by elastase in its globular domain, and by pepsin in its N-terminal domain *in vitro* (279, 280). Proteolytic cleavage of Ln α2, Ln α3, and Ln α4 has been described in the C-terminal globular domain between the G3 and G4 domains as well. The enzyme(s) involved in these cleavages have not been identified, although plasminogen and bone morphogenic protein (BMP-1) (281) are involved in the cleavage of the α3 globular domains (282). This work led to structural and functional descriptions of these globular domains as being important for cell adhesion. However, functional consequences of the loss of the G4-G5 domains have not been described except for the α3 chain where cleavage was necessary for hemidesmosome formation (282). Human Ln-5 has been shown to be cleaved in its α3 chain (plasmin, BMP-1, mammalian Tolloid 1 and 2, MMP-2 and MT1-MMP), γ2 chain (mammalian Tolloid), and β3 chain (MT1-MMP) (82, 83, 281, 283). In addition, rat γ2 chain can be cleaved by MT1-MMP and MMP-2 (84) though this cleavage has not been directly observed in human Ln-5, probably due to species differences. For Ln-5, cleavage by MMPs was shown to promote an invasive phenotype in cells plated on processed laminin-5 (84, 85). It has now been shown that cleavage of rat γ2 chain by MT1-MMP promotes invasion by release of laminin fragments containing EGF-like domains that can bind to and activate the EGFR (81). This EGF-like fragment of rat γ2 has implications for motility regardless of species differences in cleavage, as all laminins contain EGF-like repeats.

We have previously shown that MT1-MMP can cleave the human Ln-5 β3 chain (83). Cleavage of the β3 chain resulted in increased migration of prostate cancer cells. This observation is of particular interest in prostate intraepithelial neoplasia (PIN), where MT1-MMP expression is increased as normal prostate epithelium progresses to PIN and then to cancer (83, 89, 149). However, while Ln-5 is expressed in normal prostate and focally in PIN, its expression is lost in prostate cancer (89). So, while MT1-MMP cleavage of Ln-5 can explain in part migration of neoplastic cells through the BL surrounding a PIN lesion, this cleavage is not involved in the escape of prostate carcinoma cells from carcinomatous glands surrounded by a BL and the metastatic invasion of prostate cancer. Prostate cancer is surrounded by a BL composed mainly of laminin-10, laminin-2, type IV collagen, and entactin (284). In order for prostate cancer to invade the stroma and intravasate into the vasculature, it must move through this Ln-10 rich BL either by proteolysis or ameboid movement (285). Ln-10 (α5β1γ1) and Ln-11 (α5β2γ1) have been previously identified as substrates for cell migration and cell adhesion (94). Ln-10 binds the α3β1, α6β1, α6β4 (96), and αvβ3 (286) integrins and is the most widely expressed laminin, being expressed in prostate, skin, placenta, kidney, pancreas, heart, and lung (88, 89, 94). Here we demonstrate, for the first time, that MT1-MMP can cleave purified human Ln-10 α5 chain and that this cleavage causes a decrease in adhesion to cleaved Ln-10 and an increase in prostate cell migration on and invasion through processed Ln-10. These data suggest that the MT1-MMP cleavage and induction of migration on Ln-10 and invasion through Ln-10 will be of importance not only to prostate cancer, but to other cancers where epithelial cells contact a Ln-10 rich BL and

will be of importance to other cancers regarding intravasation through blood vessels, which are known to express high levels of the α5 chain.

Results

Human MT1-MMP Cleaves Purified Human Laminin-10.

To investigate whether MT1-MMP could cleave Ln-10, 2 μg of Ln-10 were adsorbed onto a 96-well tissue culture plate and incubated with 2.1 nmol of recombinant MT1-MMP or APMA-activated MMP-2 for 16 hr. Samples were solubilized and electrophoresed on 6% SDS-PAGE under reducing conditions and the gel was silver-stained (Figure 17A). In all samples, the 350 kDa α5, 220 kDa β1 and 210 kDa γ1 chains were visualized. In the MT1-MMP-treated sample, we observed a decrease in the 350kDa α5 band with a concurrent appearance of a 310 kDa band, suggesting that MT1-MMP is capable of cleaving the α5 chain of Ln-10. We did not observe the 310 kDa band in MMP-2-treated Ln-10, indicating that this cleavage is specific to MT1-MMP. Minimal endogenous cleavage of Ln-10 to its 310 kDa form occurs before the purification process and can be seen as a faint band in the stained gels. This can be explained by the fact that the cells generating Ln-10, A549, express detectable levels of MT1-MMP (287). In order to fully characterize the cleavage products, we analyzed cleaved Ln-10 samples on a 4-10% gradient gel (Figure 17B) followed by Western blotting. In addition to the 310 kDa product we could detect cleavage products with apparent MWs of 190 kDa, 160 kDa, and 45 kDa.

In order to identify the cleaved fragments of Ln-10 we utilized a proteomics

approach. Cleaved Ln-10 was run on an SDS-PAGE gel and stained with Coomassie Blue. Each band that stained with Coomassie Blue was excised, digested with trypsin, and subjected to mass spectrometry. Protein bands detected by Western blotting (Figure 17B) with apparent MWs of 100 kDa, 80kDa, and faint bands between 80 kDa and 45 kDa were not visualized by Coomassie Blue staining (data not shown) and were therefore not analyzed by mass spectrometry. We found that the 310 kDa, 190 kDa, 160 kDa, and 45 kDa bands were all different cleavage products of the 350 kDa α5 chain. Trypsin digestion of the 310 kDa band gave 25 unique peptide sequences that were identical to the expected amino acid sequence of the α5 chain. The 45 kDa band gave 5 different peptide sequences consistent with the α5 chain and all 5 of these peptides clustered at the N-terminus (Figure 18A) suggesting that cleavage occurs near the N-terminal region to give rise to the 45 kDa product. Trypsin digestion of the 190 kDa and 160 kDa bands (Figure 18B) yielded 15 and 10 different peptide sequences, respectively, all of which are found in the α5 chain. Chymotrypsin digestion was also performed on the 310 kDa, 190 kDa, and 160 kDa protein bands, all of which were again identified as the α5 chain of Ln-10 (data not shown).

An inspection of the amino acid sequence of the α5 chain indicated 5 distinct, potential MT1-MMP consensus cleavage sites. MT1-MMP has previously been demonstrated to cleave at PXX↓L (ideally PXP↓L or PXG↓L) sites (288, 289). At P^{319} (290) in the α5 chain there is a PFR↓L consensus cleavage site that would give rise to cleavage products with theoretical MWs of 329 kDa and 31.4 kDa, which corresponds to the apparent MWs of the 310 kDa and 45 kDa cleavage products we observed,

accounting for post-translation modifications, such as glycosylation of the protein (Figure 18A). It is important to note, however, that cleaving at this consensus cleavage site will not give rise to a fragment containing the epitope recognized by the antibody 15H5. We therefore propose that cleavage also occurs at a second site to give rise to fragments of similar molecular weight as those that arise from the cleavage at the PFRL site. We propose that this site is at D^{440} in a CED↓L consensus cleavage site. This second cleavage site explains why the 15H5 antibody is able to detect both the 45 kDa fragment and the 310 kDa fragment because the antibody epitope is between these two consensus cleavage sites. Another consensus cleavage site is P^{1243}PG↓L and would give rise to a product with a predicted MW of 230 kDa, which corresponds to the apparent MW of the 190 kDa cleavage product. Finally, a fourth consensus cleavage site at P^{2000}SY↓L would yield a product with a predicted MW of 180 kDa, corresponding to the 160 kDa band we observed.

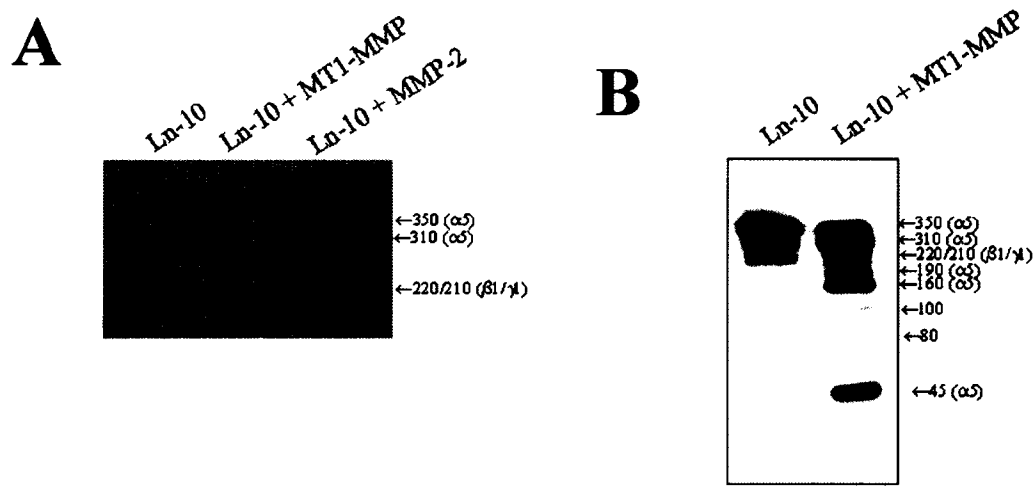

Figure 17. MT1-MMP cleavage of Ln-10 α5 chain.

A) Silver-stained gel of Ln-10 treated with MMPs. The figure shows Ln-10 treated with either MT1-MMP or APMA-activated MMP-2. MW of protein bands are indicated. B) Western blot of Ln-10 cleavage. MW and corresponding protein identification are indicated. Protein bands at 100 kDa and 80 kDa were not detected with methods other than Western blotting.

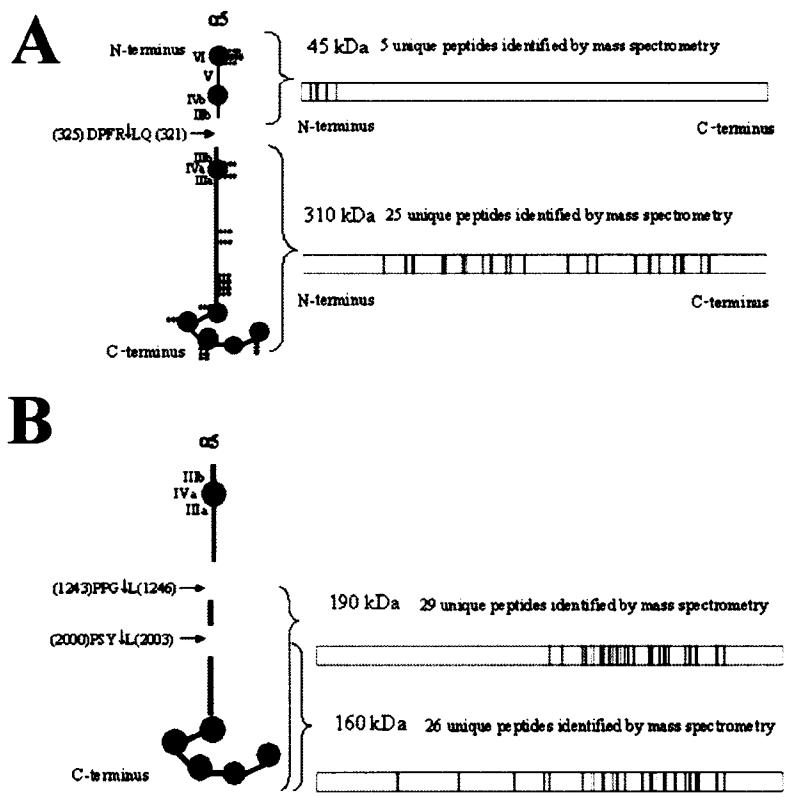

Figure 18. Identification of Ln-10 α5 chain cleavage fragments.

A) Identification of cleavage products by mass spectrometry as the α5 chain. Potential glycosylation sites are identified in gray. Structural representation of the α5 chain with potential consensus cleavage site to yield 310 kDa and 45 kDa products is indicated. The 25 peptides identified in the 310 kDa band and the 5 peptides identified in the 45 kDa band are represented by peptide coverage of full-length α5 chain. B) Structural representation of α5 chain consensus cleavage sites that could give rise to 190 kDa and 160 kDa products. Peptide coverage of the 25 and 29 peptides identified in the 190 kDa and 160 kDa bands, respectively, by mass spectrometry is represented.

MT1-MMP Functions at Physiologically Relevant Substrate: Enzyme Ratios.

We determined whether the cleavage of the α5 chain could occur at physiologically relevant substrate: enzyme ratios. For this experiment, 2 μg Ln-10 were adsorbed onto a 96-well tissue culture plate and incubated for 6 hr with decreasing concentrations of MT1-MMP (2.1-0 nmol). Samples were then separated by 4-10% gradient SDS-PAGE and Western blotted using the 15H5 antibody. The results (Figure 19) indicate that even with 0.05 mol MT1-MMP per mol Ln-10 there is an increase in the 310 kD and 45 kD cleaved products. The cleaved products continue to increase with increasing amounts of enzyme. Also, with increasing enzyme, the 190 kD band appears to decrease at 0.01 mol enzyme per mol substrate while the 160 kD band begins to increase at this same ratio of substrate: enzyme.

Decreased Adhesion of Prostate Cancer Cells to Cleaved Ln-10.

Since intact laminins are known to interact with adhesion molecules such as integrins, we examined whether cleaving Ln-10 with MT1-MMP would have an effect on adhesive interactions. Ln-10 was coated onto a 96-well plate and cleaved with 2.1 nmol MT1-MMP for 16 hr. Under these conditions, 100% of the Ln-10 is cleaved (Figure 19). ELISA for Ln-10 was performed using the 4C7 antibody to ensure that there was no significant difference in the amount of Ln-10 remaining coated on the plate with MT1-MMP treatment compared to untreated Ln-10 (data not shown). DU-145 cells, which express the adhesion receptors for Ln-10, i.e. α3β1, but do not make the ligand Ln-10, were then seeded on either cleaved or uncleaved Ln-10 for up to 1 hr. The number of

adherent cells was quantified by crystal violet absorbance. The results (Figure 20) indicate that there is a 20% decrease in adhesion of DU-145 cells to cleaved Ln-10. This indicates that cleavage of Ln-10 by MT1-MMP affects the laminin protein such that cell adhesion is no longer as effective.

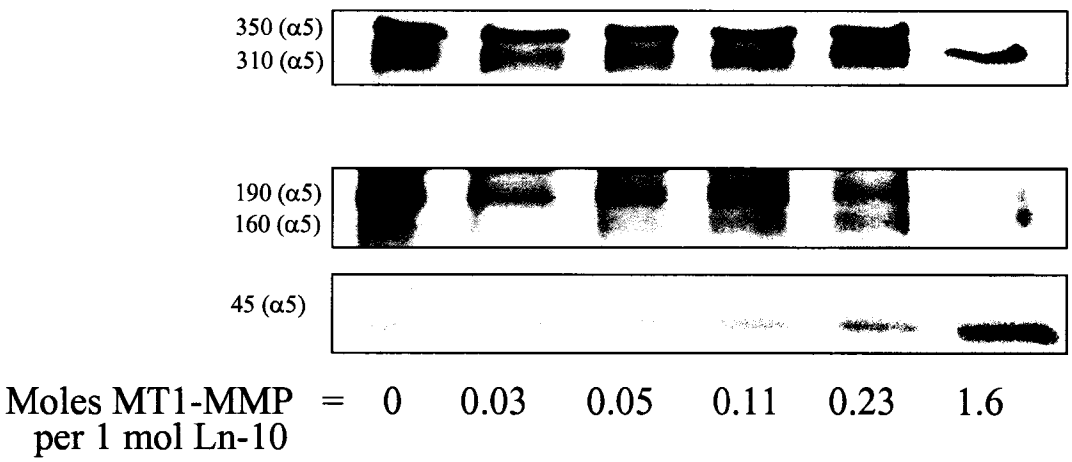

Figure 19. MT1-MMP cleavage of Ln-10 occurs at physiologically relevant substrate: enzyme ratios.

Western blot of purified Ln-10 treated with decreasing amounts of MT1-MMP. Indicated is the mol MT1-MMP per 1 mol of Ln-10 and MWs of each identified protein band. Results are representative of three experiments.

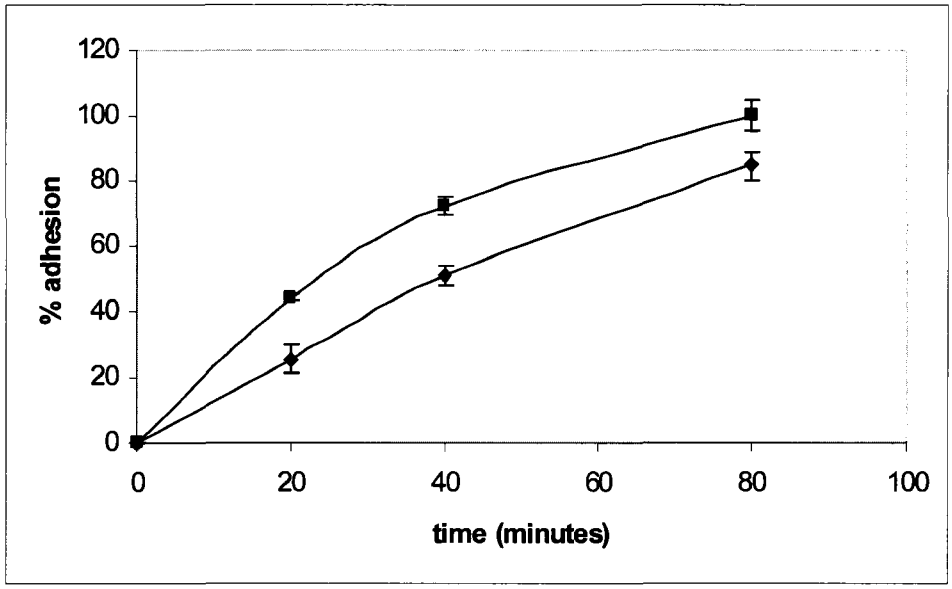

Figure 20. Cleavage of Ln-10 results in decreased adhesion of prostate cancer cells.

Adhesion assay of DU-145 cells to Ln-10 and Ln-10 treated with MT1-MMP at indicated timepoints. ♦ represents MT1-MMP cleaved Ln-10 and ■ represents uncleaved Ln-10. Results are representative of three experiments performed in triplicate. Error bars represent the standard deviation of the representative experiment. $P < 0.05$ at all time points.

Cleavage of Ln-10 α5 Chain Promotes Migration of Prostate Cancer Cells.

To examine the potential role of MT1-MMP cleaved Ln-10 in migration of DU-145 prostate carcinoma cells we used linear migration and transwell migration assays. We found that at 24 hr the DU-145 cells were about 2-fold more migratory on cleaved Ln-10 than on uncleaved Ln-10 (Figure 21A and 21B). To determine whether the increase in transmigration of DU-145 cells on cleaved Ln-10 was due to the cleavage of the α5 chain by MT1-MMP and not due to any other MT1-MMP effects, we used antisense oligonucleotides for MT1-MMP as described previously (84). We have previously shown that cells treated with antisense oligonucleotides inhibit cell surface MT1-MMP expression by 66% in DU145 cells whereas scrambled oligonucleotide had no effect (83). MT1-MMP expression in cells treated with antisense is also decreased in these experiments (Figure 22A). Migration of DU-145 cells were not affected by the scrambled oligo, but the antisense oligonucleotide did demonstrate effects on migration (Figure 21A and 21B). Both linear migration and transmigration on intact Ln-10 were reduced by approximately 70% to 80% whereas only a 30% to 40% decrease was observed on cleaved Ln-10 in the presence of the MT1-MMP antisense oligonucleotides. This demonstrates that cleavage of Ln-10 allows for increased cell motility regardless of MT1-MMP status. Blocking MT1-MMP inhibits cell motility on intact Ln-10. This indicates a role for MT1-MMP in cell motility on Ln-10 coated surfaces. The antisense-treated prostate cancer cells were still able to migrate on cleaved Ln-10 (though to a lesser extent than untreated cells). This indicates that cleaving Ln-10 was the major contributing factor to the increased motility, but MT1-MMP does play some role in

motility in addition to its ability to cleave the Ln-10 substrate.

Prostate Cancer Cells are Capable of Processing Ln-10.

Purified Ln-10 obtained from A549 cells contains some cleaved Ln-10. This indicates that MT1-MMP expressed on the surface of cells can cleave Ln-10, as A549 cells have been previously shown to express MT1-MMP (287). To confirm that cleavage of Ln-10 could occur at the cellular level, we determined that prostate cancer cell lines expressing high levels of MT1-MMP could cleave intact Ln-10. We seeded an equal number of DU-145 cells treated with antisense oligonucleotides to MT1-MMP or scrambled oligonucleotides as described in previous experiments in serum free medium on tissue culture plates coated with purified Ln-10. As a control, one well of Ln-10 coated on the plate did not receive any cells, only serum free medium. MT1-MMP expression by these cells is shown in Figure 22A. Also shown is MT1-MMP expression in A549 cells and PC3N cells. Densitometry analysis demonstrates that DU-145 cells treated with antisense oligonucleotides express lower levels of MT1-MMP compared to untreated DU-145 cells. We found the 45 kDa cleaved fragment of Ln-10 to be released from the ECM into the CM, though to a lesser extent in cells treated with antisense oligos or in the coated Ln-10 without the addition of cells (Figure 22B). In the matrix removed from the tissue culture plate, full-length Ln-10 α5 chain was observed, though the amount of full-length Ln-10 remaining was reduced in the untreated DU-145 cells or the scrambled oligonucleotide treated DU-145 cells, indicating that the Ln-10 is being cleaved by these cells (Figure 22C).

Ln-10 Cleavage Occurs in Prostate Tissue

Expression of both Ln-10 and MT1-MMP in prostate cancer have been previously reported (83, 89). Here, we show that MT1-MMP and Ln-10 are expressed in the same area of prostate cancer (Figure 23A) suggesting that MT1-MMP is available to cleave Ln-10 *in vivo*. An H&E stain of the tissue area in Figure 22A is included to show tissue morphology (Figure 23B). To investigate whether Ln-10 cleavage occurs *in vivo*, we used immunohistochemical analysis of invasive prostate cancer tissue samples with 4C7 antibody to determine whether Ln-10 cleavage could be detected. We observed a discontinuous Ln-10 BL surrounding cancer (Figure 23C), indicative of Ln-10 cleavage occurring *in vivo*. An H&E of this tissue area is also included (Figure 23D). Also, we manually microdissected snap-frozen serial sections of prostate tissue (20 μm), separating areas of high cancer concentration or areas of high normal gland concentration from surrounding stroma. These microdissected samples were analyzed by Western blotting with 15H5 antibody. We found detectable levels of the full-length 350 kDa α5 chain in both normal and cancer glands but the 310 kDa cleavage fragment was only detected in cancer samples (Figure 23E), suggesting that MT1-MMP expressed in prostate cancer is capable of cleaving the full-length Ln-10 α5 chain.

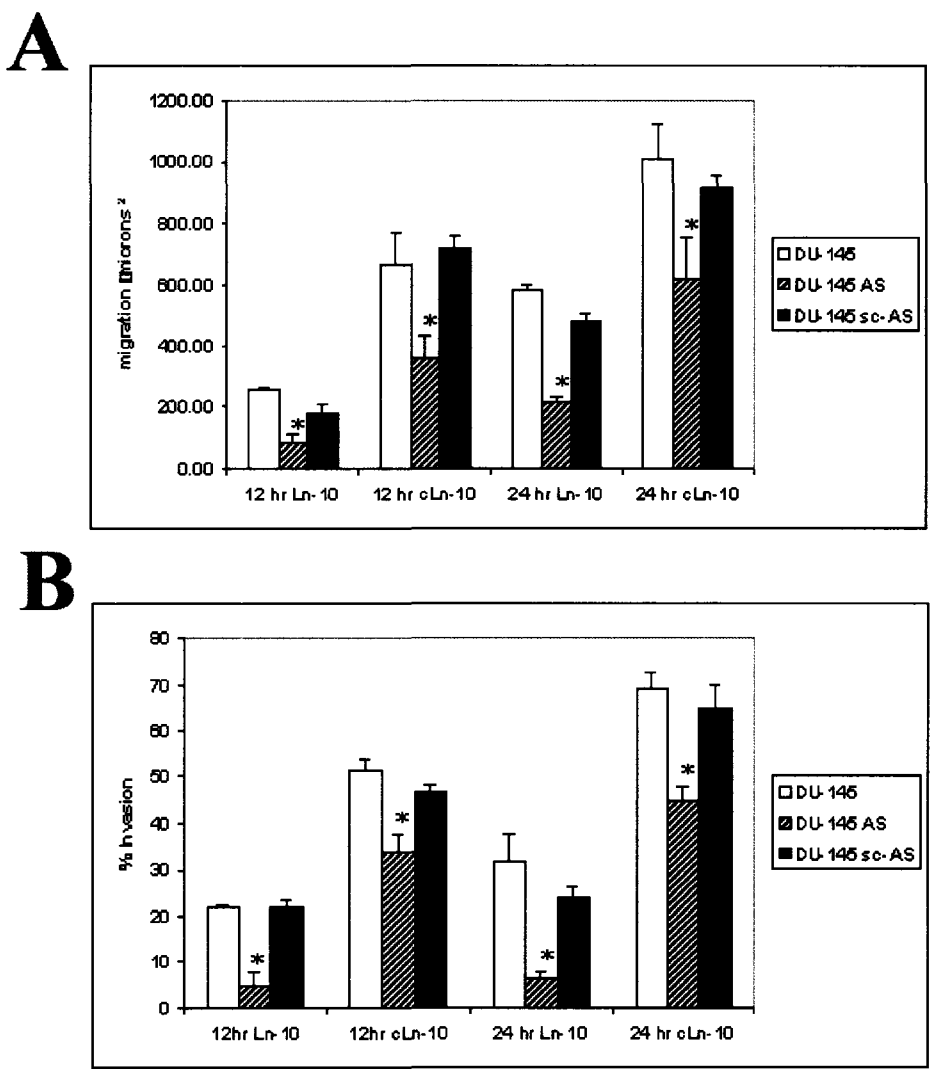

Figure 21. Ln-10 cleavage increases migration and invasion of prostate cancer cells.

A) Migration of cells on Ln-10 coated microscope slide. B) Transmigration of cells through Ln-10 coated transwell cell insert chambers coated with Ln-10 at indicated time points. Groups are: Ln-10, cleaved Ln-10 (cLn-10), Ln-10 with cells treated with antisense oligonucleotides (AS) and Ln-10 with cells treated with scrambled oligonucleotide (sc-AS). Results are representative of three experiments done in triplicate. Error bars represent standard deviation of one experiment. *P< 0.05.

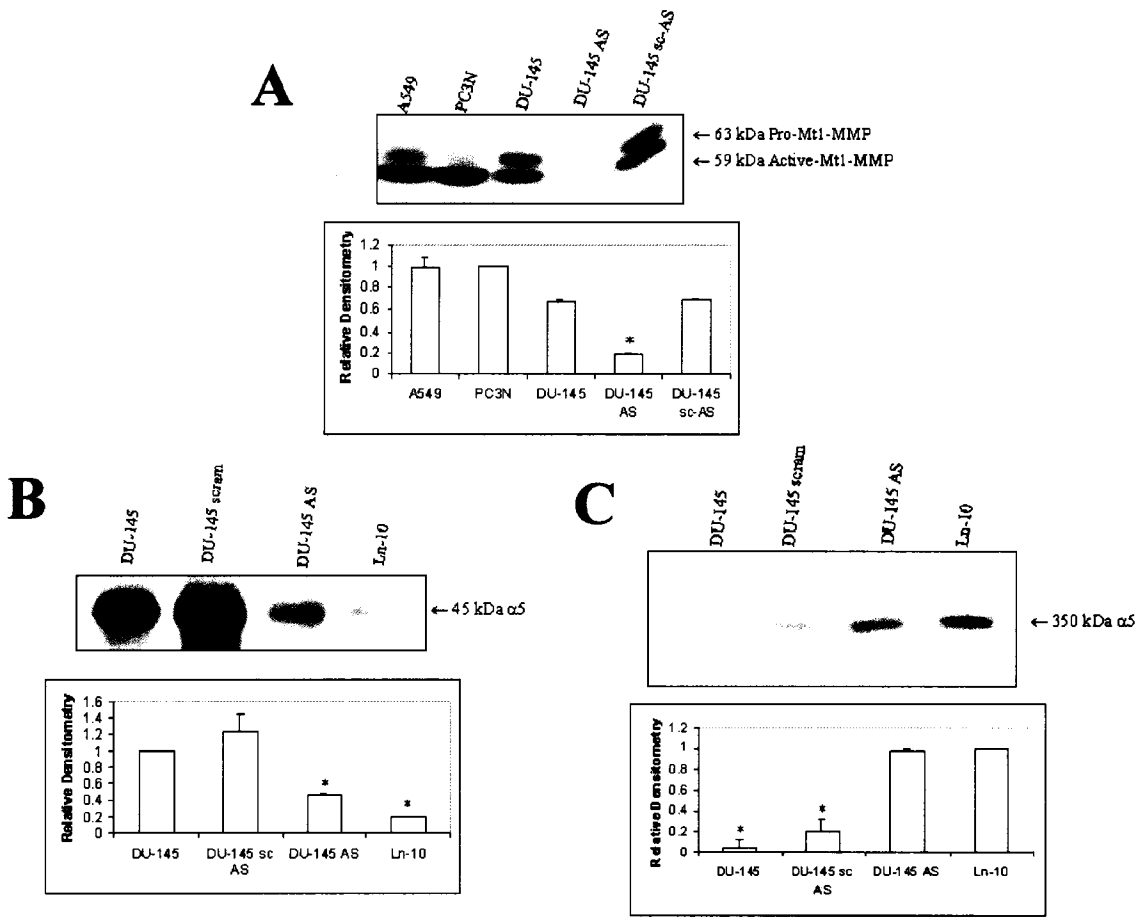

Figure 22. Prostate Cancer Cell cleavage of Ln-10.

A) Western blot for MT1-MMP of cell lysates with densitometry analysis. B) Western blot of total protein in CM for Ln-10 fragments with densitometry analysis. C) Western blot for Ln-10 of matrix after removing cells with densitometry analysis. Results are representative of three experiments. * $P<0.006$.

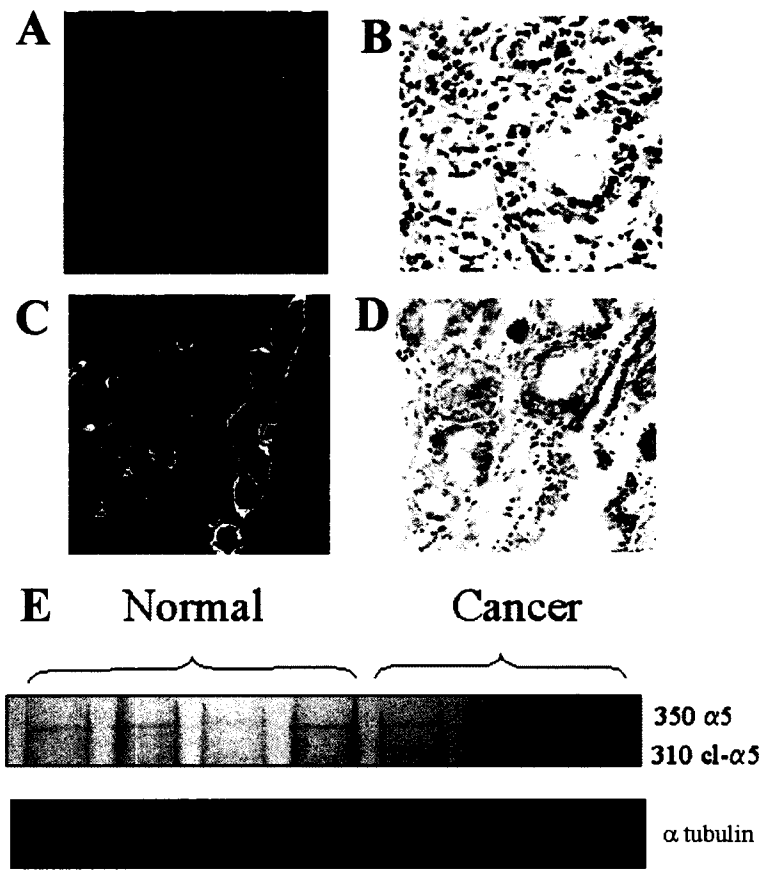

Figure 23. Cleavage of Ln-10 occurs in human prostate tissue.

A) Immunohistochemistry of area of prostate cancer demonstrating Ln-10 (green) expression surrounding cancer and MT1-MMP (red) expression in prostate cancer. B) H&E stain of the tissue area shown in A. C) Immunohistochemistry of prostate tissue with Ln-10 antibody. Areas of normal (N) and cancer (C) are indicated. Ln-10 is stained green and α6 integrin is stained red. D) H&E stain of tissue area shown in C. E) Western blot for Ln-10 and α-tubulin. Normal and cancer are indicated, as are MW and corresponding protein identification.

Discussion

We have previously shown that MT1-MMP is capable of cleaving human Ln-5 β3 chain and that this cleavage increased prostate cancer cell migration and invasion (83). In this study, we have explored a potential mechanism for the invasion of prostate cancer cells through the Ln-10-rich BL surrounding them *in vivo*. We have shown that MT1-MMP is expressed in prostate cancer along with Ln-10, and that MT1-MMP cleaves the α5 chain of Ln-10 and that this cleavage has effects on both adhesion and migration. MT1-MMP is not expressed in normal prostate but its expression increases in prostate cancer progression. Because prostate cancer cells reform a BL rich in Ln-10, it seemed likely that MT1-MMP would be involved in degrading this BL in order for prostate cancer cells to metastasize. The role of the BL in cancer biology is not completely understood, but loss of BL continuity (due to degradation) has been associated with increasing malignancy (291, 292).

The present study shows that incubation of MT1-MMP with Ln-10 generated novel cleavage products, which were identified by mass spectrometry analysis to be derived from the α5 chain. We showed that this cleavage was specific for MT1-MMP and did not occur with MMP-2 (a MMP that MT1-MMP is known to activate (293)) treatment. We did not find any evidence that either the β1 or γ1 chains of Ln-10 were cleaved with MT1-MMP treatment. We found four distinct cleavage products of different apparent molecular weights: 310 kD, 190 kD, 160 kD, and 45 kD. We propose that the 45 kD product is the N-terminal fragment from the cleavage of the 350 kD full-length α5 chain into the 310 and the 45 kD products, based on peptide coverage given by

mass spectrometry. We propose that the 190 kD and 160 kD products are additional cleavage products of the 310 kD band based on the mass spectrometry analysis. We expect that the additional cleavages by MT1-MMP give rise to approximately 150 kD, 120 kD, and 30 kD products that are not present in high enough concentrations to be detected by Coomassie or silver staining.

The Ln-α5 chain is the largest of the α chains and is considered the primordial α chain. Ln α chains contain EGF-like domains in their N-terminus and α5, being the largest α chain, has the greatest number of EGF-like repeats. Interestingly, the proposed 45 kD product contains two EGF-like repeats, and the additional processing of the 310 kD product into 190 kD and 160 kD products would yield fragments consisting almost entirely of EGF-like repeats. This provides a potential explanation for increased invasion on MT1-MMP cleaved Ln-10 if an EGF-like fragment from Ln-10 cleavage can activate the EGF receptor on the cell surface, signaling for migration (Figure 24). Essentially, the Ln-10 fragments could function as matrikines (294), enzymatic fragments of ECM that contain cryptic biologically active sites, also called matricryptins (105). In fact, an EGF-like fragment from rat Ln-5 cleavage by MT1-MMP has been shown to bind to the EGFR and stimulate downstream MAPK signaling to induce migration (81). Also, colon cancer cells have been shown to spread on Ln-10 as a result of EGFR stimulation (95) indicating that EGF-like fragments could have similar effects on prostate tumor cells. We have demonstrated that at low molar ratios of enzyme to substrate we are able to detect these laminin cleavage products. While the exact ratio of Ln-10: MT1-MMP in human tissue in unknown, we expect that due to the localization of MT1-MMP to the invading tumor

front this ratio might approach 1:1 in tissues. Our results that show cleavage is possible at 0.5 mol of MT1-MMP per 1 mol of Ln-10, concentrations which are likely to occur *in vivo*. The increased intensity of the 160 kDa band correlates to the decreased 190 kDa band. This indicates that the 190 kD product is sequentially cleaved into the 160 kD product, which corresponds to the mass spectrometry data of protein coverage of the bands.

We have also shown that cleavage of Ln-10 by MT1-MMP causes a decrease in adhesion and an increase in migration of prostate cancer cells. The increase in migration on intact Ln-10 was inhibited with MT1-MMP antisense oligonucleotide treatment, suggesting that MT1-MMP cleavage of Ln-10 is responsible for the increased motility. Since some inhibition of migration was seen with MT1-MMP antisense oligonucleotide treatment on cleaved Ln-10, we suggest that while cleavage of Ln-10 is the major cause of both migration and movement on Ln-10, some other effects of MT1-MMP are necessary for this motility. In fact recent studies have suggested and shown a possible role of MT1-MMP in downstream signaling for cell migration (295-297). While proteolysis of the ECM is essential for migration and invasion, excessive proteolysis can degrade ECM and disrupt cell-matrix interactions, actually inhibiting migration. Therefore, although we observed a decrease in adhesion to MT1-MMP treated Ln-10, cells eventually did adhere, indicating that the strength of adhesion was modified in MT1-MMP-cleaved Ln-10 and not that the Ln-10 was degraded. Indeed, strength of adhesion is one mechanism that controls the speed of cell motility (298). In this study, increased migration and invasion was correlated with a decrease in adhesion of cells to

substrates coated with cleaved Ln-10.

We have demonstrated that this processing of Ln-10 can occur at the cellular level in cells that express high amounts of MT1-MMP on their cell surface. DU-145 cells were shown to cleave Ln-10 in tissue culture and this cleavage was inhibited with antisense oligonucleotides to MT1-MMP, indicating that endogenous MT1-MMP is responsible for Ln-10 cleavage. Finally, we have demonstrated that Ln-10 cleavage occurs in prostate tissues. In areas of normal glands, full-length α5 chain was detected, while in areas of cancer, both the full-length α5 chain and the 310 kD cleaved α5 chain fragment were detected. Also detected was a discontinuous Ln-10 BL surrounding prostate cancer, further indicating that Ln-10 cleavage occurs *in vivo*. Although our initial experiments to demonstrate Ln-10 cleavage and identify cleavage fragments used the recombinant catalytic domain of MT1-MMP, our data demonstrating that prostate cancer cells can cleave Ln-10 and that Ln-10 cleavage occurs *in vivo*, along with our linear migration and transmigration assays, verify that Ln-10 cleavage by MT1-MMP is biologically significant.

These data clearly indicate that the Ln-10 α5 chain cleavage plays an important role in tumor cell migration and invasion. This is a novel and important finding as this cleavage may enhance the invasion of prostate cancer cells *in vivo*. In addition, since Ln-10 is widely expressed, similar effects may be seen in other malignant tissues where Ln-10 and MT1-MMP are also expressed. These effects of Ln-10 cleavage are probably even more widespread with respect to metastasis since blood vessels are surrounded by Ln-10. Therefore, any cancer that metastasizes via the bloodstream and expresses MT1-

MMP can use this mechanism of Ln-10 cleavage to enter the blood vessels and to extravasate from the blood vessels at a distant location to form a metastasis. Since this study was conducted using human MT1-MMP, human Ln-10, and prostate cancer cells, these findings are particularly relevant to human prostate cancer and can lead to new approaches to intervention in preventing the metastasis of prostate cancer.

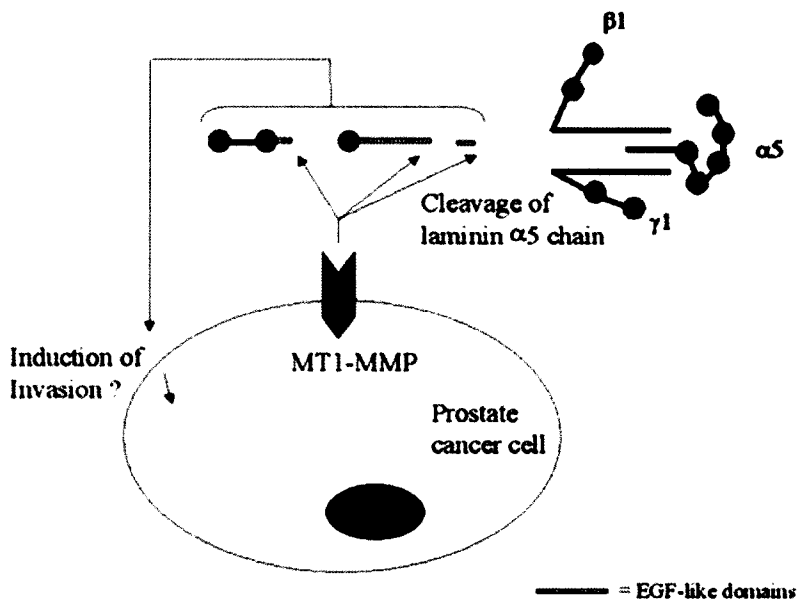

Figure 24. Schematic model of MT1-MMP dependent invasion through Ln-10.

MT1-MMP expressed on the surface of a prostate cancer cell cleaves Ln-10 in the BL forming cleavage products. Cleavage of Ln-10 disrupts the BL, allowing the prostate cancer cells to invade the BL. Additionally, the cleavage products may have effects on migration through exposure of cryptic peptides, potentially EGF-like peptides that may bind to the EGF receptor and induce signaling for migration.

VI. CONCLUDING DISCUSSION

It is well known that the extracellular environment is important for many processes including survival, growth, and differentiation. The extracellullar environment is also important for cancer invasion and metastasis. A coordinated response among extracellular matrix protein, proteolytic enzymes, cell adhesion molecules (CAMs), growth factors and cytokines must exist for a tumor cell to metastasize successfully. CAMs respond to the extracellular environment initiating responses in tumor cells that lead first to modulation of the extracellular environment via proteolysis of ECM proteins and second to induction of invasion through the BM (49-53). Understanding the coordination of these efforts will lead to improved diagnostics and prognostics, and eventually to better treatment for advanced cancers.

Integrins mediate cell-matrix adhesion and their involvement in cell migration might be considered contradictory. Yet, integrins are required for cell movement to occur. This is because the dynamic regulation of integrins is able to bridge the extracellular space with the intracellular environment. Integrins receive signals from the BM and through interactions with the actin cytoskeleton are able to generate a force that results in cell motility (155).

Cadherins mediate cell-cell contacts amongst cells, and their involvement in cell migration is important. Cadherin expression switches from predominantly E-cadherin in epithelial cells, which mediates more stable cell-cell contacts, to predominately N-cadherin in cancer cells, which mediates more transient contacts, allowing for increased motility (165).

Of course, cell-cell contacts are still relatively important during the metastatic process, if for no other reason than for a higher probability of survival both in the bloodstream and in the new site of tumor growth. Cell aggregates are more likely to survive the metastatic process than single cells, making expression of N-cadherin important, even if the interactions between cells are not as tight as interactions formed by E-cadherin. This also explains the importance of other cell adhesive proteins, such as 90K in metastasis. Since 90K can mediate cell adhesion through the cross-linking of cell-surface galectin and through interactions with β1 integrins, 90K is a prime example of a protein that is capable of assisting cancer cells in their survival during metastasis though forming cell aggregates (195). The fact that 90K is found in the circulation further justifies this claim.

Interestingly, all of these cell adhesion molecules have in common their ability to up-regulate the expression of MMPs, proteins that are known to be involved in the metastatic process. Therefore, each of these cell adhesion molecules, in their own way, are capable of not just modulating the response of the cell to its environment, but also help the cell modulate its environment through upregulation of proteolytic enzymes. It is the upregulation of MMPs that allow ECM barriers to be broken down, so that a cell might migrate through and eventually form a metastasis (97).

Therefore, the ECM is the true site of cues for migration. Through cell adhesion molecules, cells are able to sense changes in the extracellular environment. Thus the extracellular environment is able to signal for a cell to migrate. And yet, the tumor cell itself is capable of initiating this modulation of the extracellular space. How these events

are coordinated in a cancer cell is still not understood, but progress in each individual step is becoming increasingly clear.

In this report I have discussed how cell adhesion molecules are able to upregulate MMP-7 in both oral SCC and prostate cancer. In oral SCC, matrilysin expression was induced in a co-culture of fibroblasts with SCC-25 cells via integrins and cadherins. We co-cultured these two cell types together in an effort to mimic the extracellular environment that an oral SCC might be found in, with interactions between the tumor cells and surrounding stromal cells occurring. Initially, we hypothesized that the induction of matrilysin we saw in this co-culture was due to a factor secreted by the fibroblasts, such as a growth factor or a cytokine. This was based on previous studies in prostate caner cells, in which matrilysin was induced by cytokines secreted by prostate fibroblasts in a co-culutre (201). However, what we discovered was that the cell-cell communication between these two cell types was absolutely necessary for the induction of matrilysin. Simply growing the cells in each others' conditioned medium was not sufficient to induce an MMP response. Neither was growing the cells in the same culture dish, separated by a filter-bottom barrier. We, therefore, decided to investigate cell-cell and cell-matrix interactions as the mediators of this matrilysin induction. Increasing the degree of cell-cell contact by increasing the density of the cells resulted in a correlating increase in matrilysin expression. Therefore, we knew that cell-cell contact was involved in matrilysin induction. We found that it was the cell-cell contact between the two different cell types that mediated this response, because increasing the density of SCC-25 cells alone had no result on net matrilysin expression. Using blocking antibodies to

cadherins, we were able to reduce the amount of matrilysin in the co-culture, but we could not bring the matrilysin expression back down to basal levels seen in the SCC-25 cells alone. This was true for N-cadherin blocking antibodies, E-cadherin blocking antibodies, and a mixture of the N- and E- cadherin antibodies together. We also chose to look at cell-matrix interactions via integrins as mediating this matrilysin induction and found that blocking beta-1 integrin also decreased matrilysin expression, but again, not to basal levels. However, when we inhibited all three adhesion molecules, we were able to bring matrilysin expression levels back to the basal level of expression. This indicated that integrins and cadherins were involved in the upregulation of matrilysin expression. We hypothesize that this regulation is not necessarily independent of each cell adhesion molecule involved. Cross-talk between CAMs is known to exist (233) and we propose that signaling through beta-catenin may coordinate the response matrilysin in the SCC-25 cell line when cocultured with stomal fibroblasts.

However, we cannot ignore the fact that cadherin interactions between these two cell types and interactions of the integrins with the active matrix molecules may incite the release of growth factors by the cell types in either a paracrine or autocrine loop. A separate hypothesis is that the cell-cell communication between these two cell types was able to induce the release of a factor by the SCC-25 cells that was able to induce matrilysin via an autocrine pathway. However, our preliminary studies on cytokine-induction on SCC-25 cells did not indicate that IL-1β or IL-6 were capable of inducing matrilysin expression. So, although other cytokines may be regulated by this cell-cell and cell-matrix communication between the fibroblast cells and SCC-25 cells, we know

that these CAM molecule interactions are required for induction of matrilysin in this system. This mechanism for matrilysin induction describes how the response of CAMs to the environment (in this case, stromal cells) initiates a metastatic response through upregulation of MMPs.

Another mechanism for MMP induction cues from the extracellular environment is from proteins that may be secreted by tumor cells or released from the ECM. In prostate cancer, matrilysin is known to be unregulated by various cytokines. Of interest is exactly where these cytokines come from. One explanation is through prostate fibroblasts, which express IL-1β an IL-2, two cytokines that have been shown to upregulate matrilysin in prostate cancer. Matrilysin expression in prostate cancer seems to be increased in areas of prostate surrounded by inflammatory cell infiltrates, which would be expected to have a high concentration of cytokines (120). In an effort to identify a potential new biomarker for prostate cancer, we discovered a high rate of expression of the protein 90K in prostate cancers and prostate cancer cell lines. We, therefore, chose to look at a possible role for 90K in the induction of matrilysin. 90K is considered to be involved in the host immune response to tumors and other infections, and therefore is capable of inducing various cytokines while fulfilling this role. Interestingly, some of the cytokines it induces are the cytokines that we have previously demonstrated to increase matrilysin expression (201, 246). We chose to examine matrilysin expression in LNCaP cells, a prostate cell line that can be induced to express IL-1β. We treated these prostate cancer cells with purified 90K protein and were able to discern an induction of matrilysin. Matrilysin induction was maximal at 48 hours after

treatment, indicating that time for new protein synthesis was required for this induction. We were able to block this induction through the use of antibodies that can immuno-deplete 90K from the medium and through IL-6 neutralizing antibodies. We, therefore, propose that matrilysin induction in prostate cancer cells though IL-6 can be mediated by the expression of 90K in prostate. This information gives new credence to the idea that 90K is a good prognostic indicator, as it has been associated with poor prognosis in many different cancer types. Possibly the reason for this poor prognosis is through the ability of 90K to induce MMP-7 in these cancers, which in turn can lead to a more invasive phenotype.

Now that two different mechanisms of cell adhesion proteins (integrins and cadherins) and an ECM-associated protein (90K) mediating a metastatic response through MMP induction have been identified, I will now discuss one aspect by which MMPs allow cells to invade. In particular, I will address another cell-matrix contact, mediated by the transmembrane metalloprotease MT1-MMP interacting with and cleaving a laminin-10 matrix. Most MMPs are secreted MMPs, and therefore have the capacity to interact with matrix proteins and cleave them at sites distant from where they are produced. The transmembrane metalloproteases are different. Since they are embedded in the membrane via their transmembrane domain, they must act locally to degrade ECM components. In prostate cancer, there is a well-described down-regulation of certain integrins, while others continue to be expressed throughout progression. The two integrins that continue to be expressed happen to be the integrins that bind laminin-10 (284). This integrin-laminin interaction brings the cell in direct contact with laminin-10,

and puts laminin-10 at a prime location to be degraded by proteases. I have shown that MT1-MMP, which is upregulated in prostate cancer progression, is capable of cleaving laminin-10 into 4 distinct fragments. Cleavage of laminin-10 by MT1-MMP was also able to induce prostate cancer cell migration on and invasion through laminin-10 coated surfaces. Interestingly, prostate cancer cells were also capable of cleaving laminin-10, and laminin-10 cleavage was demonstrated to occur *in vivo* in human prostate cancer. We also identified areas of human prostate cancer where the laminin-10 BM seemed to have been broken down, indicating the MT1-MMP cleavage of laminin-10 is a real mechanism by which prostate cancer may become invasive. We propose that instead of MT1-MMP induced cleavage of laminin-10 being only a mechanism to break down a barrier to invasion, fragments of laminin-10 containing EGF-like repeats produced by cleavage may also have functional effects on surrounding cells. The laminin-10 fragments may function as matrikines capable of inducing signals for migration through the EGF receptor as has previously been described for rat laminin-5 (81).

This suggests that laminin-10 cleavage may be a good indicator for invasive prostate cancer. If the cleaved fragments of laminin-10 are secreted into either the serum or the prostatic fluid of a patient with prostate cancer, being able to detect high levels of these fragments may be a good indicator that the patient has aggressive cancer that needs to be treated aggressively.

Taken together, these studies represent the dynamic regulation of the extracellular environment of a tumor cell. CAMs react to changes in the BM by signaling for a cell to secrete proteolytic enzymes to further change the BM and by signaling for changes in the

expression of CAMs to allow the cell to become more motile. The signaling pathways by which these changes take place are not yet completely understood, but may include signaling via focal adhesion kinase, β-catenin, rac, and ERK, amongst others. The resulting differences in the extracellular environment (degraded BM, increased MMPs) are very likely to be important clues clinicians can use to classify a tumor, either a prostate tumor, an oral SCC, or any number of tumors, as aggressive tumors capable of initiating a coordinated metastatic response. Assays that can detect degraded fragments of ECM (i.e. laminin-10 α5 chain fragments), increased MMPs (i.e. matrlysin in prostate or oral SCC, or MT1-MMP in prostate), or increased expression of the proteins the regulate MMPs (i.e. 90K in prostate, or integrins and cadherins in oral SCC) will be important tools for prognosis of different cancers in the future.

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Dear Dr. Carpenter,

I am submitting my dissertation to the Graduate College at the University of Arizona in partial fulfillment of the requirements for the degree of Doctor of Philosophy. I intend to include text and all figures from a previously published manuscript in *Experimental Cell Research* Volume 270, Issue 2, 1 November 2001, Pages 259-267, entitled "Integrin- and Cadherin-Mediated Induction of the Matrix Metalloprotease Matrilysin in Cocultures of Malignant Oral Squamous Cell Carcinoma Cells and Dermal Fibroblasts."

The University of Arizona requires a letter that permits me to include published data in my dissertation. Permission is also requested for microfilming and publication of the dissertation by University Microfilm Incorporated.

Thank you in advance for your help in this matter

Sincerely,

Elisabeth I. Bair

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5 November 2004

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Ms Elisabeth Bair

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APPENDIX B: PUBLISHED MANUSCRIPTS AND ABSTRACTS

Bair EL, Chen ML, McDaniel K, Sekiguchi K, Cress AE, Nagle RB, Bowden, GT. Membrane-Type-1 Matrix Metalloprotease (MT1-MMP) Cleaves Laminin-10 and Promotes Prostate Cancer Cell Invasion. *Neoplasia* (in press).

Bair EL, Massey CP, Tran NL, Borchers AH, Heimark RL, Cress AE, Bowden GT. "Integrin- and Cadherin- Mediated Induction of the Matrix Metalloprotease Matrilysin in Co-cultures of Malignant Oral Squamous Cell Carcinoma Cells and Dermal Fibroblasts." *Experimental Cell Res.* 2001 Nov 1; 270 (2): 259-67.

Udayakumar TS, **Bair EL**, Nagle RB, Bowden GT. "Pharmacological Inhibition of FGF Receptor Signaling Inhibits LNCaP Prostate Tumor Growth, Promatrilysin and PSA Expression." *Mol Carcinog.* 2003 38(2):70-7.

Udayakumar, TS, Chen, ML, **Bair, EL**, von Bredow, DC, Cress, AE, Nagle, RB, Bowden, GT. "Membrane Type-1-Matrix Metalloproteinase Expressed by Prostate Carcinoma Cells Cleaves Human Laminin β3 Chain and Induces Cell Migration." *Cancer Res.* 2003 May 1; 63 (9): 2292-2299.

Bair EL, Chen ML, Sekiguchi K, Cress AE, Nagle RB, Bowden GT. "Membrane Type-1 Matrix Metalloprotease (MT1-MMP) Cleaves Laminin-10 and Promotes Prostate Cancer Cell Invasion" Therapeutic Targeting of Human Prostate Cancer Symposium 2004, Tucson, AZ.

Bair EL, Chen ML, Sekiguchi K, Laferté S, Cress AE, Nagle RB, Bowden GT. "Laminin-10 α5-chain Associates with Mac-2 Binding Protein *in vivo* and is Cleaved by MT1-MMP to Promote Prostate Cancer Cell Invasion" American Society for Cell Biology Annual Meeting 2003, San Francisco, CA.

Bair EL, Chen ML, Sekiguchi K, Cress AE, Nagle RB, Bowden GT. "Cleavage of Laminin-10/11 by Membrane-Type-1 Matrix Metalloprotease (MT1-MMP) Increases the Invasive Potential of Prostate Cancer Cells" American Association for Cancer Research 2003, Washington, D.C.

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