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Breast epithelial cell function is greatly influenced by interactions with the underlying basal lamina. Matrilysin, a matrix metalloproteinase, has previously been shown to be expressed in both adenomas and carcinomas of the human breast. We have tested the hypothesis that cell-ECM interactions regulate the expression of matrilysin in human breast carcinoma cells *in vitro*. We have used northern analysis and *in situ* hybridization to determine levels and localization of several members of the MMP family in human tumors implanted into nude mouse mammary glands. Tumors from mammary glands injected with the human breast adenocarcinoma cell line MDA-MB-468 were shown to express matrilysin; a MMP that is primarily expressed in normal and neoplastic cells of epithelial origin. Stromelysin-1 was induced in the stroma of the host mammary gland in the region immediately surrounding the tumor. Northern analysis revealed that gelatinase A, which was not produced by MDA-MB-468 *in vitro*, was expressed in the tumor. This combination of MMPs along with stromelysin-3, which has been extensively studied in breast cancer, may lead to the eventual metastasis of mammary tumors to the regional lymph nodes and distant sites. We also describe a new antibody against human matrilysin which indicates that matrilysin is expressed in epithelial cells as expected and appears to be apically secreted in endometrium, breast and prostate. This lumenal secretion of an MMP that degrades extracellular matrix molecules may have important implications as to its function in normal and neoplastic tissues.

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Introduction

Breast cancer is the most commonly diagnosed female cancer in the United States population with an estimated 182,000 new cases in 1993 (32% of all female cancers diagnosed in 1993) (Parker *et al.*, 1996). Metastatic spread to the regional lymph nodes has long been used as a predictor of the extent of disease and with reasonable success. When the tumor has invaded the regional lymph nodes the 5-year survival rate drops from 93% to 73% and when distant metastasis is found the rate falls to 19% (Parker *et al.*, 1996). These data make it clear that the process of tumor cell invasion and metastasis are important in the progression of breast cancer from a benign to a malignant state. The process by which neoplasms metastasize is composed of a complex series of events (Liotta and Stetler-Stevenson, 1991 ; Stetler-Stevenson *et al.*, 1993). One of the initial steps in tumor cell invasion is the degradation of the basal lamina (BL) and local invasion of the surrounding tissue. To move through the BL, cells must secrete proteinases that are able to degrade the components of the BL including, collagen type IV, laminin, tenascin and entactin.

The matrix metalloproteinases (MMPs) are a multigene family of secreted proenzymes whose substrates are the proteins that make up the BL and the extracellular matrix (Matrisian, 1992). Tight regulation of the expression and activity of MMPs and their specific inhibitors, TIMPs, maintain cells in a non-invasive phenotype (Liotta and Stetler-Stevenson, 1991). A change altering the balance in favor of MMP activity may be one of the initial steps in tumor cell invasion. Matrilysin is the smallest known member of the MMP family containing only the signal sequence, the pro-peptide and the active site. Matrilysin has been shown to be expressed in several different human tumor types including: breast, prostate, colon, gastric, rectal and head and neck (for review see(Powell and Matrisian, 1995)). Matrilysin degrades casein, gelatins I, III, IV and V, fibronectin, laminin, elastin and entactin. Matrilysin also interacts with other proteinases; specifically, matrilysin can cleave activated interstitial collagenase to increase collagenases activity five fold (Quantin *et al.*, 1989). Matrilysin can proteolytically cleave pro-urokinase to yield low-molecular weight pro-urokinase that is unable to bind to its specific transmembrane membrane receptor (Marti *et al.*, 1992). Matrilysin mRNA has been shown to be expressed in a high percentage of both adenomas and carcinomas of the breast (Heppner *et al.*, 1996; Wolf *et al.*, 1992). *in situ* analysis indicates that the mRNA is localized to the transformed epithelial cells of the breast (Heppner *et al.*, 1996).

It is of interest to understand the regulation of matrilysin in breast cancer where invasion and metastasis has such a large effect on prognosis. We hypothesize that alterations in the interactions between breast epithelial cells and the BL can modify the invasive and/or metastatic phenotype of the transformed breast epithelial cells. The interface that exists between a rapidly growing tumor and the surrounding tissue is a very dynamic region consisting of tumor cells interacting with extracellular matrix molecules, basement membrane, growth factors and normal stromal fibroblasts. Events that occur in this region can affect many characteristics of the tumor

including growth, local invasion and metastasis. In order to more clearly define this complex interaction we have investigated the roles that matrix metalloproteinases (MMPs) play at the interface between the tumor and normal stroma. Matrix metalloproteinases are a multi-gene family of extracellular proenzymes that degrade components of the extracellular matrix (ECM) and basement membrane (BM). MMP expression has been correlated with increased local invasion and metastasis in many animal models of human tumors and the use of both natural and synthetic inhibitors of MMPs have shown promise in treating cancer patients. Work from our laboratory and others have shown that many of these MMPs are produced by the stroma within and surrounding the tumor. Our laboratory has focused on two prototypical MMPs, matrilysin and stromelysin-1. Matrilysin is the only MMP expressed primarily by epithelial cells and tumors of epithelial origin and stromelysin-1 is expressed by stromal fibroblasts and in some late stage tumors that have undergone epithelial to mesenchymal transition (EMT) a further step in tumor progression (Thompson *et al.*, 1991). It is for these reasons that we have focused our investigations on matrilysin produced by tumor cells and stromelysin-1 production by stromal fibroblasts.

Results

We have used species specific in situ hybridization probes to determine which tissue compartment, tumor or stroma, expresses the MMPs in a xenograft model system of human breast cancer. This model system involves orthotopic injection of MCF-7, MDA-MB-468 and MDA-MB-231 cells into the mammary glands of adult nude mice. The tumors that developed were fixed and sectioned for in situ hybridization using the species specific MMP RNA probes for matrilysin, stromelysins 1,2,3, interstitial collagenase, collagenase-3, MT1-MMP and gelatinase A. This methodology has allowed us to make some important observations regarding the environment at the tumor/stoma border. The three cell lines used represented tumors that exhibit increasing progression (MCF-7<MDA-468<MDA-231). We have shown that MCF-7 tumors express no detectable transcripts for the MMPs surveyed, where as, MDA-468 and MDA-231 both express the membrane bound MMP, MT1-MMP. MDA-468 tumors also express matrilysin as opposed to expression of interstitial collagenase expression by the MDA-231 tumors. This increase in expression of MMPs with increasing progression is consistent with previous reports on the process of EMT. It is interesting to note however that the majority of MMPs produced at the interface are derived from the surrounding stroma with stromelysin-1 and collagenase-3 being the most prominent (Table 1, Figure 1).

Table 1. Xenograft in situ summary

	MCF-7		MDA-MB-468		MDA-MB-231	
MMP	*Human*	*Mouse*	*Human*	*Mouse*	*Human*	*Mouse*
Matrilysin	(-)	(-)	Tumor cells	(-)	(-)	(-)
Stromelysin-1	(-)	Stroma near tumor	(-)	Stroma near tumor	(-)	Stroma near tumor
Stromelysin-2	(-)	(-)	(-)	Infiltrating Immune cells	(-)	(-)
Stromelysin-3	(-)	(-)	(-)	Tumor stroma and host stroma	(-)	(-)
Interstitial Collagenase	(-)	NA	(-)	NA	Tumor cells	NA
Collagenase-3	ND	Stroma near tumor	ND	Stroma near tumor	ND	Host stroma
MT1-MMP	(-)	ND	Tumor cells	ND	Tumor cells	ND
Gelatinase A	(-)	Stroma near tumor	(-)	Stroma near tumor	(-)	Stroma near tumor

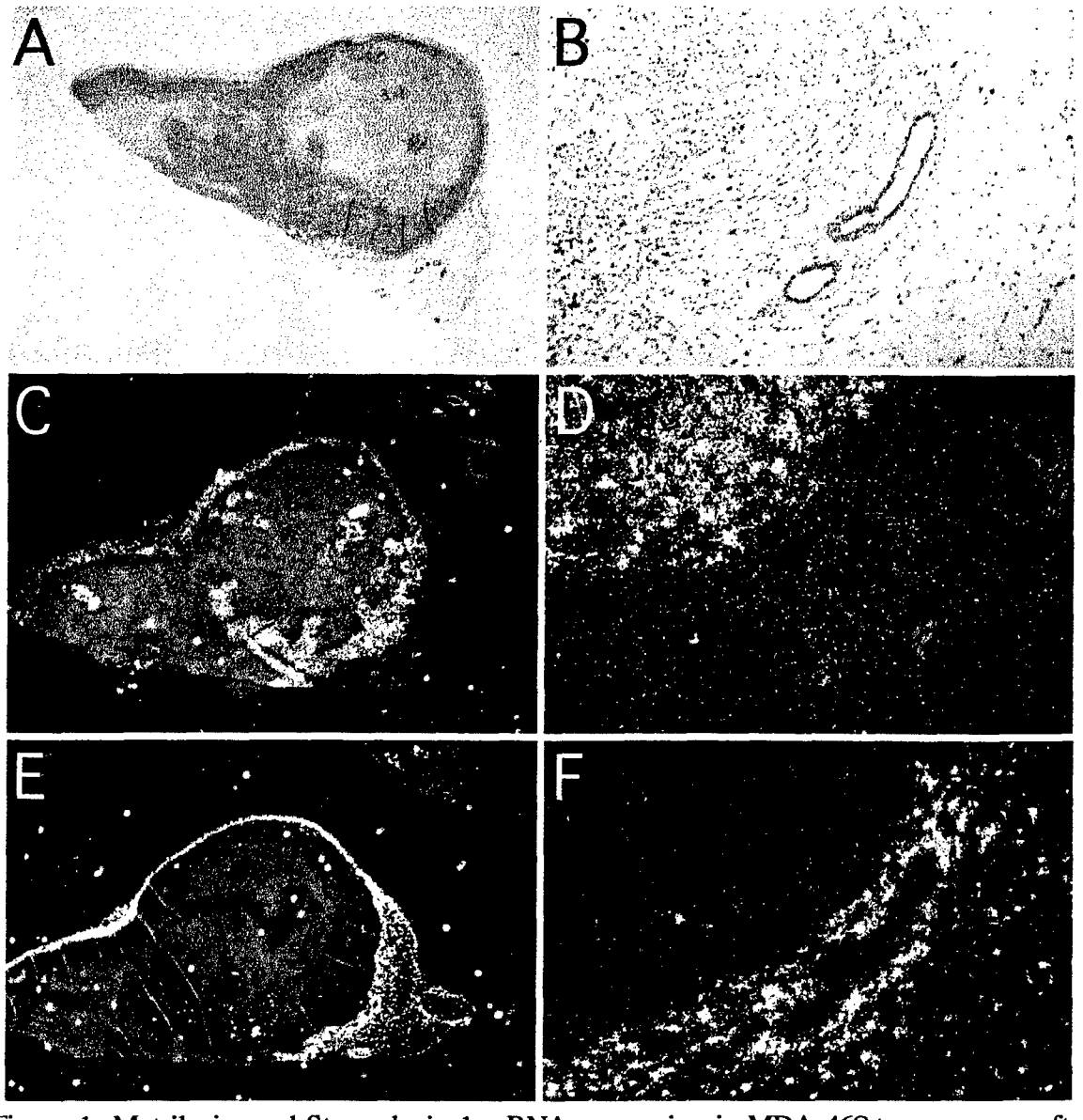

Figure 1. Matrilysin and Stromelysin-1 mRNA expression in MDA-468 tumor xenografts. Low (A,C,E) and high power (B,D,F) photographs of an MDA-468 tumor from a nude mouse mammary gland hybridized with matrilysin (C,D) or stromelysin-1 (E,F). Hemetoxylin staining is shown in A and B.

The MDA-468 tumor produced the most complex pattern of MMP expression, in addition to stromelysin-1 and collagenase-3, the stroma also produced stromelysin-3 (seen in human breast cancer) and gelatinase A and a novel observation of stromelysin-2 expression in a few isolated cells within the tumor, potentially infiltrating immune cells. This expression of stromal MMPs within and immediately adjacent to tumors has been hypothesized by some to be an example of EMT at the interface of the tumor and normal stroma. Using the human matrilysin and mouse stromelysin-1 probes on MDA-468 tumors has clearly shown that EMT has not occurred at this very focal level. Our data indicate a progression from the differentiated MCF-7 cells to the more malignant MDA-469 and 231 tumors with the appearence of more tumor derived MMPs. The expression of MT-1-MMP is of particular interest given its role on the activation of gelatinase A which was expressed int he stroma surrounding all three tumor xenografts (Table 1).

The induced expression of MMPs in the stroma surrounding a tumor can be due to at least two factors, tumor derived growth factors and cytokines which have been shown to induce MMPs and a wound response to the expanding tumor. We have also investigated the potential mechanism of stomal MMP induction by tumor cells. by using conditioned media from MDA-468 cells to treat human fibroblast cultures we were able to show that there was a diffusable factor produced by MDA-468 cells that was capable of inducing both stromelysin-1 and collagenase-3 in the fibroblasts (year 2 progress report). Literature and the approximate size of the factor indicated that

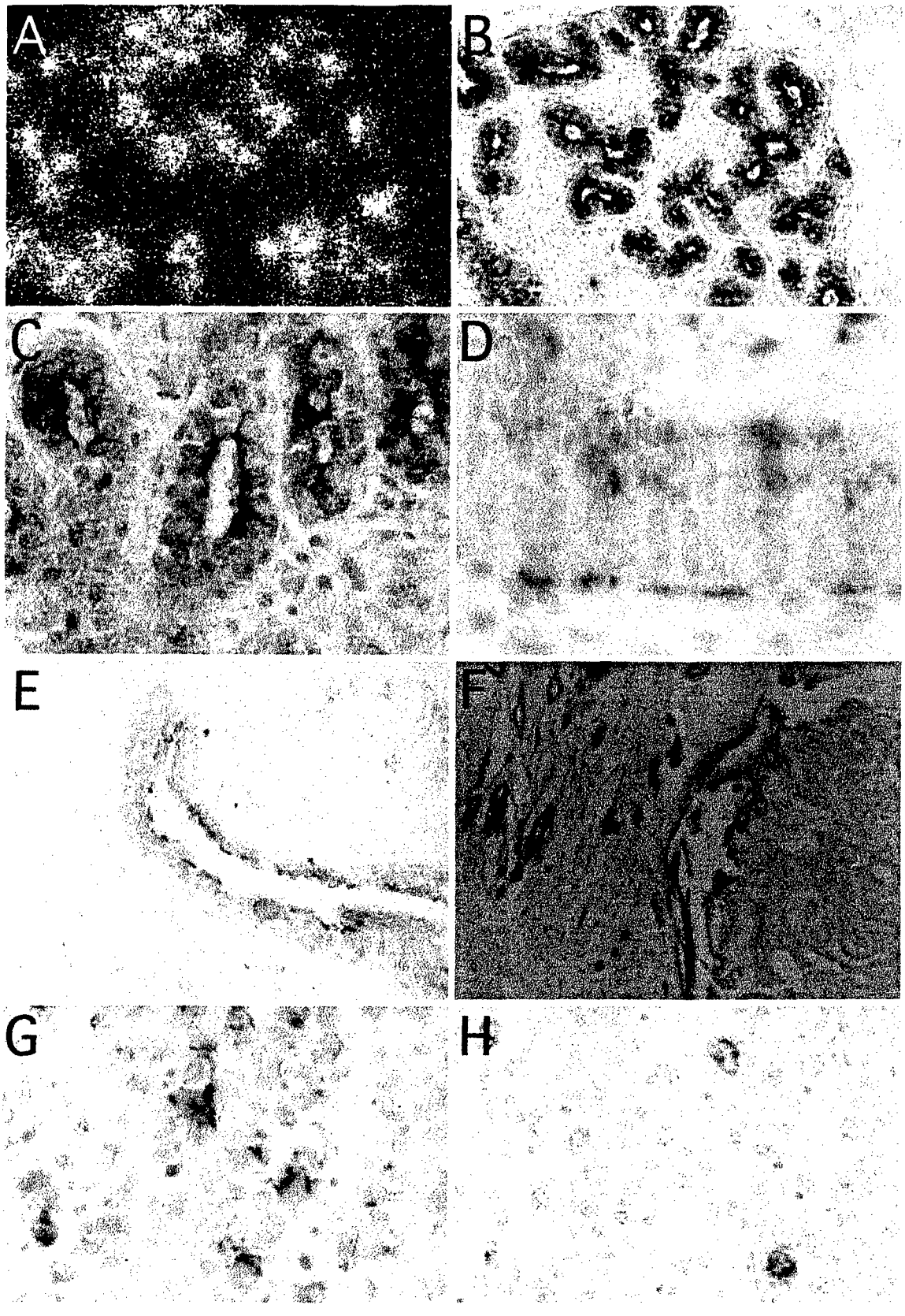

Figure 2. Characterization of the rat anti-human matrilysin antibody and localization in human breast cancer cases. A. In situ hybridization of matrilysin on a section human breast tissue from a reduction mammoplasty. B. A serial section from (A) stained with the monoclonal antibody against human matrilysin showing colocalization and specificity. C. High power view of the localization of matrilysin in panel B, notice the apical staining in the epithelium. D. Localization of matrilysin in human endometrium (late proliferative phase), notice the distinct basal localization along with some apical production indicating regulated secretion. E and F. Matrilysin localization in normal ducts and glands adjacent to expanding lesions (generally papilomas) in the breast. G. Matrilysin staining

of an MDA-468 tumor xenograft, notice theundirected secretion pattern associated with this higher grade tumor. Although most of the tumor produces the mRNA (Figure 1D) matrilysin protein iwas only detected in about 40% of the cells. H. Matrilysin staing in an invasive human breast carcinoma with staining very similar to that in the MDA-468 xenografts.

it might be TGFα which is produced by MDA-468 and has been shown to induce stromelysin-1 in fibroblasts. However, the factor does not appear to be TGFα beacuse neutralizing antibodies to TGFα do not block the inductive activity of MDA-468 conditioned media (data not shown). At this point, the identity of the diffusable factor is un known, but it size is between 30 and 60 kDa based on Centricon filtration. We cannot rule out the possibility that tumor growth and invasion can cause a stromal wound response which would include the induction of MMPs by fibroblasts. The in vitro data indicate that factor secreted by the tumor cells is at least partially responsible for the host expression of MMPs.

We have begun to investigate matrilysin protein production in archival samples of human breast tumors. This involved the production of a new matrilysin antibody for use in immunohistochemistry. A single monoclonal antibody was found to stain a known matrilysin producing tumor and subsequent western analysis indicated that it does recognize human matrilysin (Figure 2).

We have stained a series of 30 patient samples of various histologies and analysis of these samples is ongoing (Table 2). These data have shown that approximately 80% of these patient samples contain some matrilysin staining. This staining can be broken down into two catagories: within the tumor cells and in normal epithelium near a tumor border. In most cases there was either normal or tumor staining, but not both, with the exception being medulary carcinomas. There was a trend in switching of matrilysin localization with hyperplastic leasions only staining in the normal epithelium and the majority of the in situ ductal carcinomas (DCIS) staining the tumor cells. This differential staining in DCIS may be a potential stratificaion mechanism for determining the level of progression of a DCIS lesion.

Table 2. Summary of matrilysin staining of human breast neoplasms.

Diagnosis	*Matrilysin positive*	*Matrilysin negative*	*In Normal epithelium*	*In neoplastic tissue*
DCIS	10	3	4	6
Medulary Ca	3	1	3	2
Complex Sclerosing lesion	5	1	3	2
Hyperplastic lesions	4	0	4	0

One of the more interesting observations from these studies is that matrilysin appears to be secreted apically from polarized neoplastic epithelium (Figure 2). This was surprising since the primary substrates of matrilysin are components of the BM. This localization indicates that matrilysin may function differently than previously expected, potentially processing membrane bound proteins to a soluble form at the lumenal cell surface. Data from another grantee in our laboratory indicates that in a transgenic mouse model of breast cancer that matrilysin production can hasten the onset of tumors in the MMTV-Neu mouse system. It was theorized that matrilysin may be releasing growth factors from either the cell surface or the extracellular matrix. We have evidence from another system that matrilysin may function in the apoptotic pathway.

Matrilysin is induced in the rodent prostate during castration induce involution. With the observation that matrilysin was secreted into the lumen of noraml breast epithelium, we decided that this may focus our search for a lumenal substrate for matilysin. Apoptosis in the involution of the prostate is partially dependant on the Fas/Fas ligand system and it was known that TNFα was released from the cell surface by matrilysin. TNFα and FasL are in the same family of transmembrane proteins and both can intduce apoptosis in different systems. We therefore asked the question: Is FasL a substrate of matrilysin? We looked at the consensus cleavege site for matrilysin and found that there was a site in FasL near the transmembrane region that is highly conserved between human, rat and mouse (Figure 3 A and B). This was highly suggestive that FasL could be a substrate. We then performed an in vitro cleavage assay and found that matrilysin could cleave recombinant FasL in a dose dependent manner (Figure 3C).

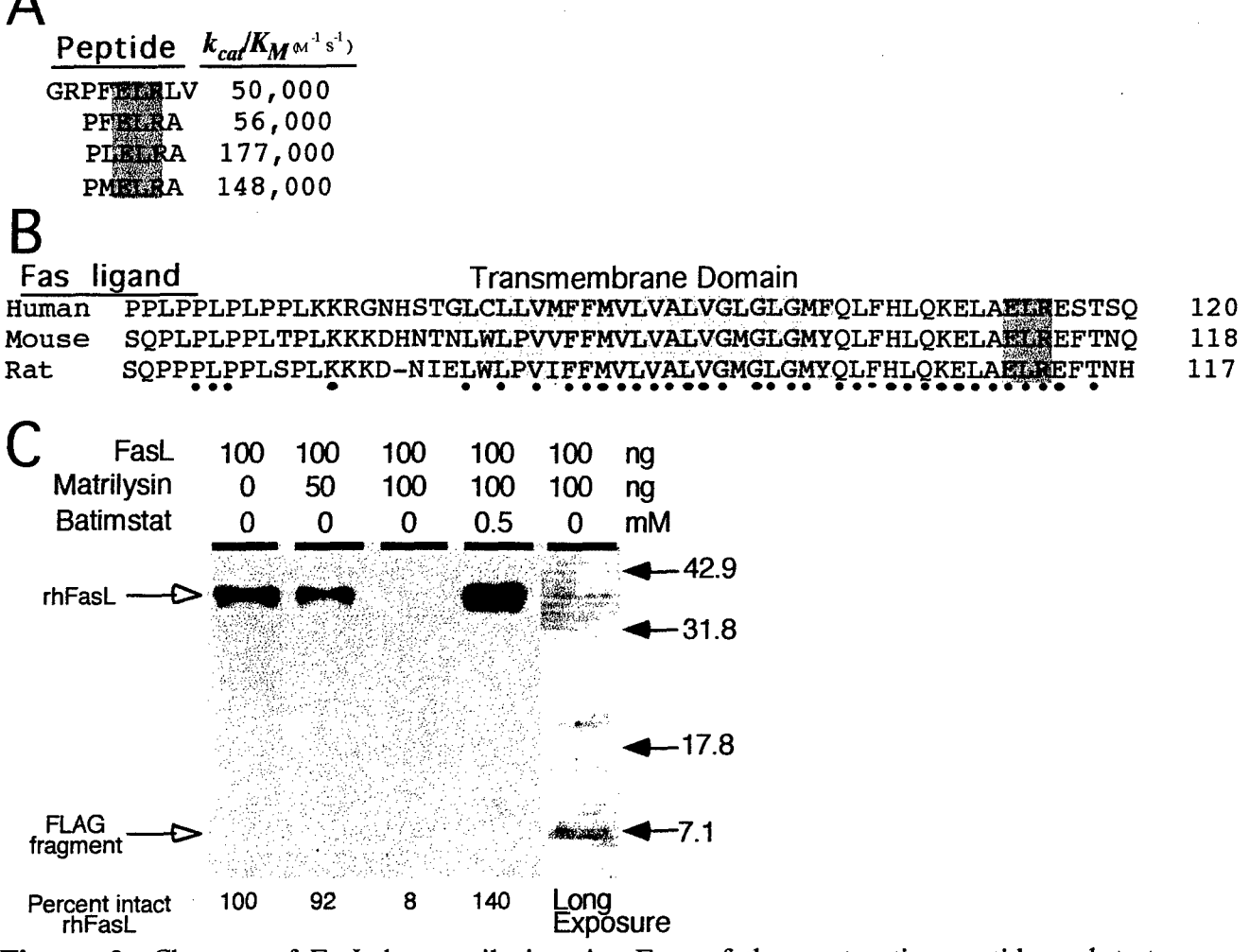

Figure 3. Cleavage of FasL by matrilysin. A. Four of the most active peptides substrates identified by phage display were aligned to indicate the core homology at the cleavage site and the *kcat/KM* values are given 13. B. Human, mouse and rat FasL peptide sequences were aligned to identify potential matrilysin cleavage sites in the extracellular domain of FasL. The core sequence identified by Smith et al. 13(dark shading) was present 10 amino acids from the transmembrane domain (light shading) in a highly conserved portion of the protein. The symbol ● indicates conserved amino acids among human, rat and mouse FasL. C. 100 ng of N-terminal FLAG-tagged rhFasL was incubated with varying amounts of APMA activated matrilysin with or without 0.5 mM Batimastat for 30 minutes at 37°. The reactions were analyzed by western blotting for the FLAG-tag on the rhFasL. The FLAG-tagged fragment was apparent upon a longer exposure. The amount of intact rhFasL in matrilysin treated samples relative to the untreated sample was determined by densitometry and the percent remaining is indicated at the bottom of each lane.

This cleavage was inhibited by the spcific MMP inhibitor Batimastat. Experiments are ongoing to determine the precise role of matrilyin in the involution of the prostate. This data supports the theory that matrilysin may function as a growth factor releasing enzyme for epithelial cells and the effects are dependant on the system and stimulus that induces matrilysin. Thus, matrilysin expression in the normal breast epithelium near an expanding tumor may represent the mechanical stress placed upon those cells and an attempt to initiate the apoptotic pathway. Whereas, matrilysin expression in the tumor may function to give a growth advantage by releasing growthfactors and cytokines from the matrix or cell surface.

Summary

We have been able to show using species specific in situ probes that MMPs can be used as indicators of EMT and progression in human beast tumor cell lines and may be useful in determining the level of progression of human tumors based on the number and types of MMPs

produced by tumor cells. Our data clearly show that the normal stromal tissue surrounding a tumor can respond to the presence of a tumor with the production of other stromally derived MMPs. This may be in response to growth factors and cytokines produced by the tumor or a failed attempt to repair a wound in the tissue; this expression could favor local and metastatic spread of the tumor. New insights into matrilysin expression in human breast tumors indicates that both normal and neoplastic cells can produce matrilysin protein and secrete it apically and this localization may point to a novel function for matrilysin in normal and neoplastic breast epithelium.

The expression and localization of MMPs in this model of breast cancer indicates that there is potential for the use of MMPs in the determination of tumor progression. The switch in localization of the stromal MMPs is of particular interest. Previous studies have indicated that stromal MMPs are expressed in tumor cells as they become more mesenchymal like. The use of species specific probes has allowed us to demonstrate that there may be a difference in the stroma intermixed with the tumor and the stroma surrounding the tumor. The effects on intratumor stroma may be mediated by cell-cell contact whereas the alterations at the host /tumor interface may be due to growth factors and cytokines produced by the tumor cells or a wound response to the growing neoplasm. We extended these studies by focusing on the one MMP that is produced by normal epithelium and neoplasms derived from epithelium., matrilysin. We have produced a rat monoclonal antibody to human matrilysin which recognizes both the latent and activated forms of matrilysin from both human and mouse. This antibody has provided significant insight to the role that matrilysin plays in both normal epithelium and tumors. In normal epithelium matrilysin is secreted into the lumen and is proposed to process proteins in the mammary fluid or on the apical surface of the epithelial cells. This is contrary to what would be expected for a matrix metalloproteinase designed to degrade extracellular matrix molecules in the stroma and basement membrane. In the breast this may be a rather benign function since we saw expression in samples from breast reductions. However our laboratory does have evidence that over expression of matrilysin can speed the onset of mammary tumors in transgenic mice containing the Neu transgene (Lori Rudolph). In the limited number of human cases we have studied matrilysin appears to be increasingly produced by tumor cells as progression proceeds. The loss of differentiation and polarity has the affect of switching the substrates available to matrilysin from lumenal proteins to matrix proteins which could give the unpolarized tumor cells an invasive advantage in breaching the basement membrane and invading locally. A more extensive IHC study of human breast tumors is underway in a case/control study at our institution. The finding that FasL is a substrate for matrilysin is an important observation, not only for breast cancer but also other tumors. We have evidence that matrilysin and FasL are expressed in the same cells in the prostate during involution and that this coexpression has a functional consequence in the rate of apoptosis of prostate epithelial cells. Thus, the new localization of matrilysin has opened a number of avenues for investigation into the non-matrix degrading effects of MMPs in breast and other tumors.

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