Matriptase and HAI-1 Are Expressed by Normal and Malignant Epithelial Cells *in Vitro* and *in Vivo*

Michael Oberst, Joanna Anders, Bin Xie,
Baljit Singh, Miguel Ossandon, Michael Johnson,
Robert B. Dickson, and Chen-Yong Lin

From the Lombardi Cancer Center, Georgetown University
Medical Center, Washington, District of Columbia

Matriptase and its cognate, Kunitz-type serine protease inhibitor, HAI-1, comprise a newly characterized extracellular matrix-degrading protease system that may function as an epithelial membrane activator for other proteases and latent growth factors. Both enzyme and inhibitor have been detected in breast cancer cells, immortalized mammary epithelial cells, and human milk, but not in cultured fibroblasts nor in fibrosarcoma cells. To test the hypothesis that this system is expressed by normal breast epithelium, invasive breast cancers, and other cancers of an epithelial origin (carcinomas) but not in cancers of a mesenchymal origin, we have expanded our expression analysis of matriptase and HAI-1 *in vitro* and *in vivo*. Matriptase and HAI-1 were detected at the protein and mRNA levels both in hormone-dependent and hormone-independent cultured breast cancer cells, and this expression correlated with the expression of the epithelial markers E-cadherin or ZO-1. However, none of the breast cancer cell lines tested that express the mesenchymal marker vimentin express matriptase or HAI-1, consistent with an epithelial-selective expression of this system. Expression of matriptase, as determined by Western blot analysis, was observed in primary human breast, gynecological, and colon carcinomas, but not in stromal-derived ovarian tumors and human sarcomas of various origins and histological grades. The epithelial-selective expression of matriptase and HAI-1 was further confirmed in human breast cancers by immunohistochemistry and *in situ* hybridization, where the expression of the protease and the inhibitor were found in the carcinoma cells and in surrounding normal breast epithelia. The expression of the matriptase/HAI-1 system by malignant epithelial cells *in vivo* suggests a possible role for this protease in multiple aspects of the pathophysiology of epithelial malignancy, including invasion and metastasis. (Am J Pathol 2001, 158:1301–1311)

It has long been proposed that metastasis is a multistep process. This includes the breakdown of the basement membrane, detachment of cancer cells from the primary tumor, invasion into the stroma, intravasation into blood vessels, survival in the blood stream, extravasation through target organ blood vessels, and the establishment and proliferation of cancer cells in remote tissues. To accomplish these events, cancer cells must acquire an enhanced ability to migrate through and degrade extracellular matrix components. An array of extracellular matrix-degrading proteases and cell motility factors have been characterized and implicated in cancer invasion and metastasis.¹ Among the protease systems, the plasmin/urokinase-type plasminogen activator (uPA) system,^{2–6} and the matrix metalloproteases^{7–12} have received the most attention. Although these extracellular matrix-degrading proteases have been implicated in breast cancer invasion and metastasis, they are mainly expressed by stromal components of human breast tumors.^{9,11,13–16} The stromal origins of these extracellular matrix-degrading proteases in breast cancer suggests that malignant invasion is an event that depends at least in part on a stromal-epithelial interaction.¹⁷ Furthermore, growth and motility factors secreted by stromal cells may also contribute to the ability of cancer cells to migrate through the extracellular matrix. Hepatocyte growth factor (HGF)/scattering factor (SF) is one of these mesenchymal cell-derived proteins. On binding to the c-Met receptor on the surfaces of epithelial cells, HGF can dissociate epithelial colonies and scatter cells. This activity is thought to be important in the modulation of cancer cell motility and invasion.^{18–20} Matriptase has been shown to activate the latent form of HGF/SF to produce the active growth and motility factor that can bind to and activate the c-Met receptor.²¹ In addition, matriptase has been characterized as an *in vitro* activator of uPA, linking it to the activation of other protease systems important for cancer cell invasion and metastasis.²²

To test the hypothesis that epithelial-derived cancer cells within a tumor may be a major source of the synthesis and presentation of a protease(s) important for multiple aspects of tumor behavior, including growth and

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Address reprint requests to Dr. Chen-Yong Lin, Lombardi Cancer Center, Georgetown University Medical Center 3970 Reservoir Rd. NW, Washington, DC 20007. E-mail: lincy@gunet.georgetown.edu.

metastasis, we have isolated and characterized a membrane-bound, trypsin-like, serine protease termed matriptase and have identified its integral membrane, Kunitz-type inhibitor, called HAI-1 (hepatocyte growth factor activator inhibitor-1) from T-47D human breast cancer cells and from human milk.^{23–26} Matriptase is a mosaic, transmembrane, trypsin-like serine protease with two potential regulatory modules: two tandem repeats of a CUB (C1r/s, Uegf, and bone morphogenic protein-1) domain and four tandem repeats of a LDL receptor domain²⁵ (also see updated sequence, GenBank accession no. AF118224). This protease is identical in sequence to the protease termed the membrane-type serine protease-1, MT-SP1,²⁷ and has a high percentage of sequence identity with the mouse serine protease epithin,²⁸ the apparent mouse homologue of matriptase. Matriptase was detected in some breast cancer cell lines and in immortalized luminal epithelial cells of the mammary gland, but not in cultured fibroblasts nor in HT1080 fibrosarcoma cells.²⁶ Thus, it was proposed that matriptase is produced by epithelial cells *in vivo*. The matriptase inhibitor, an integral membrane serine protease inhibitor with two Kunitz domains separated by an LDL receptor domain, was initially identified as an inhibitor (HAI-1) of hepatocyte growth factor activator.²⁹ This inhibitor is expressed primarily by simple columnar epithelium in multiple human tissues *in vivo*.³⁰

In this study, we characterize the expression of matriptase in human primary breast cancer and in human breast cancer cell lines. Data from the breast cancer cell lines suggest that the expression of matriptase correlates with markers of an epithelial phenotype. Results from primary tumors indicate that the protease is expressed by tumors of epithelial origin. Matriptase is expressed predominantly by the epithelial elements of carcinomas and not by fibroblasts. The *in vivo* epithelial origin of matriptase and its expression by breast cancer cells highlight the potential role of matriptase in the activation of latent growth factors and proteases at the breast epithelial cell surface and in the development, growth, invasion, and metastasis of breast cancer.

Materials and Methods

Cell Lines and Culture Conditions

All breast and ovarian cancer cell lines were obtained from the Lombardi Cancer Center Tissue Culture Shared Resources. Cells were maintained in culture by growth in Iscove's minimal essential media (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum at 37°C and 5% CO₂, with the exception of the MCF-10A cell line, grown in Dulbecco's modified Eagle's medium/HAM F-12 (Life Technologies, Inc.) supplemented with 0.5% fetal bovine serum, 0.5 mg/ml hydrocortisone, 10 µg/ml insulin, and 10 ng/ml epidermal growth factor, and the 184 A1N4 human mammary epithelial cell line, grown in Iscove's minimal essential media supplemented with 5% fetal bovine serum, 0.5 mg/ml hydrocortisone, 5 µg/ml insulin, and 20 ng/ml epidermal growth factor.

Monoclonal Antibodies (mAbs)

Anti-matriptase and anti-HAI-1 mAbs were prepared as described previously.^{24,26} Immunohistochemistry-competent anti-matriptase mAb S5 (IgG1) was prepared by hybridoma technology using formalin-treated matriptase, isolated from T-47D cells, as immunogen. A panel of mAbs was selected by its ability to stain paraffin-embedded breast cancer sections. Monoclonal Ab S5 was selected from these mAbs for its ability to recognize matriptase by immunoblot.

Extraction of Proteins from Frozen Human Tumors

Frozen human tumors from various sites were obtained from the Histopathology and Tissue Shared Resource at the Lombardi Cancer Center, Georgetown University. The tumor specimens were kept frozen with liquid nitrogen and ground to a fine powder by mortar and pestle. The specimens were extracted using RIPA buffer (150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), and 50 mmol/L Tris, pH 8.0). The insoluble debris was removed by centrifugation, and the protein concentration was determined by bicinchoninic acid protein assay (Pierce, Rockford, IL).

Western Blotting

Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred overnight to polyvinylidene fluoride, and subsequently probed with mAbs as indicated. Immunoreactive polypeptides were visualized using horseradish peroxidase-labeled secondary antibodies and the ECL detection system (New England Nuclear, Boston, MA).

Northern Blotting

Total RNA was extracted from cell lines with RNazol reagent (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. Ten micrograms of total RNA from each cell line was resolved by electrophoresis on a 1.2% phosphate-buffered agarose gel containing 2 mol/L formaldehyde. RNA was subsequently transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) and hybridized to ³²P-labeled riboprobes at 65°C for 36 hours, followed by three washes in 0.1× standard saline citrate (SSC)/0.1% SDS at 80°C for 30 minutes each to remove unbound probe. To generate the labeled riboprobes, the coding sequences of matriptase and HAI-1 were cloned into the pcDNA3.1 vector (Invitrogen, San Diego, CA), and were linearized with an appropriate restriction enzyme just 5' of the coding sequences. These linearized vectors were used in *in vitro* SP6 RNA polymerase reactions with ³²P-UTP (3,000 Ci/mmol; New England Nuclear) to generate labeled antisense riboprobes. To control for approximately equal loading of RNA from each cell line, labeled riboprobes directed against the message of the ribo-

somal protein 36B4 were generated in a similar manner, except that a vector containing ~500 bp of the coding sequence was used. Signals on hybridized membranes were visualized by exposure to X-OMAT AR imaging film (Eastman-Kodak, Rochester, NY) for 4 to 12 hours at -80°C.

Immunohistochemistry

Paraffin-embedded sections of human primary breast cancers were obtained from the Histopathology and Tissue Shared Resource at the Lombardi Cancer Center, Georgetown University. Briefly, 5- μ m tumor sections were heated in an oven to 56°C for 3 hours and then dewaxed in xylene. Slides were then rehydrated by immersion in a decreasing gradient of ethanol in water. Endogenous peroxidase activity was quenched by immersion in 1.5% H₂O₂/methanol for 10 minutes, followed by washes in water and phosphate-buffered saline (PBS). Sections were blocked for 30 minutes in blocking buffer (2% goat serum/5% bovine serum albumin in PBS) before incubation with the primary antibody. Sections were incubated in the presence of the matriptase-specific mAb clone S5 (IgG1) at a concentration of 1 μ g/ml, the HAI-1-specific mAb clone M58 (IgG1) at a concentration of 5 μ g/ml or mouse IgG1 at a concentration of 5 μ g/ml in blocking buffer for 1 hour at room temperature. After incubation in the primary antibody, sections were washed in PBS to remove unbound antibody, and then were incubated with a biotinylated goat anti-mouse secondary antibody. After washes in PBS, the staining was completed by incubation with streptavidin-horseradish peroxidase and diaminobenzidine colorimetric reagents from the BioGenex immunohistochemistry kit (San Ramon, CA) according to the manufacturer's protocol. As a control, nonrelevant mouse IgG1 was used in place of the specific mAbs at the equivalent dilutions. The colorimetric reaction for the control slides was developed for the same amount of time as the experimental slides, and did not show any development of the color reagent.

In Situ Hybridization

Probes for use in *in situ* hybridization were prepared by generating digoxigenin-labeled sense and antisense RNA riboprobes using the Dig-RNA labeling kit (Boehringer-Mannheim, Mannheim, Germany) according to a modified manufacturer's protocol. Briefly, a 650-bp *Bam*HI-*Sac*II fragment of the matriptase sequence corresponding to the 5' end of the matriptase cDNA was cloned into the pBluescript SK-vector (Stratagene, La Jolla, CA). This vector was subsequently linearized with *Sac*II or *Bam*HI and used as a template for the synthesis of sense and antisense digoxigenin-labeled riboprobes, respectively, with T7 or T3 RNA polymerase (Life Technologies, Inc.), according to the manufacturer's protocol, using digoxigenin-11-UTP. Synthesized probes were purified by G50 column chromatography to remove unincorporated nucleotides, including unincorporated digoxigenin-11-UTP. The concentration of the labeled riboprobes

was determined spectrophotometrically. The accuracy of the concentration assignment was confirmed by analysis of the riboprobes by 1% agarose/2 mol/L formaldehyde gel electrophoresis, followed by ethidium bromide staining. The equal efficiency of digoxigenin incorporation into sense and antisense probes was confirmed by dot-blotting of equal amounts of probe onto Hybond-N nylon membranes, followed by detection of labeled riboprobe with an alkaline-phosphatase-conjugated anti-digoxigenin antibody and colorimetric substrate (not shown). In addition, the efficiency of digoxigenin incorporation was confirmed by dot blotting equal amounts of denatured double-stranded vector containing the full-length sequence of matriptase and probing these blots with digoxigenin-labeled sense or antisense probes for matriptase. Equal signals were observed for equal amounts of sense or antisense probe used in the hybridization to membrane-bound plasmid (not shown). For detection of matriptase mRNA in paraffin-embedded breast cancer sections, 20 ng of labeled sense or antisense riboprobe was used in a standard protocol provided by Boehringer Mannheim. Briefly, 5- μ m paraffin-embedded breast cancer tissue sections were deparaffinized, rehydrated, treated with 0.2 mol/L HCl, permeabilized with proteinase K, and postfixed with 4% paraformaldehyde before prehybridization in 50% formamide/1 $\times$ SSC at 65°C and hybridization at 65°C in hybridization buffer for 12 hours in a humidified chamber. After hybridization, unbound probe was removed by two washes in 2 $\times$ SSC, two washes in 1 $\times$ SSC, and two washes in 0.1 $\times$ SSC at 42°C. Bound probe was detected by use of an alkaline-phosphatase-conjugated anti-digoxigenin antibody that produces an insoluble blue precipitate in the presence of a nitroterazolum blue/X-phosphate color solution. Sense and antisense probes were hybridized and washed under identical conditions, and the colorimetric reactions were stopped at the same time for sense and antisense hybridized sections.

Results

In Vitro Expression of Matriptase and HAI-1 Correlates with the Expression of Epithelial Markers in Breast Cancer Cell Lines

To determine the expression of matriptase and HAI-1 in breast cancer cell lines, Northern blotting was performed using RNA from a panel of breast cancer cell lines (Figure 1). Expression of matriptase and HAI-1 in human breast cancer cell lines was completely concordant; matriptase and HAI-1 were found in four of four estrogen receptor-positive (ER+) and three of nine estrogen receptor-negative (ER-) breast cancer cell lines. Both were expressed in two of two ER- immortalized breast epithelial cell lines tested. Neither was expressed by a primary breast fibroblast cell line (data not shown). Matriptase and HAI-1 were detected in one of three human ovarian cancer cell lines tested (Figure 1). The expression of matriptase and HAI-1, or lack thereof, by all of the cell

Figure 1. Expression analysis of matriptase and HAI-1 in immortalized human breast epithelial, human breast cancer, and ovarian cancer cell lines. Ten micrograms of total RNA were examined for each breast or ovarian cell line by Northern blot analysis using matriptase-specific (**A**) or HAI-1-specific (**B**) riboprobes. Analysis of cell lines included two immortalized breast epithelial lines (MCF-10A and A1N4), four ER+ breast cancer cell lines (MCF-7, ZR-75-1, T47D, BT474), nine ER- breast cancer cell lines (SKBR3, MDA-MB-468, -453, -436, -435, -157, and -231, BT549, and Hs578t), and three ovarian cancer cell lines (SKOV3, PA-1, and OVCAR-3). Matriptase expression always correlated with HAI-1 expression, and both were found in two of two immortalized breast epithelial cell lines, four of four ER+ breast cancer cell lines, three of nine ER- breast cancer cell lines, and in one of three ovarian cancer cell lines.

lines was confirmed at the protein level by Western blot analysis (data not shown).

When the expression of matriptase and HAI-1 were compared with markers of an epithelial morphology (E-cadherin, ZO-1) or of a generally mesenchymal morphology (vimentin), the expression of the two proteins correlated with epithelial cell markers (Table 1) and never with vimentin.³⁴⁻³⁶ The protease and inhibitor were found in

all ER+ cell lines and in a smaller number of ER- cell lines tested. However, this trend toward expression in ER+ tumor cells and absence in ER- cells was not observed in our studies of primary breast tumors (see below). In primary breast tumors, the expression of both have been found in both ER+ and ER- tumors, with no trend toward either an ER+ or ER- status. Matriptase and HAI-1 do not seem to be regulated at the transcrip-

Table 1. Expression of Matriptase and HAI-1 in Breast Cancer Cell Lines and Comparison with Markers of Differentiation and *in Vitro* Invasiveness

Cell line	ER	Matriptase	HAI-1	E-cadherin	ZO-1	Vimentin	<i>In vitro</i> invasion*
A1N4	-	+	+	NA	NA	NA	NA
MCF-10A	-	+	+	NA	NA	NA	NA
MCF-7	+	+	+	+	+	-	+
T47D	+	+	+	+	+	-	+
ZR-75-1	+	+	+	+	-	-	+
BT-474	+	+	+	+	-	-	+
SKBR3	-	+	+	-	+	-	+
MDA-MB-468	-	+	+	-	-	-	+
MDA-MB-453	-	+	+	-	-	-	+
MDA-MB-157	-	-	-	-	NA	NA	NA
MDA-MB-436	-	-	-	-	+	+	++
MDA-MB-435	-	-	-	-	-	+	++
MDA-MB-231	-	-	-	-	-	+	+++
BT-549	-	-	-	-	-	+	+++
Hs578t	-	-	-	-	-	+	+++

The expression of matriptase and HAI-1 in immortalized human breast epithelial cell lines and cancer cell lines as determined in Figure 1 are compared with the expression of E-cadherin and zona occludens-1 (ZO-1)—markers typical of an epithelial differentiation, and vimentin—a marker typical of mesenchymal differentiation, as determined by others.³⁴⁻³⁶

Matriptase and HAI-1 are expressed in the same cells that express either E-cadherin or ZO-1, or which lack expression of vimentin.

Abbreviations: ER, estrogen receptor status; NA, data not available.

**In vitro* invasiveness as assessed *in vitro* by invasion into the extracellular matrix preparation Matrigel, as determined by Sommers et al.³⁴

Figure 2. Expression analysis of matriptase in mammary tissues. Samples of proteins were extracted using RIPA buffer from normal breast tissue surrounding the breast tumor of three different patients (**lanes 13 to 15**) and tumors of 10 different patients (**lanes 3 to 12**). Proteins (50 μ g per lane) were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and probed by anti-matriptase mAb 21-9 (**A**) and anti-HAI-1 mAb M19 (**B**). The positions of matriptase (70 kd), HAI-1 (55-kd membrane-bound form and 50-kd fragment), and the 95-kd matriptase/HAI-1 complex were indicated according to the samples from the cell-conditioned medium (**lanes 1**) and the membrane fractions (**lanes 2**) of T-47D breast cancer cells.

tional level by estrogen or progesterone, as no change in mRNA levels for matriptase or HAI-1 were observed in estrogen-depleted MCF-7 cells when treated with 100 nmol/L 17 β -estradiol or with 100 nmol/L 17 β -estradiol plus 100 nmol/L progesterone (data not shown).

Expression Analyses of Matriptase and HAI-1 by Immunoblot Analysis of Human Primary Tumors

The correlation between matriptase/HAI-1 expression and epithelial markers in cultured cells suggests that the matriptase/HAI-1 system could also be expressed *in vivo* by normal epithelial cells and by epithelium-derived cancer cells. To test this hypothesis, we examined various epithelium-derived and nonepithelium-derived frozen human tumor specimens and the surrounding tissues of these tumors by protein immunoblotting. The epithelium-

derived tumors included 10 breast, nine ovarian, four uterine, and eight colon carcinomas. Noncancerous tissues surrounding these carcinomas were also included in the lysates tested. Nonepithelial tumors included three stroma-derived ovarian tumors and 10 sarcomas with various origins and histological grades.

Breast Tumors

Expression of matriptase in 10 infiltrating human breast carcinomas (nine ductal carcinomas and one colloid carcinoma; five were ER+ specimens and five ER-) was examined by Western blot and compared to three samples of noncancerous breast tissue surrounding a tumor. In these surrounding normal tissues, matriptase was detected at very low levels or below the detection sensitivity (Figure 2A, lanes 13 to 15). In contrast, higher levels of expression of matriptase was observed in all 10 of the primary breast carcinomas examined (Figure 2A, lanes 3 to 12), consistent with the higher epithelial cell-derived component of these specimens. The expression of HAI-1 was also observed in these 10 human breast specimens, but under the detection sensitivity by Western blot in the surrounding noncancerous tissue, which is composed primarily of stromal (nonepithelial) elements. Expression of HAI-1 fluctuated widely among these specimens (Figure 2B).

Gynecological Tumors

We further investigated the expression of matriptase in gynecological tumors (Figure 3). There are more than 25 major types of ovarian neoplasms. These are classified into three groups based on cell of origin: those of the germinal surface epithelium, the gonadal stroma, and germ cells. We examined ovarian tumors both of epithelial origin and of stromal origin. Among the nine tumors of epithelial origin, matriptase was detected at moderate to high levels (Figure 3A, lanes 3 to 11). In contrast,

Figure 3. Expression analysis of matriptase in gynecological tumors. **A** and **B**: Samples of proteins (50 μ g per lane), which were extracted by RIPA buffer from nine ovarian carcinomas (**lanes 3 to 11**) and three stromal-derived tumors, including two fibrothecomas (**lanes 12 and 13**) and one granulosa cell tumor (**lane 14**), were analyzed by immunoblot using anti-matriptase mAb 21-9 and anti-HAI-1 mAb M19. The positions of matriptase, HAI-1 and the 95-kd matriptase/HAI-1 complex were indicated according to the samples from the cell-conditioned medium (**lanes 1**) and the membrane fractions (**lanes 2**) of T-47D cells. **C** and **D**: Samples of proteins from four uterine carcinomas (**lanes 3, 5, 7, and 8**) and two patient-matched normal tissues surrounding tumors (**lanes 4 and 6**) were probed by immunoblot using anti-matriptase mAb 21-9 and anti-HAI-1 mAb M19. The positions of matriptase, HAI-1, and the 95-kd matriptase/HAI-1 complex are indicated, as described above.

A. Matriptase

B. HAI-I

Figure 4. Expression analysis of matriptase in human colon tumors. Protein samples (50 μ g per lane) that were extracted by RIPA buffer from nine colon carcinomas (lanes 3 to 10) and four normal colon specimens (lanes 11 to 14) were examined by Western blot using anti-matriptase mAb 21-9 (A) and anti-HAI-1 mAb M19 (B). The positions of matriptase, HAI-1, and its 95-kD matriptase/HAI-1 complex, were indicated according to the samples from the cell-conditioned medium (lanes 1) and the membrane fractions (lanes 2) of T-47D cells.

matriptase was not detected in three sex cord/stromal tumors, including a granulosa cell tumor (Figure 3A, lane 14), and two fibrothecomas (Figure 3A, lanes 11 and 12). The negative results in sex cord/stromal tumors again suggest that expression of matriptase is restricted to tumors of an epithelial origin. Expression of HAI-1 varied widely among these ovarian carcinomas (Figure 3B, lanes 3 to 11). In one of these specimens, HAI-1 was below the detection sensitivity (Figure 3B, lane 10), whereas matriptase was detected at a high level (Figure 3A, lane 10). HAI-1 was not detected in the three sex cord/stromal tumors. A minor, nonspecific band with a migration similar to that of HAI-1 could not be depleted with anti-HAI-1 mAb; this band was also observed in both matriptase and HAI-1 Western blots (Figure 3, A and B; lanes 12 to 14).

We also examined the expression of matriptase and HAI-1 in four uterine carcinomas (Figure 3, C and D; lanes 3, 5, 7, and 8) and in two patient-matched normal tissues (Figure 3, C and D; lanes 4 and 6). Expression of matriptase was observed strongly in three of four (Figure 3C; lanes 3, 5, and 7), and weakly in one of four (Figure 3C, lane 8) cancer specimens, whereas the two normal tissues were below the detection limit (Figure 3C, lanes 4 and 6). HAI-1 expression was observed at a high level in one specimen (Figure 3D, lane 5) and at low levels in the other three specimens (Figure 3C; lanes 3, 7, and 8).

Colon Tumors

Eight colon carcinoma specimens (Figure 4A, lanes 3 to 10) and five normal colon specimens (Figure 4A, lanes 11 to 14) were also examined. Expression of matriptase fluctuated among colon carcinomas as well as among normal colon tissues. In contrast to breast and gynecological carcinomas, expression of matriptase in some normal colon tissues was as high as that seen in cancer specimens, consistent with the high percentage of epithelial cells present in normal colon tissues relative to that of normal breast and ovarian tissue. Expression of HAI-1 also varied among these specimens (Figure 4B), but tended to correlate with matriptase expression.

Sarcomas

Ten human sarcomas were examined for the expression of matriptase (data not shown). These included three high-grade osteosarcomas, three well-differentiated (low grade) liposarcomas, two malignant fibrous histiocytomas that were clinically metastatic, one dermatofibrosarcoma protuberance (a low-grade sarcoma), and one high-grade leiomyosarcoma that was most likely of uterine origin. Matriptase was below the detection limit or barely detectable for all of the sarcomas. HAI-1 was below the detection limit for all 10 sarcomas.

From this preliminary screening, matriptase was detected in all 31 human carcinomas tested. In contrast, expression of matriptase and HAI-1 was negligible or not detected in all of the 13 nonepithelial tumors tested. These results suggest that matriptase is selectively expressed in epithelium-derived tumors *in vivo*, consistent with the observation that matriptase was detected in cultured cells that tended to express epithelial markers, but not in cells with a mesenchymal marker. Matriptase and HAI-1 are found at higher levels in breast and gynecological cancer tissue when compared to normal surrounding tissue; however, this is likely because of the increased epithelial cellularity of cancer tissue *versus* normal tissue. This observation is supported by the fact that matriptase was detected at high levels in some normal colon tissues for which the epithelial element represents a major portion of normal colon tissue.

Matriptase Protein and mRNA Are Detected in Normal and Cancerous Epithelial Cells in Human Breast Tumor Sections

To further determine which cell types express matriptase protein and mRNA in primary tumor specimens, immunohistochemistry and *in situ* hybridization using digoxigenin-labeled riboprobes were performed using formalin-fixed, paraffin-embedded human breast carcinomas. Matriptase protein was detected in breast cancer cells (Figure 5; A, B, and C) as well as in surrounding normal breast epithelial cells with comparable intensity (Figure 6, A and B). Within normal breast epithelium and in surrounding hyperplastic ducts, the ducts stained intensely, whereas mild or no staining was observed in surrounding terminal duct lobular units (TDLUs). The localization of matriptase to the breast cancer cell component of the breast tumors was confirmed by *in situ* hybridization using digoxigenin-labeled riboprobes (Figure 7). The localization of HAI-1 protein was also determined by immunohistochemistry in the primary breast tumors and in surrounding normal breast tissue. The inhibitor co-localized with that of matriptase in the tumor cell compartment (Figure 5; D, E, and F). Within surrounding normal breast tissue, focal staining was observed for HAI-1 in the TDLUs, with no staining seen in myoepithelial cells (Figure 6, C and D). Normal ducts surrounding TDLUs show variable staining for HAI-1. These results are consistent with the expression of HAI-1 by epithelial elements of breast and other tissues as found by others.³⁰

Figure 5. Analysis of matriptase and HAI-1 protein expression in human breast carcinomas by immunohistochemistry. Human breast carcinomas were stained by immunohistochemistry using mAbs directed specifically against matriptase (S5) or HAI-1 (M58). Positive staining for matriptase and HAI-1 are observed as a brown precipitate (diaminobenzidine) within the sections, and nuclei were counterstained with hematoxylin. A metastatic breast adenocarcinoma shows both cytoplasmic and membranous staining for matriptase [original magnifications, $\times 100$ (A) and $\times 400$ (B)] and HAI-1 [original magnifications, $\times 100$ (D) and $\times 400$ (E)] in the breast epithelial cells. No staining is noted in stromal components of the tumor. A colloid breast carcinoma likewise shows a similar staining pattern for matriptase [original magnification, $\times 400$ (C)] and HAI-1 [original magnification, $\times 400$ (F)].

The subcellular localization of the immunohistochemical staining for matriptase and for HAI-1 was observed in both the cytoplasm and at the cell membrane. The latter observation is consistent with the fact that the two are integral membrane proteins, and the former may be explained by the internalization of the proteins or the synthetic pool of these molecules. These results are consistent with the localization of matriptase at the cell surface and in the cytoplasm of cultured breast cancer cells stained by immunofluorescent techniques.²³

Tissue Concentration of Matriptase in Breast Carcinomas

The tissue concentrations of matriptase in six breast carcinomas and two surrounding noncancerous breast tissues were determined by immunoblot (Figure 8). The

concentration of matriptase in the six carcinomas ranged from 13 to 24 ng/mg tissue protein, with the exception of a colloid carcinoma specimen, which contained 7 ng/mg tissue protein. The relatively low matriptase protein level in the colloid carcinoma may be explained by the high proportion of nonepithelial elements in this tumor type. The concentration of matriptase in the two normal surrounding breast tissue specimens was 2 and 3 ng/mg tissue protein, respectively. Again, this lower value in normal breast tissue is consistent with the lower epithelial representation of this tissue relative to breast carcinomas.

Discussion

Matriptase is a mosaic, transmembrane serine protease isolated from human breast milk and initially identified in

Matriptase

HAI-1

Figure 6. Analysis of matriptase and HAI-1 protein expression in normal and hyperplastic human breast epithelium by immunohistochemistry. Intense staining for matriptase is seen in the duct and mild or no staining in the surrounding terminal duct lobular units (TDLU) of this area of normal breast epithelium surrounding a breast carcinoma [original magnification, $\times 20$ (A)]. A duct with usual ductal hyperplasia shows intense staining for matriptase, and the surrounding TDLUs show mild staining [original magnification, $\times 40$ (B)]. Focal staining for HAI-1 is noted in the TDLU, and no staining is seen in the surrounding duct [original magnification, $\times 40$ (C)]. A high-power view of the same lobule shows preferential staining of the lobular cells, and no staining is seen in the myoepithelial cells [original magnification, $\times 200$ (D)].

human breast cancer cell-conditioned media by gelatin zymography.²² Matriptase is identical to the membrane-type serine protease-1 (MT-SP1), and is likely to be the human homologue of the mouse serine protease epithin based on its high degree of sequence identity and syntenic chromosomal location (human chromosome 11 and mouse chromosome 9).^{28,31} The purified serine protease domain of MT-SP1 has recently been shown to cleave and activate the urokinase plasminogen activator and the protease-activated receptor-2 (PAR-2).²² Active uPA cleaves and activates the serine protease plasmin, and this may lead to degradation of the extracellular matrix and activation of other protease systems involved in the spread of cancer cells, such as matrix metalloprotease-2 and matrix metalloprotease-9.³² In addition, matriptase can cleave HGF/SF to an active form able to activate the c-Met receptor and induce cell scattering.²¹ Many studies have implicated HGF in the growth and motility of various cell types, as well as in the angiogenesis and growth of tumors.³³ Therefore, this protease may play a role in the growth and/or invasion of human breast cancer via its activation of pro-uPA and pro-HGF. To further characterize the expression of matriptase, and its cognate inhibitor HAI-1, in breast cancer cell lines *in vitro* and

in human primary breast cancers and other cancers and normal tissues *in vivo*, we have expanded our expression analysis of matriptase in this study.

Matriptase was initially identified by gelatin zymography only in ER+ hormone-dependent breast cancer cells, including T-47D, MCF-7, and ZR-75-1 and BT474,²³ but not in the ER- hormone-independent cell lines MDA-MB-231, MDA-MB-435, MDA-MB-436, and BT-549.²³ To test the hypothesis that matriptase may be expressed exclusively by ER+ breast cancer cell lines, we screened additional ER+ and ER- cell lines for expression of matriptase and HAI-1 by Western and Northern blotting. We found that both matriptase and HAI-1 protein and mRNA are expressed in three ER- cell lines, SKBR3, MDA-MB-453, and MDA-MB-468, but not in numerous other ER- cell lines. Thus, the expression of matriptase and HAI-1 does not always correlate with the expression of the estrogen receptor in cultured breast cancer cells. The expression of matriptase and HAI-1 *in vivo* in primary breast tumors differs from the expression pattern in cultured cells in that all ER- tumors examined to date have been found to express matriptase and HAI-1, as have all ER+ tumors. Therefore, matriptase and HAI-1 expression does not seem to rely on the expression

Figure 7. Analysis of the expression of matriptase in invasive primary breast tumors by *in situ* hybridization. A matriptase-specific antisense probe (**A** and **C**) and the corresponding control sense probe (**B** and **D**, respectively) were hybridized to paraffin-embedded sections of primary breast tumors as described in Materials and Methods. The matriptase antisense probe shows reactivity with the cancer cells within the sections and lack of reactivity with stromal elements such as fibroblasts and adipocytes (**A** and **C**). Control sense probes do not show any reactivity with the breast cancer sections (**B** and **D**), demonstrating the specificity of the labeled antisense probe.

status of the estrogen receptor either *in vitro* or *in vivo*. Furthermore, both genes do not seem to be transcriptionally regulated by estrogen or progesterone, as neither is induced by the exposure of estrogen-stripped MCF-7 cells to 17β -estradiol nor to 17β -estradiol plus progesterone.

The expression of matriptase did correlate with the expression of markers of an epithelial phenotype (E-cadherin- or ZO-1-positive) and did not correlate with the expression of a mesenchymal phenotype (vimentin-positive). The co-expression of matriptase and HAI-1 negatively correlates with the previously determined *in vitro* invasion of these cells into Matrigel.³⁴ This data may be

interpreted as indicating that matriptase is not involved in augmenting the invasive phenotype of cultured breast cancer cells. However, the significance of this observation with regard to invasion *in vivo* in human carcinomas is unclear, because most of the breast cancer cell lines tested originated from pleural effusions or ascites in human cancer patients with metastatic disease, and are therefore by definition invasive. Additionally, all of the cell lines tested have been cultured for many passages, allowing significant opportunity for phenotypic and genetic drift. Furthermore, we found the expression of matriptase and HAI-1 in 10 of 10 primary invasive breast cancers examined. This observation and the fact that primary invasive breast cancers rarely express mesenchymal markers such as vimentin further suggests that the lack of expression of matriptase and HAI-1 *in vitro* in a subset of invasive breast cancer cell lines may be a tissue culture artifact.

Although no absolute correlation exists between matriptase/HAI-1 expression and estrogen receptor expression in cultured breast cancer cells, a reverse correlation between matriptase/HAI-1 and vimentin, a mesenchymal marker, was observed among these breast cancer cells. This tendency toward lack of expression in cells that express a mesenchymal phenotype is consistent with our previous study that matriptase was not detected in cultured human fibroblasts and HT-1080 fibrosarcoma cells.²⁶ The *in vivo* expression analyses for matriptase and HAI-1 seem to support this *in vitro* corre-

Figure 8. The tissue concentration of matriptase in human breast tumors and the surrounding tissues. The tissue concentration of matriptase was determined by immunoblot. The concentration of a purified matriptase standard was determined by comparison with a bovine serum albumin standard curve resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. Different amounts of purified matriptase were then used (80 pg, 200 pg, 400 pg, and 800 pg; **lanes 1 to 4**) to generate a standard curve for the immunoblot. Protein samples (50 μ g) from six human breast tumors (**lanes 5 to 10**) and two surrounding tissues (**lanes 11 and 12**) were examined by immunoblot and compared to the standard curve for matriptase. The final concentration of each specimen was calculated and indicated.

lation. Expression of matriptase and HAI-1 was not detected, or found at negligible levels, in stromal-derived ovarian tumors and various human sarcomas. In contrast, matriptase and HAI-1 were detected in all of the human carcinoma specimens in this study.

The detection of matriptase mRNA by *in situ* hybridization in primary human breast tumors revealed that the matriptase/HAI-1 system is synthesized by epithelial cells and epithelium-derived cancer cells *in vivo*. The lack or negligible amount of matriptase in stromal-derived ovarian tumors and primary human sarcomas, including osteosarcoma, liposarcoma, leiomyosarcoma, malignant fibrous histiocytoma, and dermatofibrosarcoma protuberans, further confirms that epithelial cells, rather than mesenchymal cells, are the major sources of matriptase *in vivo*. These results are consistent with our earlier observation that matriptase and HAI-1 are produced *in vitro* by breast cancer cells and milk-derived, immortalized luminal epithelial cells of the mammary gland, but not by cultured foreskin fibroblasts nor the fibrosarcoma cell line HT1080.²⁶

When assayed by Western blotting of tumor cell lysates and normal tissue lysates, matriptase protein is present in tumor tissue and in normal breast tissue. Direct comparison of matriptase protein levels between tumor and surrounding normal tissue by Western analysis, however, is of limited value because of cellularity issues, because tumors tend to contain primarily epithelial cells whereas normal breast tissue is composed primarily of stromal tissue with a smaller epithelial component. When examined by immunohistochemistry, there is no obvious difference in the expression of matriptase between tumor cells and normal breast epithelial cells (Figure 6). This is similar to the observation for cultured immortalized/non-tumorigenic breast epithelial cells and tumorigenic breast cancer cell lines for which no obvious overall difference exists in the level of expression of matriptase mRNA and protein.

These results suggest that if the catalytic activity of the serine protease matriptase is important for the growth and/or invasion of breast cancer cells in human breast tumors, then the increased activity of the protease in breast cancer is likely because of mechanisms other than a simple increase in matriptase protein or mRNA. An increase in matriptase activity could be manifested in multiple ways, for example, by an increase in the matriptase:inhibitor ratio within a tumor, tipping the balance in favor of the protease relative to the inhibitor, or by an increase in the activation of matriptase on the cell surface by proteolytic cleavage. Like many other proteases, matriptase requires proteolytic cleavage from a one-chain latent form to a two-chain active form,³⁷ an event that is not measured by the immunohistochemistry or *in situ* hybridization assays presented in this paper. Future studies will include the examination of the active form of matriptase (two-chain, active matriptase). This may be done by immunocytochemistry using a mAb that recognizes only the two-chain, active form of matriptase,³⁷ or by activity assays of tumor extracts, to assess the relative activity of matriptase in breast cancers of various stages and grades, and in normal breast tissue. Furthermore, we will assess the matriptase:HAI-1 ratio within a large series of primary human breast

carcinomas of various stages and grades to determine the relevance of the protease:inhibitor ratio in breast tumors.

A significant imbalance of matriptase and HAI-1 expression was observed in some of the tumor specimens analyzed by Western blot in this study. We have determined the ratio of matriptase to HAI-1 expression by comparing their intensity on Western blot after normalizing the signals with that of the control sample from T-47D cells (lane 1 in Figures 2, 3, and 4) (data not shown). These ratios fluctuate among the breast and gynecological tumors. For example, two infiltrating carcinomas (Figure 2, lanes 4 and 6) had a relatively low ratio, whereas other samples, all invasive carcinomas, had a relatively higher ratio. Among the gynecological tumors, a high ratio was observed in some specimens (Figure 3, A and B, lanes 10 and 11; and Figure 3, C and D, lanes 3 and 7), but not in others. The matriptase to HAI-1 ratio among the colon tumors analyzed showed a more consistent value, with one exception (Figure 4, lane 9). Taken together, these results suggest that the ratio of matriptase to HAI-1 varies among tumors of breast and gynecological origin, and warrants further study to determine whether a trend in the matriptase to HAI-1 ratio, assessed in a much larger set of tumors, correlates with pathological grade or stage of the tumors, or with clinical measures of outcome such as disease-free and overall survival or response to chemotherapy. Such studies are currently in progress.

In summary, we have characterized the expression of matriptase and HAI-1 both in cultured human breast cancer cells and in primary human breast carcinomas and other primary human cancers. We have found that matriptase and HAI-1 are expressed concomitantly by both ER- and ER+ breast cancer cell lines. The expression of these proteins correlates with the expression of epithelial markers and not with markers of a mesenchymal phenotype in cultured breast cancer cells. In primary breast cancers and in normal breast epithelial tissue, matriptase and HAI-1 are expressed by the epithelial component of the tissue, and not by stromal elements such as fibroblasts and adipocytes. The expression pattern of matriptase and HAI-1 in primary breast cancers suggests that this protease system is an epithelial-derived system that may activate stromal-derived proteases such as uPA, and growth/motility factors such as HGF/SF, on the surface of breast cancer cells, enhancing their growth and/or invasive properties. Therefore, matriptase may represent an important link in our understanding of how stromally derived proteases and growth/motility factors may be activated on the surface of normal breast epithelial cells or breast cancer cells. Within a breast tumor, such activity may contribute to the tumorigenic and metastatic properties of breast cancer cells.

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