Membrane Type-1-Matrix Metalloproteinase Expressed by Prostate Carcinoma Cells Cleaves Human Laminin-5 β3 Chain and Induces Cell Migration

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INTRODUCTION

BM is a dense sheet of proteins of the ECM that separates the connective tissue from the epithelium. Structural components of the BM are laminins, type IV and type VII collagens, nidogens, and proteoglycans such as perlecan and fibulins (1, 2). In addition to mechanical support, the BM influences cell proliferation, differentiation, adhesion, migration, and gene expression (3). Ln-5 is an epithelium-specific subtype, and is a component of the epithelial cell adhesion complex containing hemidesmosomes and anchoring filament in the BMs (3). Laminins constitute a family of glycoproteins expressed in the BM of various tissues including the prostate (4), and are associated with a variety of biological activities, including cell differentiation, cell shape and movement, maintenance of tissue phenotypes, and promotion of tissue survival (5). Laminins are composed of three chains, α, β, and γ chains, and are arranged in a cruciform structure. At this time 11 different isoforms have been identified (laminins 1, 2, and 4–12). Evidence for the role of Ln-5 in maintaining the integrity of the BM has come from the identification of mutations in the Ln-5 genes observed in the Herlitz’s variant of JEB, a blistering and usually lethal skin disease caused by disruption of the epidermal-dermal junction. Mutations resulting in the failure of expression of any of the Ln-5 (α3β3γ2) chains results in a complete absence Ln-5 and structural modifications in hemidesmosomes (6). The expression and distribution pattern of hemidesmosomal-associated proteins were retained in the normal and PIN lesions, whereas the carcinoma cells uniformly lacked hemidesmosomal structures. The loss of critical cellular attachments may play a role in influencing the progression potential of prostate carcinoma (7, 8). These analyses revealed that high-grade PIN is an early event in prostate cancer progression, and BM attachment factors may have a major role in the progression of this premalignant lesion to an invasive and metastatic prostate cancer.

An important step in the metastatic process of prostate cancer is the proteolysis of ECM proteins, which allows for migration of neoplastic cells through the BM into the interstitial stroma (9). Members of the MMP gene family have been implicated in the physiological as well as pathologic remodeling of the ECM in events ranging from organogenesis to tumor metastasis (10–12). The MMPs can be divided into five families including the collagenases (MMP-1, 8, and 13), gelatinases (MMP-2 and 9), the stromelysins (MMP-3, 7, 10, 11, and 12), the membrane type (MT-1, 2, 3, 4, 5, and 6 MMP) and nonclassified MMPs (13). Human MT1-MMP (MMP-14) is a key enzyme in the initiation of ECM protein breakdown. It has been originally described as a 66-kDa protein from a human placenta cDNA library (14) and found to activate human MMP-2 (Gelatinase A). The enzyme is expressed widely in human tissues under normal and pathological conditions, and it has been proposed to occur with enhanced levels in human tumor tissues. TIMP-2 has been shown to modulate MMP-2 by regulating MT1-MMP activation (15, 16). Increased expression of MMP in prostate cancer is one of the major factors that contributes to tumor cell invasion and metastasis (17). MT1-MMP expression has been studied in prostate cancer-derived cell lines (18). These authors showed that PC-3, DU-145, and TSU-PR1 cells expressed MT1-MMP on their cell surface, and also expressed mRNA for MT1-MMP. They found no expression of MT1-MMP in LNCap cells. In another study, MT1-MMP, MT3-MMP, TIMP-1, and TIMP-2 were found both in the stromal and in the epithelial cells (19). Upadhyay et al. (20) reported that in benign prostate glands, basal cells uniformly expressed MT1-MMP, whereas secretory cells were rarely positive. In contrast, they found in high-grade PIN lesions that secretory cells showed cytoplasmic expression. These authors found in prostate cancers that expression was heterogeneous and varied from no expression to very intense expression in select glands. They found significant association between the pattern of MMP-2 and MT1-MMP expression within the epithelial components of the cancer glands in individual
specimens. These authors suggested that the regulation of these enzymes was altered during the earliest stages of prostate cancer (20).

It was shown recently that rat Ln-5 can serve as a substrate for MT1-MMP and MMP-2 (21, 22). It was also reported that cells that migrate constitutively on Ln-5 correlated with MT1-MMP expression and were inhibited by MMP inhibitors. In this study, we have hypothesized that prostate adenocarcinoma cell invasion through the basal lamina is attributable in part to MT1-MMP-induced cleavage of basal lamina and enhanced motility of the cancer cells. We also determined the nature of the MT1-MMP cleavage of human Ln-5 and how this altered Ln-5 changes the migration of prostate carcinoma cells. Experiments were also designed to examine the role of MT1-MMP expressed on the surface of prostate tumor cells in cleaving Ln-5 and enhancing the migration of prostate tumor cells.

**MATERIALS AND METHODS**

**Cell Culture and Reagents.** Cell lines DU-145, PC3-N (Variant of PC3) PPC, MCF-7, and keratinocyte cell line HaCaT were maintained in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin, both from Invitrogen (Carlsbad, CA). All of the cells were maintained in a humidified incubator at 37°C and 5% CO₂. DMEM was obtained from Invitrogen. Fetal bovine serum was obtained from JRH Biosciences (Lenexa, KS). Recombinant human MT1-MMP and rabbit anti-MT1-MMP antibody were purchased from Chemicon (Temecula, CA). Ln-5 β3-antibody, antikalinin clone 17, a monoclonal antibody directed against the COOH terminal of β3, was purchased from BD Transduction Laboratories (Lexington, KY).

**Purification of Human Ln-5 and Cleavage Ln-5 by Recombinant MT1-MMP.** Human Ln-5 was purified from human HaCat cell-conditioned medium as described previously (23). Briefly, the human keratinocyte cell line HaCaT was grown in 175-cm² culture flasks as described above. After the cells reached confluence, the conditioned medium was harvested. Endogenous protease activity was minimized by the addition of 5 mM EDTA, 50 μM phenylmethylsulfonyl fluoride, and 50 μM N-ethylmaleimide, respectively. The HaCaT conditioned medium was passed through a BM165-Sepharose CL-4B affinity column prepared by coupling the anti-Ln-5 mAb 3 chain monoclonal antibody BM165 to cyanogen-activated Sepharose CL-4B (Pharmacia Bio-tech). The BM165 antibody was provided by Dr. Robert E. Burgeson (Massachusetts General Hospital, Boston, MA; Ref. 4). Ln-5 was eluted from affinity column with 0.1 M glycine (pH 7.2), neutralized by addition of Tris-HCl (pH 8.0), and dialyzed against PBS. The protein concentration was determined with advanced protein assay reagent (Cytoskeleton Inc.). To study cleavage of Ln-5 by MMP-1, 1 μg of purified human Ln-5 was applied onto a 96-well plate and then incubated with recombinant MT1-MMP (1 or 2 μg/ml) for 12–16 h at 37°C in 50 mM Tris (pH 7.5), 0.005% Brij-35, and 10 mM CaCl₂. After incubation, each reaction mixture was electrophoresed on 6% SDS-PAGE under reducing conditions and stained with Bio-Safe Coomassie (Bio-Rad Laboratories, Hercules, CA) for mass-spectrometry analysis or transferred to a blot for Western analyses.

**Identification of the MT1-MMP-cleaved Ln-5 by Mass Spectrometry.** The cleaved product of Ln-5 by MT1-MMP was identified using the Proteomics Core facility of the Southwest Environmental Health Sciences Center and Arizona Cancer Center at the University of Arizona. The cleaved Ln-5 was first separated by SDS-PAGE on a 6% gel. After staining with Coomassie Blue, the 80-kDa bands were excised, cut into small pieces (1 × 1 mm), and subjected to in-gel digestion using trypsin or chymotrypsin as described previously (24). The extracted peptides after digestion were analyzed by mass spectrometry to assign peptide sequence to the spectra (25). SEQUEST analyses were performed against the publicly available nonredundant database.

**Western Blot Analysis.** Ln-5 after incubation with MT1-MMP was electrophoresed on 6% SDS-PAGE under reducing conditions. The reaction mixture was then transferred to Immobilon transfer membranes as described earlier (26). Monoclonal antibody (Clone-17; Transduction Laboratories) against human Ln-5-β3 chain was used as a primary antibody, and horseradish peroxidase-conjugated goat antimouse antibody (Pierce, Rockford, IL) was used as secondary antibody. Signals were detected with an enhanced ECL Western blotting detection reagent (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and by exposing the blot to Kodak (Rochester, NY) autoradiographic film. Western analysis was performed for antisense down-regulation of MT1-MMP cell surface expression in DU-145, PC3N, and PPC cells. Total cellular proteins were isolated after incubation of DU-145 cells with MT1-MMP antisense as described elsewhere (21).

**Cell Migration and Invasion Assays.** The migration assay was performed as described by Berens et al. (27) and Amberger et al. (28). In brief, Teflon-printed microscope slides (CSM, Inc., Phoenix, AZ), subdivided into 10 wells, were precoated overnight with purified human Ln-5 (1 μg/well) at 4°C. Five wells were used as control and the other 5 wells were treated with MT1-MMP (2 μg/ml) for 12–16 h in a humidified incubator at 37°C and 5% CO₂. After incubation, the excess liquid in the wells was removed, and the wells were rinsed with PBS and covered with 70 μl of serum-free medium. The cell sedimentation manifold was placed on the slide, and 1 μl of cell suspension (2000 cells) was placed in each cylinder and placed at 37°C for 4 h at 5% CO₂, which allows the cells to attach before removal of the manifolds. After incubation for 4 h, the cell sedimentation manifold was removed. The migration area was recorded using Axiocam camera scans with CCD sensor, attached to an inverted microscope (Carl Zeiss, Göttingen, Germany). The cell migration area was quantified with an image analysis system (Axioplan 2; Carl Zeiss). The initial area of sedimentation was used as a migration reference point, and the area was normalized to the initial area. The migration was measured in microns. Each experiment was performed at least three times in triplicate. Human Ln-5-MMP antisense oligonucleotide sequences and their scrambled oligonucleotides were synthesized as described before (21). Briefly, these oligonucleotides were freshly dissolved in PBS and added to the DU-145 cells at 20 μM. The DU-145 and PC3N cells were pretreated for 2 days with antisense and scrambled MT1-MMP oligonucleotides before the cells were tested in migration assays. The oligonucleotides were also added during the migration assays. Invasion assay was carried out as described previously (29). In some experiments, purified Ln-5 (1 μg) was precoated as described above, and cells were plated onto the coated Ln-5 and incubated for 48 h. After incubation the cells were removed with 5 mM EDTA, and the coated Ln-5 was aspirated using a solubilizing buffer for PAGE gel analysis and immunoblots.

**Time-Lapse Video Microscopy.** The migration of prostate cancer cell line DU-145 on different substrates was monitored using differential interference contrast optics on an inverted Olympus IMT2 microscope (Olympus America, Melville, NY) equipped with a BiopTechs Delta T live cell system (BiopTechs, Butler, PA) and under a humidified 5/95% CO₂/O₂ atmosphere. Images were obtained with a grayscale CCD camera (ORCA-100; Hamamatsu, Japan) and analyzed using image analysis software (SimplePCI 4.0; C Imaging Inc., Cranberry Township, PA). Time-lapse imaging of migrating cells on fibronectin (1 μg/0.5 cm²), Ln-5 (1 μg/0.5 cm²), and MT1-MMP (2 μg/ml) -cleaved Ln-5 was performed by coating these substrates overnight at 4°C on a Delta T dish (0.15 mm; Biotech, Inc.). After coating the substrates the dishes were rinsed with sterile PBS, and the cells were plated in serum-free DMEM for 2 h with a speed over each migrating cell was calculated by manually measuring the distance traveled by the cell nuclei over the 18-h time period using the SimplePCI software. The average rate of migration of the cell (10 cells/experiment) in microns for 18 h was calculated.

**MT1-MMP-expressing Prostate Cancer Cell Lines Cleaved of Purified Human Ln-5.** The DU-145, PC3-N, and PPC cells were grown as described above. Ln-5 was coated 2 μg/well in a six-well plate and allowed to attach overnight at 4°C. After precoating with Ln-5 the well was rinsed with sterile PBS twice, and the cells (2 million cells/well) were plated on these precoated wells for 48 h. After 48 h the cells were removed by adding 1 ml of 5 mM EDTA to each well for 30 min. After removing the cells the wells were rinsed...
three times with PBS. Fifty μl of sample buffer was added to solubilize the coated Ln-5. The sample from each well was electrophoresed, transferred, and probed with Ln-5 antibodies as described in the Western analyses section. For inhibition experiments, the cells were treated with TIMP 2 (50 nm; Chemicon) for 60 min before adding the cells on Ln-5 substrate.

**Immunohistochemistry.** Human tissue sections were obtained from 28 cases of radical prostatectomies. Tissue samples were snap-frozen at the time of surgery and stored at −80°C until use for studies on MT1-MMP. All of the carcinomas were assigned Gleason Grade according to published criteria (30). Rabbit polyclonal antibodies raised against the synthetic peptide RECPYAIRENKE derived from the protein sequence of MT1-MMP were obtained from Dr. William Stetler-Stevenson (National Cancer Institute, Bethesda, MD). For detection of MT1-MMP, frozen sections that had been placed on positively charged slides were fixed in acetone for 5 min, rehydrated in PBS, and incubated with primary antibody diluted in 1% BSA in PBS for 1 h at room temperature. The intensity of the staining was graded from 0 (negative staining) to 3+ (intense staining). The pattern of staining was either qualified as “diffuse” meaning all cells of the glandular structure were positive or “focal” meaning single cell or group of cells were positive.

**RESULTS**

**Human MT1-MMP Cleaved Purified Human Ln-5.** To examine the nature of MT1-MMP cleavage of human Ln-5, 1 μg of Ln-5 was absorbed onto a 96-well plate and then incubated with recombinant MT1-MMP (2 μg/1 μg/ml) for 16 h at 37°C. MT1-MMP/Ln-5 reaction mixture was electrophoresed on 6% SDS-PAGE under reducing conditions and then stained with Coomassie Blue. The figure shows in Lane 1, Ln-5 (4 μg) and in Lanes 2 and 3, Ln-5 (4 μg) with MT1-MMP (2 μg/ml and 1 μg/ml), respectively. Numbers 1–4 in Lane 2 represent the α3 (165 kDa), γ2 (150 and 105 kDa), and β3 (140 kDa) chains of Ln-5, and band number 5 is the MT1-MMP-cleaved product of Ln-5. B, identification of MT1-MMP-cleaved product by mass spectrometry as Ln-5 β3 chain. Trypsin and chymotrypsin digests of the 80-kDa band were performed before subjecting the peptides to tandem mass spectrometry analyses. Both enzymes digested the 80-kDa cleaved product of Ln-5, yielding unique peptides. These peptides were subjected to tandem mass spectrometry. The trypsin digest of the 80-kDa band identified 8 peptides (underlined) and the chymotrypsin digest recognized 9 peptides (boxed). All of the peptides identified were within the sequence of Ln-5 β3 chain.
tides, which were identical with the sequence in human Ln-5 β3-chain (Fig. 1B). The recovered peptides represent 10.3% of protein coverage. Both trypsin and chymotrypsin digestion of MT1-MMP-cleaved product of Ln-5 did not show any other peptides that were identical to the other chains of Ln-5.

The MT1-MMP-cleaved Product of Ln-5 Was Recognized by a Monoclonal Antibody Specific for Ln-5 β3 Chain. Next we determined whether the novel 80-kDa-cleaved product of Ln-5 could be recognized by an antibody specific for Ln-5 β3-chain with immuno blot analysis. The purified Ln-5 was precoated overnight at 4 °C and treated with MT1-MMP as described in “Materials and Methods.” Western analysis of the MT1-MMP/Ln-5 mixture probed with a monoclonal antibody specific for Ln-5 β3 chain (antibody raised against Ln-5 β3 sequence 1009–1170; Transduction Laboratories) showed a 140-kDa Ln-5 β3 chain and a distinct 80-kDa band. The dose response of Ln-5 treated with MT1-MMP showed a gradual increase in the cleavage of Ln-5 with increasing dose. The cleaved product was observed at the concentration of 0.125 μg/ml, and there was an increase in cleaved product of Ln-5 with 1 and 2 μg/ml. The band immediately below the 140-kDa β3 chain of Ln-5 at a molecular weight of ~120 kDa was identified to be a partially cleaved product of Ln-5 β3 chain by mass spectrometry.

Fig. 2. MT1-MMP-cleaved human Ln-5 (80 kDa) was recognized by antibodies specific for β3 chain. A, MT1-MMP/Ln-5 reaction mixture was electrophoresed on 6% SDS-PAGE under reducing conditions and then transferred to a blot for Western analyses. The dose response of Ln-5 treated with MT1-MMP (0.125–2 μg/ml) showed an increase in the cleavage of Ln-5 with increasing dose. The cleaved product was observed at the concentration of 0.125 μg/ml, and there was an increase in cleaved product of Ln-5 with 1 and 2 μg/ml. The band immediately below the 140-kDa β3 chain of Ln-5 at a molecular weight of ~120 kDa was identified to be a partially cleaved product of Ln-5 β3 chain by mass spectrometry.

Linear Migration and Invasion Assays Showing Cleaved Ln-5 β3 Is Promigratory in DU-145 Cells. To examine the potential role of MT1-MMP-cleaved Ln-5 β3 chain in migration of DU-145 prostate carcinoma cells we used a linear migration assay developed by Berens as described in “Materials and Methods.” We found that at 18 h the DU-145 cells were 2-fold more migratory on MT1-MMP-cleaved Ln-5 than they were on uncleaved Ln-5. At 24 h the cells were still more migratory on MT1-MMP cleaved than uncleaved Ln-5 (Fig. 3A). Similar results were observed in invasion assays using transwell inserts where the DU-145 cells showed 2-fold increase in invasion in cleaved Ln-5 compared with intact Ln-5 (data not shown). To determine whether this increase in migration of DU-145 cells was because of the cleavage of Ln-5 β3-chain by MT1-MMP, we used antisense
MT1-MMP cleaved Ln-5 β3 and prostate cancer cells

oligonucleotide for MT1-MMP (21). The cells treated with antisense MT1-MMP but not with scrambled oligonucleotides revealed that expression of surface MT1-MMP was inhibited by 66% in DU-145 prostate cancer cells (Fig. 3B). Migration of DU-145 cells was stimulated by the presence of MT1-MMP and inhibited (~50%) by the presence of blocked oligonucleotides. The MT1-MMP scrambled oligonucleotide did not inhibit the cell migration (Fig. 3A). To strengthen our conclusion that MT1-MMP cleavage of Ln-5 is critical in prostate carcinoma cell migration we also investigated the migratory potential of MT1-MMP antisense and scrambled oligonucleotide-treated DU-145 cells on MT1-MMP-cleaved Ln-5. The results showed that the MT1-MMP antisense oligonucleotide-treated DU-145 cells migrated on cleaved Ln-5 and showed a 2-fold increase compared with the intact Ln-5, and the migration of DU-145 treated with MT1-MMP-scrambled oligonucleotide showed a 2-fold increase in migration similar to DU-145 migration on MT1-MMP-cleaved Ln-5 (Fig. 3A).

Migration of DU-145 Cells Was Enhanced on Ln-5 Treated with MT1-MMP by Time-Lapse Photography. Next we tested whether MT1-MMP-cleaved Ln-5 could induce an increase in the rate of random migration of DU-145 cells. To do this, we performed time-lapse video analysis of DU-145 cells migrating on Delta T-dish coated with purified Ln-5 or MT1-MMP-cleaved Ln-5, or fibronectin as substrate. The migration of each cell was calculated by manually measuring the distance traveled by the cell nuclei over the 18-h time period using the SimplePCI software. The average rate of migration of the cell (10 cells/experiment) in microns for 18 h was calculated as described in “Materials and Methods.” The experiments revealed that in MT1-MMP, cleaved Ln-5 showed a 3-fold increased rate of migration of DU-145 cells compared with uncleaved Ln-5 (Fig. 3B). To determine the specificity of the migration of DU-145 cells on Ln-5 we compared with fibronectin. DU-145 cells migrate very poorly on fibronectin compared with Ln-5 or MT1-MMP-treated Ln-5.

Distribution of MT1-MMP in Prostate Tissue. All 28 of the carcinoma cases (100%) examined stained diffusely positive for MT1-MMP protein (Fig. 4A, panel a). The intensity of the staining varied between cases (between 1+ and 3+; mean 1.7±). No staining was found in control slides in which the primary antibody had been omitted. MT1-MMP was found focally stained in 14 of 17 PIN lesions (Fig. 4A, panel b). It was observed diffusely in 2 of 17 cases. Staining intensity in PIN was weaker than in cancer. More intense staining of high-grade PIN was observed in areas in which the lesions were in transition to becoming invasive. MT1-MMP was also observed in basal cells of normal ducts (92%) and in basal cells of normal glands (34%). In 3 of 9 positive cases, MT1-MMP was also detected in luminal cells.

Cleavage of Ln-5 by MT1-MMP-expressing Prostate Cancer Cell Lines. To confirm that the MT1-MMP cleavage of Ln-5 was physiologically relevant we determined whether the prostate cancer cells expressing abundant MT1-MMP could cleave intact Ln-5. First we did a Western blot analysis with an antibody specific for MT1-MMP using different prostate cancer cell lines including PrEC (normal cells), LNCaP, DU-145, JCA-1, TSU-PR1, PC3, PC3N (PC3 variant), PPC, and MCF-7 (breast cancer cell line). The results showed that PC3N, PPC, and MCF-7 cells expressed higher amounts MT1-MMP than other prostate cells (Fig. 4B). DU-145, PC3N, and PPC cell lines were used to study the cleavage of the intact human Ln-5.

Human Ln-5 (1 µg/well) was coated on six-well plates and the MT1-MMP-expressing cells were placed on the coated surface. We found the PPC, PC3N, and DU-145 cells cleaved the intact Ln-5, and gave rise to an 80-kDa fragment, which was recognized by the Ln-5 β3 monoclonal antibody (Fig. 4C). The densitometry analysis of the 80-kDa bands showed that there was a 2–3-fold increase between DU-145 cells and PPC or PC3N cells (Fig. 4D). This clearly showed that the cells expressing abundant MT1-MMP are capable of cleaving the intact Ln-5. Furthermore, TIMP-2 that has been shown to inhibit MT1-MMP (19) blocked the cleavage of intact Ln-5 in PPC, PC3N, and DU145 cells.

Migration of MT1-MMP-expressing Prostate Cancer Cells on Ln-5 Substrate. We evaluated the possible role of the MT1-MMP expressed by the prostate cells to cleave and migrate on a Ln-5-coated substrate. We also attempted to block the migration by using an antisense MT1-MMP as described in “Materials and Methods.” For this experiment we selected the prostate cancer cells, which were strongly positive for MT1-MMP (PC3N and PPC). Using the linear migration assay developed by Berens, we found that at 18 and 24 h both of these prostate cancer cells PPC and PC3N, which were strongly positive for MT-MMP expression by Western analyses, migrated on Ln-5, and the migratory potentials of these prostate cancer cells were inhibited (~50%) by the presence of blocked oligonucleotides in PC3N cells and by ~40% in PPC cells (Fig. 4E). The MT1-MMP scrambled oligonucleotide (control) did not inhibit the migration. The cells treated with antisense MT1-MMP but not with scrambled oligonucleotides revealed that expression of surface MT1-MMP was inhibited by 63% in PC3N prostate cancer cells and 23% in PPC prostate cancer cells (Fig. 3B).

DISCUSSION

Previous work from our laboratory showed that prostate carcinoma progression might be influenced by the biochemical nature of the BM surrounding the primary carcinoma cells. Reports also indicated that the regulation of MT1-MMP (20) and BM attachment factor Ln-5 (7) was altered during the earliest stages of prostate cancer. We investigated the distribution of MT1-MMP in prostate carcinoma, PIN lesions, and adjacent histologically normal-appearing tissue. MT1-MMP protein was present in 100% of the carcinoma cases. Increased expression of MT1-MMP was observed at the point of transition of high-grade PIN to invasive carcinoma indicating that the expression of MT1-MMP might be an important step in the initial progression of prostate carcinoma. The expression pattern of Ln-5 was also studied in prostate normal, PIN lesions, and cancer glands (7, 31), and found that Ln-5 is expressed in normal prostate glands and PIN but not in invasive cancer. This and other published evidence clearly indicate that high-grade PIN is an early event in prostate cancer progression, and the MMPs and BM attachment factors have a major role in the progression of this premalignant lesion to an invasive prostate cancer. The role of the BM in cancer biology is not well understood. Based mostly on morphological data, the loss of BM continuity has been associated with increasing malignancy (3). Studies also showed that various proteolytic enzymes have altered distribution and activity in malignant tissues, and it has been suggested that BM degradation is a prerequisite for invasion. Proteolytic modification of specific laminin isoforms may provide for highly localized regulation of cellular behavior. Laminins associate with cells through a variety of mechanisms including binding to specific integrins such as α3β1, α6β1, and α6β4 (32). Proteolysis may alter receptor recognition sites or expose cryptic epitopes with novel biological functions. A previous report from our laboratory also showed that DU-145 prostate carcinoma cells, when transfected with matrilisin (MMP-7) -expressing vector, were significantly more invasive than vector-only transfected cell lines as assayed by a severe combined immunodeficient mouse model of tumor cell invasion. These results suggest a functional role for MMPs in the initial invasion of prostate cancer through the epithelial basal lamina and into the surrounding stroma (33).
The present findings show that the incubation of the recombinant catalytic domain of human MT1-MMP with purified human Ln-5 generated a novel 80-kDa MT1-MMP-cleaved Ln-5/β3-product. We identified that the 80-kDa cleaved product of Ln-5 was β3 by mass spectrometry both by trypsin and chymotrysin digestion. Two-dimensional gel and Western analyses (data not shown) demonstrated that the cleaved product of Ln-5 was a single protein with a mass of 80 kDa. We do not have any evidence that either α3 or γ2 chains of human Ln-5 are cleaved with human MT1-MMP. The Ln-5 β3 antibody recognized a band (~120-kDa) that gave 13 different peptides with 13.5% protein coverage by amino acid count. On the basis of the mass spectrometry data we conclude that this 120-kDa band might be a partially cleaved product of 140-kDa Ln-5/β3 chain.

Furthermore, we demonstrated that the cleaved Ln-5/β3 chain influences the migration of prostate cancer cells and also observed that DU-145 cells were more invasive on MT1-MMP-cleaved Ln-5/β3 chain than intact Ln-5 coated substrate (data not shown). Reports have shown that laminins undergo proteolytic cleavage, and post-transla-
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It was reported recently that rat MT1-MMP cleaves rat Ln-5 α2 chain at two putative sites (21). One cleavage site generated an ~100-kDa fragment called γ2'. This could be the naturally occurring processed form of the precursor 165-kDa peptide; however, this has not been proven. The other fragment is an 80-kDa protein, γ2x, and is thought to be the same fragment as the one generated by MMP-2 cleavage; however, this MT1-MMP-cleaved rat Ln-5 γ2 chain fragment has not been sequenced. It is not clear that human MT1-MMP and MMP2 actually cleave at the same site in the rat Ln-5 α2 chain. Giannelli et al. (22) have sequenced the NH₂ terminus of the MMP2-cleaved α2 chain of rat Ln-5 and found cleavage at residue 587. This cleavage site immediately precedes two closely spaced cysteines in domain III of the α2 chain (Cys-Pro-Ala-Cys), which are thought to be involved in joining the α3, β3, and γ2 laminin subunits. These published data suggest that state of cleavage of the Ln-5 α3 or α2 chains determines whether Ln-5 can cause interacting cell migration or stable cell adhesion (8, 22, 38). Koshikawa et al. (21) have shown that Ln-5 γ2 degradation associates with the overexpression of MT1-MMP and cell migration. The cleavage of the γ2 chain of rat Ln-5 has shown to be mediated by MMP-2 and/or MT1-MMP in a dose-dependent manner (21, 22) and not by other proteases such as plasmin or MMP-9 (22). The degraded fragments identified by Koshikawa et al. (21) after the cleavage of rat Ln-5 γ2 chain by MT1-MMP corresponds in size to what we observed after MT1-MMP-mediated degradation of human Ln-5 β3 chain. In our study we used human Ln-5 and human MT1-MMP, and the study by Koshikawa et al. (21) was carried out using rat Ln-5 and rat MT1-MMP. Of interest the amino acid sequence of rat Ln-5 γ2 cleavage site is not present in the human Ln-5 α2 chain, which may explain why we did not observe cleavage of human Ln-5 γ2 by MT1-MMP.

We examined the potential role of MT1-MMP-cleaved Ln-5 β3 chain in migration of DU-145 prostate carcinoma cells. Our data suggested there was a significant increase in the migration of DU-145 cells on MT1-MMP-cleaved Ln-5. We also determined that this increase in migration of DU-145 cells was because of the cleavage of Ln-5 by MT1-MMP by using antisense and scrambled (control) oligonucleotides specific for MT1-MMP (21). The results showed that migration of DU-145 cells was inhibited by the presence of blocked oligonucleotides, and the scrambled oligonucleotides had no effect. These data support our hypothesis that MT1-MMP cleavage of human Ln-5 enhances the migration of prostate carcinoma cells on Ln-5. We also investigated whether MT1-MMP has a more direct role in prostate carcinoma cell migration by determining the migratory capability of MT1-MMP antisense-treated DU-145 cells on cleaved Ln-5. We found that the MT1-MMP antisense-treated DU-145 cells migrated on cleaved Ln-5 and showed a 2-fold increase compared with the intact Ln-5. The results suggest that cleavage of Ln-5 is important in DU-145 cell migration, and MT1-MMP does not play a more direct role in cell migration. Random migration of DU-145 cells by time-lapse video analyses revealed that in MT1-MMP-treated Ln-5, a 3-fold increased rate of migration of DU-145 cells compared with intact Ln-5. Another ECM protein fibronectin was used to compare the specificity of DU-145 cells migrating on Ln-5. We found that DU-145 cells migrate very poorly on fibronectin compared with Ln-5. Gilles et al. (39) demonstrated using MCF10A mammary epithelial cells that the acquisition of a migratory phenotype is accompanied by an overexpression of MT1-MMP and a localization of the protein in the lamellipodia, and at the basal surface of the cells in contact with the ECM substrate.

Physiological relevance of MT1-MMP-cleaved Ln-5 β3 chain was demonstrated by using cells that express high levels of MT1-MMP (PC3N and PPC) to cleave intact Ln-5. We also show that pretreatment of the cells with TIMP-2 inhibits the MT1-MMP-cleaved product of Ln-5 β3 chain. Furthermore, we show that these cells expressing high levels of MT1-MMP could cleave and migrate on intact Ln-5 substrate, which was blocked using antisense MT1-MMP oligonucleotides. These findings are in agreement with the reports that human tumor cells constitutively expressing MT1-MMP display a higher migrating ability toward exogenously provided rat Ln-5 than MT1-MMP-negative cells (21, 39). The importance of Ln-5 has been demonstrated by studies on JEB, a recessive inherited blistering disease of the skin in which defective expression of Ln-5 causes disruption of the epidermal-dermal junction, often with fatal consequences (40–42). Two groups have reported thus far that Ln-5 β3 chain plays a major role in hemidesmosomal formation in JEB. Vaillot et al. (43) have shown that in human JEB transplanted with laminin-β3 transgene synthesized the β3 protein that assembled with the endogenous Ln-5 α3 and γ2 chains into a biologically active Ln-5 that was secreted, processed, and deposited into the ECM. They also reported that re-expression of Ln-5 induced cell spreading, nucleation of hemidesmosome, and enhanced adhesion to the culture substrate. In another study Robbins et al. (44) demonstrated that restoration of Ln-5 β3 gene expression in postnatal JEB cells corrected critical features such as Ln-5 β3 chain expression, hemidesmosome formation, and the correct localization of other basement proteins. Hao et al. (8) suggested that failure of hemidesmosome formation results in a less stable epithelial-stromal junction, which may allow malignant cells more potential to invade and spread through adjacent structures. Taken together these findings clearly indicate that Ln-5 β3 and γ2 chains are important in hemidesmosome formation and BM stability. Any gene mutations in these chains could lead to loss or change in the integrity of the BM arrangements, such as loss of hemidesmosome or change in the adhesive property, which may influence the migratory potential of the cell. To our knowledge, no prior studies are available on MT1-MMP and Ln-5 β3 chain cleavage. This is a novel and important finding where we have shown that β3 chain is cleaved by MT1-MMP, and this cleavage enhances migration of prostate cancer cells. These findings with human Ln-5 and human MT1-MMP are particularly relevant to human prostate cancer and can lead to new approaches for intervention in preventing progression of prostate cancer.

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Membrane Type-1-Matrix Metalloproteinase Expressed by Prostate Carcinoma Cells Cleaves Human Laminin-5 β3 Chain and Induces Cell Migration

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