Membrane Type 1 Matrix Metalloprotease Cleaves Laminin-10 and Promotes Prostate Cancer Cell Migration[1]

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Abstract

Disruption of the extracellular matrix by proteases is crucial for tumor invasion. Laminin-10 (Ln-10) has previously been identified as a substrate for cell migration and cell adhesion, and is present in the basal lamina (BL) of both normal prostate and prostate cancer. Here, we investigate a role for membrane type 1 matrix metalloprotease (MT1-MMP) in modifying this Ln-10–rich BL. MT1-MMP is a transmembrane member of the MMP family that has been demonstrated to be upregulated as prostate cancer progresses from normal to prostate intraepithelial neoplasia to invasive cancer, suggesting a role for MT1-MMP in the invasion of prostate cancer. We show that MT1-MMP cleaves the α_5 chain of purified human Ln-10 from its 350-kDa form into 310-, 190-, 160-, and 45-kDa fragments. This cleavage causes a decrease in DU-145 prostate cancer cell adhesion to purified Ln-10, and an increase in transmigration of DU-145 cells through cleaved Ln-10. We also show that prostate cancer cells expressing membrane-bound MT1-MMP cleave the α_5 chain of Ln-10. Ln α_5-chain cleavage is also observed in human prostate cancer tissues. These findings suggest that prostate cancer cells expressing high levels of MT1-MMP have increased invasive potential through their ability to degrade and invade Ln-10 barriers.

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Introduction

Remodeling of the extracellular matrix (ECM) through proteolysis of ECM proteins is an important step in the metastatic progression of cancer, allowing for invasion of neoplastic cells through the basal lamina (BL) and into the stroma [1]. Proteolysis creates paths for migration, releases signaling molecules such as growth factors bound in the ECM, and generates biologically active ECM fragments [2–6]. Matrix metalloproteases (MMPs) are a family of zinc-dependent enzymes that degrade components of the ECM and have been implicated in the pathologic remodeling of ECM in tumor invasion and metastasis [7–9]. Membrane-type MMPs, of which there are currently six members, are not secreted MMPs; they contain a transmembrane domain that anchors them into the cell membrane. Membrane type 1 matrix metalloprotease (MT1-MMP), a member of the transmembrane metalloproteases, was first described as a 66-kDa activator of pro-MMP-2 (gelatinase A) [10,11] but has also been found to proteolytically cleave ECM proteins including gelatin, fibronectin, K-elastin, vitronectin, collagens, and laminin-5 [10,12–17]. MT1-MMP is expressed in a wide variety of human tissues under both normal and pathologic conditions, although its expression is enhanced in tumor tissues [18]. MT1-MMP has been demonstrated to be upregulated in the progression of prostate cancer, and its expression is correlated with an increase in the invasiveness of tumor cells [11,19–22], indicating that it may have a role in the invasion and metastasis of this cancer.

Laminins are ECM glycoprotein components of all BL. They play essential roles in tissues such as providing the major structure of BL [12,23], attaching cells to the ECM through interactions with cell surface components [24], and interacting with cellular receptors such as integrins to induce intracellular signaling [25,26]. Each laminin consists of three distinct chains (α, β, and γ) arranged in a cruciform structure. All laminin chains share structural similarity, each consisting of small globular domains, epidermal growth factor (EGF)-like repeats, and an α helical coiled coil in the long arm [27].

Being the major components of BL, laminins are structural barriers that separate connective tissues from epithelia and must be penetrated by tumor cells during invasion and metastasis. This occurs through proteolytic degradation of the BL and

Abbreviations: Ln, laminin; MMP, matrix metalloprotease; MT1-MMP, membrane type 1 matrix metalloprotease; ECM, extracellular matrix; BL, basal lamina; PIN, prostate intraepithelial neoplasia; EGFR, EGF receptor.

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mobilization of tumor cells through the degraded BL. Some laminins have previously been shown to be proteolytically processed [28–32], which may aid in this mobilization.

We have previously shown that MT1-MMP can cleave the human Ln-5 β_3 chain [17]. Cleavage of the β_3 chain resulted in increased migration of prostate cancer cells. However, although Ln-5 is expressed in normal prostate and focally in prostate intraepithelial neoplasia (PIN), its expression is lost in prostate cancer [33], meaning that Ln-5 cleavage in prostate cancer is involved only in the initial invasion of neoplastic cells from the PIN lesion. Prostate cancer is surrounded by a BL composed mainly of laminin-10, laminin-2, type IV collagen, and entactin [34]. In order for prostate cancer to invade the stroma and intravasate into the vasculature, it must move through this Ln-10–rich BL either by proteolysis or by ameboid movement [35]. Ln-10 ($\alpha_5\beta_1\gamma_1$) and Ln-11 ($\alpha_5\beta_2\gamma_1$) have been previously identified as substrates for cell migration and cell adhesion [36]. Ln-10 binds the $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$ [37], and $\alpha_v\beta_3$ [38] integrins and is the most widely expressed laminin, being expressed in the prostate, skin, placenta, kidney, pancreas, heart, and lungs [33,36,39]. Here we demonstrate, for the first time, that MT1-MMP can cleave purified human Ln-10 α_5 chain, and that this cleavage causes a decrease in adhesion to cleaved Ln-10 and an increase in prostate cell transmigration and linear migration through processed Ln-10. We also demonstrate that this cleavage occurs *in vivo* in human prostate cancer. These data suggest that the MT1-MMP cleavage and induction of migration on Ln-10 will be of importance not only to prostate cancer but to other cancers where epithelial cells contact a Ln-10–rich BL and will be of importance to other cancers regarding intravasation through blood vessels, which are known to express high levels of the laminin α_5 chain.

Materials and Methods

Cell Culture and Reagents

The human prostate cancer cell lines DU-145 and PC3-N (variant of PC3) and the human lung cancer cell line A549 were maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), penicillin (100 U/ml; Invitrogen Life Technologies), streptomycin (100 μg/ml; Invitrogen Life Technologies), and 0.2 mM L-glutamine (Invitrogen Life Technologies). All cells were maintained at 37°C in 5% CO_2 and passaged with trypsin/EDTA (Invitrogen Life Technologies) when confluent. Recombinant human MT1-MMP catalytic domain, polyclonal MT1-MMP antibody (AB815), was obtained from Chemicon (Temecula, CA). Rabbit polyclonal antibodies (Ab470) raised against the synthetic peptide RECPYAIREGNEK derived from the protein sequence of MT1-MMP were obtained from Dr. Stetler-Stevenson (NCI, Bethesda, MD). Laminin-10/11 α_5 chain antibody, 15H5, was purified as described previously [40], and 4C7 was a gift from Dr. Eva Engvall (The Burnham Institute, La Jolla, CA).

Immunohistochemistry

For detection of Ln-10, frozen prostate tissue sections (3 μm) were placed on positively charged glass slides, fixed in acetone for 5 minutes, and incubated with primary antibody in PBS for 30 minutes at room temperature. Antibody detection was performed by incubating slides with fluorescent-labeled secondary antibodies (Alexa 485 and 565; Molecular Probes, Eugene, OR). For detection of MT1-MMP, slides were fixed in 2% formaldehyde for 7 minutes, 50 mM NH_4Cl for 5 minutes, and 0.2% Triton X-100 for 3 minutes. Slides were analyzed on a Zeiss LSM 410 UV (Carl Zeiss, Oberkochen, Germany) dual-laser confocal microscope using the argon/krypton ion laser operating at 488 and 568 nm. Tissue sections were also stained with hematoxylin and eosin (H&E) to identify tissue structures.

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

Human Ln-10 was purified as described previously [40]. Briefly, the human lung carcinoma cell line A549 was grown in 175-cm^2 culture flasks. After the cells reached confluence, the CM were harvested. Endogenous protease activity was minimized by the addition of 5 mM EDTA, 50 μM phenylmethysulfonyl floride, and 50 μM *N*-ethylmaleimide. The A549 CM was passed through a 4C7-Sepharose CL-4B affinity column prepared by coupling the anti–Ln-10 α_5 chain monoclonal antibody 4C7 to cyanogen-activated Sepharose CL-4B (Amersham Biosciences, Piscataway, NJ). Ln-10 was eluted from the affinity column with 0.1 M glycine (pH 2.7) and neutralized by addition of Tris–HCl (pH 8.0). The protein concentration was determined with Advanced Protein Assay Reagent (Cytoskeleton, Inc., Denver, CO).

Cleavage of Ln-10 by MT1-MMP

Purified Ln-10 was adsorbed and dried onto a 96-well plate well and incubated with recombinant catalytic domain of MT1-MMP (from 0.034 to 2.1 nmol) for 6 to 18 hours at 37°C in 50 mM Tris, pH 7.5, 0.005% Brij-35, and 10 mM $CaCl_2$ as described previously for Ln-5 [16,17]. After incubation, each mixture was solubilized off the plate with a sample buffer and electrophoresed on a 4% to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gradient under reducing conditions. Gels were analyzed by either silver staining method, Coomassie blue staining method, or Western analysis as described previously [41] with mouse monoclonal antibody 15H5 to the α_5 chain of Ln-10.

Mass Spectrometry Analysis of Cleaved Fragments

Cleaved Ln-10 samples were separated by SDS-PAGE. After staining with Biosafe Coomassie Brilliant Blue (Bio-Rad, Hercules, CA), bands were excised and identified by mass spectrometry analysis as described previously [17] using the Proteomics Core facility of the Southwest Environmental Health Sciences Center at the University of Arizona. Briefly, the protein bands were excised, cut into small pieces (1 × 1 mm), and subjected to in-gel digestion using trypsin or chymotrypsin. The extracted peptides after digestion were analysed by liquid chromatography tandem mass

spectrometry using a quadropole ion trap Finnigan LCQ class mass spectrometer equipped with a Michrom (Auburn, CA) MAGIC 2002 high-performance liquid chromatography and a nanoelectrospray ionization source (University of Washington, Seattle, WA). The peptides were eluted from a pulled tip capillary column packed with Vydac (Hesperia, CA) C18 material. The gradient was from 0% to 65% solvent B (98% methanol/2% water/0.5% formic acid/0.01% triflouroacetic acid) over 60 minutes at a flow rate of 200 to 300 nl/min. Tandem mass spectrometry spectra of the peptides were analyzed with the SEQUEST program (Turbo Sequest) to assign peptide sequence to the spectra. SEQUEST analyses were performed against the publicly available nonredundant database.

Adhesion Assays

Ln-10 (1 μg) was adsorbed and dried onto a 96-well plate well and treated with MT1-MMP (2 μg/ml) for 18 hours at 37°C. Wells were then blocked with 1% BSA in PBS for 30 minutes. DU-145 cells (0.5×10^5) in serum-free medium (SFM) were then added to the wells and adhesion at 20-minute intervals was determined. Briefly, unattached cells were removed by aspiration and washing with PBS, and attached cells were stained for 10 minutes with 5% crystal violet/20% methanol. Fixed cells were then washed with ddH_2O until no more dye was leached. After air drying, dye was eluted with 0.1 M citric acid and absorbance was read on a plate reader at 570 nm. By quantifying the absorbance, the percentage of attached cells was calculated.

Linear Migration and Transmigration Assays

The migration assays were performed as described previously [42]. In brief, Teflon-printed microscope slides (CSM, Inc., Phoenix, AZ) subdivided into 10 wells were precoated overnight with purified human Ln-10 (1 μg/well) at 4°C. Five wells were used as control and the other five wells were treated with MT1-MMP (2μg/ml) for 16 hours in a humidified incubator at 37°C and 5% CO_2. After incubation, the excess liquid in the wells was removed, and the wells were rinsed with PBS and covered with 70 μl of SFM. The cell sedimentation manifold was placed on the slide, and 1 μl of cell suspension (2000 cells) was placed in each cylinder and incubated at 37°C for 4 hours in 5% CO_2, which allows the cells to attach before removal of the manifold. After the manifold was removed, the initial sedimentation area was recorded using an Axiocam camera scanner with CCD sensor, attached to an inverted microscope (Carl Zeiss, Göttingen, Germany). Cell migration area was quantified at each time point with an image analysis system (Axioplan 2; Carl Zeiss). The initial area of sedimentation was used as a migration reference point and the migration area was normalized to this initial area. Migration was measured in microns. Each experiment was performed at least three times in triplicate. Transmigration assays were preformed as described previously [43], with modifications. Briefly, 0.8-μm filter bottom cell culture inserts (Corning-Costar, Action, MA) were coated with 1 μg of purified Ln-10 treated with either 2 μg/ml MT1-MMP or PBS. DU-145 cells were then seeded inside the insert and transmigration through the insert was quantified by crystal violet–methanol staining of migrated cells. One group of DU-145 cells used in both migration assays were pretreated for 2 days with 10 μM human antisense oligonucleotide or scrambled antisense sequences against human MT1-MMP as described previously [16]. These cells were then seeded in either the migration assay manifold or the cell culture inserts, with the antisense oligonucleotides (10 μM) added to the medium for the linear migration assays and to both the upper and lower chambers of the tissue culture plate for the transmigration assays.

Cell-Mediated Cleavage of Ln-10

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

Results

Human MT1-MMP Cleaves Purified Human Laminin-10

To investigate whether MT1-MMP could cleave Ln-10, 2 μg of Ln-10 was adsorbed onto a 96-well tissue culture plates and incubated with 2.1 nmol of recombinant MT1-MMP or APMA-activated MMP-2 for 16 hours. Samples were solubilized and electrophoresed on 6% SDS-PAGE under reducing conditions and the gel was silver-stained (Figure 1*A*). In all samples, the 350-kDa α_5, 220-kDa β_1, and 210-kDa γ_1 chains were visualized. In the MT1-MMP–treated sample, we observed a decrease in the 350-kDa α_5 band with a concurrent appearance of a 310-kDa band, suggesting that MT1-MMP is capable of cleaving the α_5 chain of Ln-10. We did not observe the 310-kDa band in MMP-2–treated Ln-10, indicating that this cleavage is specific to MT1-MMP. Minimal endogenous cleavage of Ln-10 to its 310-kDa form occurs before the purification process and can be seen as a faint band in the stained gels. This can be explained by the fact that the cells generating Ln-10, A549, express detectable levels of MT1-MMP [44]. In order to fully characterize the cleavage products, we analyzed cleaved Ln-10 samples on a 4% to 10% gradient gel (Figure 1*B*) followed by Western blotting. In addition to the 310-kDa product, we could detect cleavage products with apparent M_W of 190, 160, and 45 kDa.

In order to identify the cleaved fragments of Ln-10, we utilized a proteomics approach. Cleaved Ln-10 was run on an SDS-PAGE gel and stained with Coomassie blue. Each band that stained with Coomassie blue was excised, digested with trypsin, and subjected to mass spectrometry. Protein bands detected by Western blotting (Figure 1*B*) with

apparent M_W of 100 and 80 kDa, and faint bands between 80 and 45 kDa were not visualized by Coomassie blue staining (data not shown) and were therefore not analyzed by mass spectrometry. We found that the 310-, 190-, 160-, and 45-kDa bands were all different cleavage products of the 350-kDa α_5 chain. Trypsin digestion of the 310-kDa band gave 25 unique peptide sequences that were identical to the expected amino acid sequence of the α_5 chain. The 45-kDa band gave five different peptide sequences consistent with the α_5 chain and all five of these peptides clustered at the N-terminus (Figure 1*C*), suggesting that cleavage occurs near the N-terminal region to give rise to the 45-kDa product. Trypsin digestion of the 190- and 160-kDa bands (Figure 1*D*) yielded 15 and 10 different peptide sequences, respectively, all of which are found in the α_5 chain. Chymotrypsin digestion was also performed on the 310-, 190-, and 160-kDa protein bands, all of which were again identified as the α_5 chain of Ln-10 (data not shown).

An inspection of the amino acid sequence of the α_5 chain indicated five distinct potential MT1-MMP consensus cleavage sites. MT1-MMP has previously been demonstrated to cleave at PXX↓L (ideally PXP↓L or PXG↓L) sites [45,46]. At P^{319} (GenBank accession no. AF443072) [47] in the α_5 chain, there is a PFR↓L consensus cleavage site that would give rise to cleavage products with theoretical M_W of 329 and 31.4 kDa, which corresponds to the apparent M_W of the 310- and 45-kDa cleavage products we observed, accounting for posttranslation modifications, such as glycosylation of the protein (Figure 1*C*). It is important to note, however, that cleaving at this consensus cleavage site will not give rise to a fragment containing the epitope recognized by the antibody 15H5. We therefore propose that cleavage also occurs at a second site to give rise to fragments of similar molecular weight as those that arise from the cleavage at the PFRL site. We propose that this site is at D^{440} in a CED↓L consensus cleavage site. This second cleavage site explains why the 15H5 antibody is able to detect both the 45-kDa fragment and the 310-kDa fragment because the antibody epitope is between these two consensus cleavage sites. Another consensus cleavage site is P^{1243}PG↓L and would give rise to a product with a predicted M_W of 230 kDa, which corresponds to the apparent M_W of the 190-kDa cleavage product. Finally, a fourth consensus cleavage site at P^{2000}SY↓L would yield a product with a predicted M_W of 180 kDa, corresponding to the 160-kDa band we observed.

MT1-MMP Functions at Physiologically Relevant Substrate: Enzyme Ratios

We determined whether the cleavage of the α_5 chain could occur at physiologically relevant substrate/enzyme ratios. For this experiment, 2 μg of Ln-10 was adsorbed onto a 96-well tissue culture plate and incubated for 6 hours with decreasing concentrations of MT1-MMP (2.1–0 nmol). Samples were then separated by 4% to 10% gradient SDS-PAGE

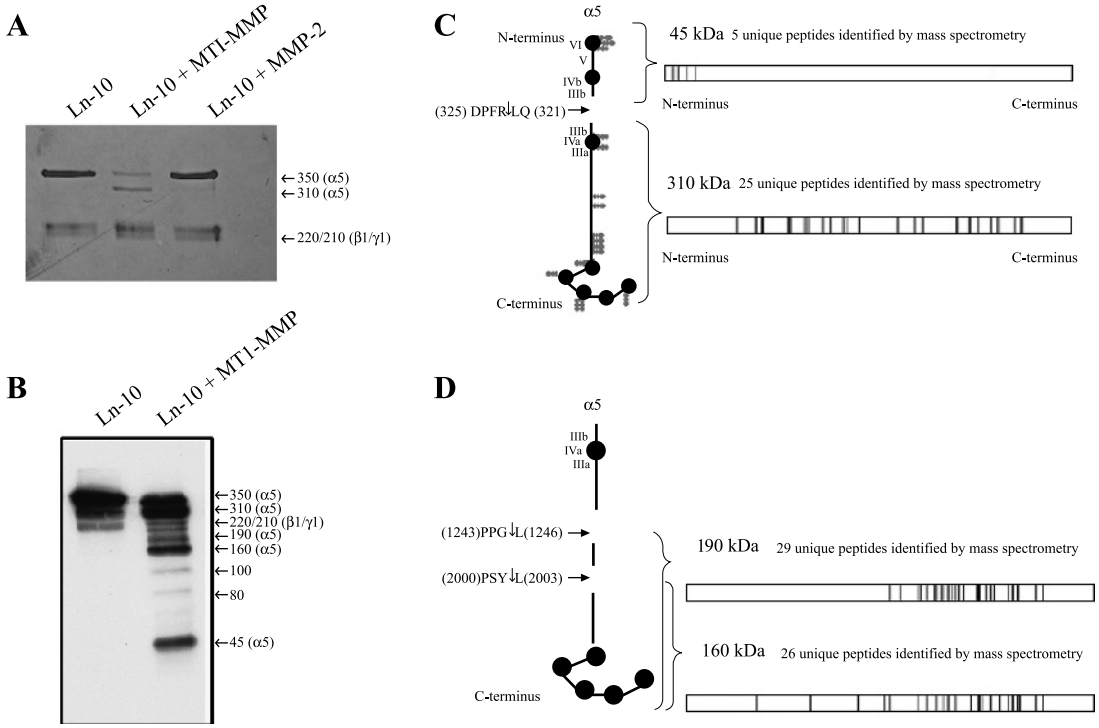

Figure 1. *MT1-MMP cleavage of Ln-10 α_5 chain and identification of cleavage fragments. (A) Silver-stained gel of Ln-10 treated with MMPs. The figure shows Ln-10 treated with either MT1-MMP or APMA-activated MMP-2. M_W values of protein bands are indicated. (B) Western blot of Ln-10 cleavage. M_W and corresponding protein identification are indicated. Protein bands at 100 and 80 kDa were not detected with methods other than Western blotting. (C) Identification of cleavage products by mass spectrometry as the α_5 chain. Potential glycosylation sites are identified in gray. Structural representation of the α_5 chain with potential consensus cleavage site to yield 310- and 45-kDa products is indicated. The 25 peptides identified in the 310-kDa band and the five peptides identified in the 45-kDa band are represented by peptide coverage of full-length α_5 chain. (D) Structural representation of α_5 chain consensus cleavage sites that could give rise to 190- and 160-kDa products. Peptide coverage of the 25 and 29 peptides identified in the 190- and 160-kDa bands, respectively, by mass spectrometry is represented.*

and Western blotted using the 15H5 antibody. The results (Figure 2) indicate that even with 0.05 mol MT1-MMP/mol Ln-10, there is an increase in the 310- and 45-kDa cleaved products. The cleaved products continue to increase with increasing amounts of enzyme. Also, with increasing enzyme, the 190-kDa band appears to decrease at 0.01 mol enzyme/mol substrate, although the 160-kDa band begins to increase at this same substrate/enzyme ratio.

Decreased Adhesion of Prostate Cancer Cells to Cleaved Ln-10

Because intact laminins are known to interact with adhesion molecules such as integrins, we examined whether cleaving Ln-10 with MT1-MMP would have an effect on adhesive interactions. Ln-10 was coated onto a 96-well plate and cleaved with 2.1 nmol of MT1-MMP for 16 hours. Under these conditions, 100% of the Ln-10 is cleaved (Figure 2). ELISA for Ln-10 was performed using the 4C7 antibody to ensure that there was no significant difference in the amount of Ln-10 remaining coated on the plate with MT1-MMP treatment compared to untreated Ln-10 (data not shown). DU-145 cells, which express the adhesion receptors for Ln-10 (i.e., $\alpha_3\beta_1$) but do not make the ligand Ln-10, were then seeded on either cleaved or uncleaved Ln-10 for up to 1 hour. The number of adherent cells was quantified by crystal violet absorbance. The results (Figure 3) indicate that there is a 20% decrease in adhesion of DU-145 cells to cleaved Ln-10. This indicates that cleavage of Ln-10 by MT1-MMP affects the laminin protein such that cell adhesion is no longer as effective.

Cleavage of Ln-10 α_5 Chain Promotes Migration of Prostate Cancer Cells

To examine the potential role of MT1-MMP–cleaved Ln-10 in the migration of DU-145 prostate carcinoma cells, we used linear migration and transwell migration assays. We found that at 24 hours, the DU-145 cells were about two-fold more migratory on cleaved Ln-10 than on uncleaved Ln-10 (Figure 4, *A* and *B*). To determine whether the increase in transmigration of DU-145 cells on cleaved Ln-10 was due to the cleavage of the α_5 chain by MT1-MMP and not due to any other MT1-MMP effects, we used antisense oligonucleotides for MT1-MMP as described previously [16]. We have previously shown that cells treated with antisense oligonucleotides inhibit cell surface MT1-MMP expression by 66% in DU145 cells, whereas scrambled oligonucleotide had no effect [17]. MT1-MMP expression in cells treated with antisense is also decreased in these experiments (Figure 5*A*). Migration of DU-145 cells was not affected by the scrambled oligo, but the antisense oligonucleotide did demonstrate effects on migration (Figure 4, *A* and *B*). Both linear migration and transmigration on intact Ln-10 were reduced by approximately 70% to 80%, whereas only a 30% to 40% decrease

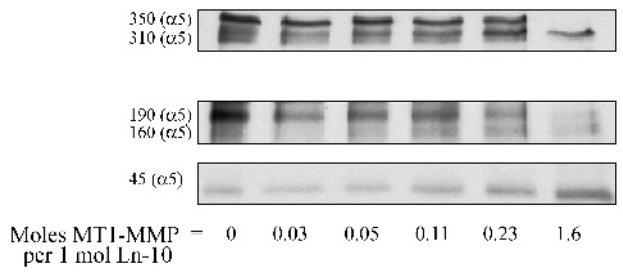

Figure 2. *MT1-MMP cleavage of Ln-10 occurs at physiologically relevant substrate/enzyme ratios. Western blot of purified Ln-10 treated with decreasing amounts of MT1-MMP. Indicated is the mole of MT1-MMP per mole of Ln-10, and M_W values of each identified protein band. Results are representative of three experiments.*

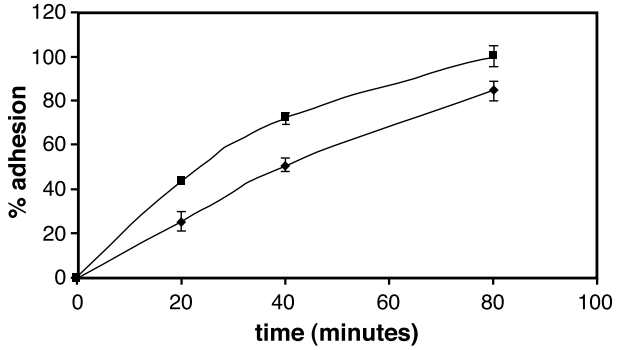

Figure 3. *Cleavage of Ln-10 results in decreased adhesion of prostate cancer cells. Adhesion assay of DU-145 cells to Ln-10 and Ln-10 treated with MT1-MMP at indicated timepoints. (♦) MT1-MMP–cleaved Ln-10. (■) Uncleaved Ln-10. Results are representative of three experiments performed in triplicate. Error bars represent the standard deviation of the representative experiment. P < .05 at all time points.*

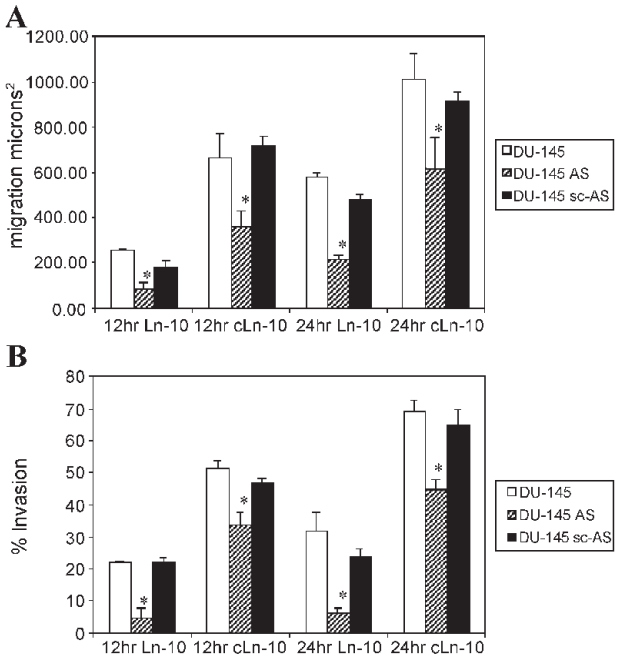

Figure 4. *Ln-10 cleavage increases migration and invasion of prostate cancer cells. (A) Migration of cells on Ln-10–coated microscope slide. (B) Invasion of cells through Ln-10–coated transwell cell insert chambers coated with Ln-10 at indicated time points. Groups are Ln-10, cleaved Ln-10 (cLn-10), Ln-10 with cells treated with antisense oligonucleotides (AS), and Ln-10 with cells treated with scrambled oligonucleotide (sc-AS). Results are representative of three experiments done in triplicate. Error bars represent standard deviation of one experiment. *P < .05.*

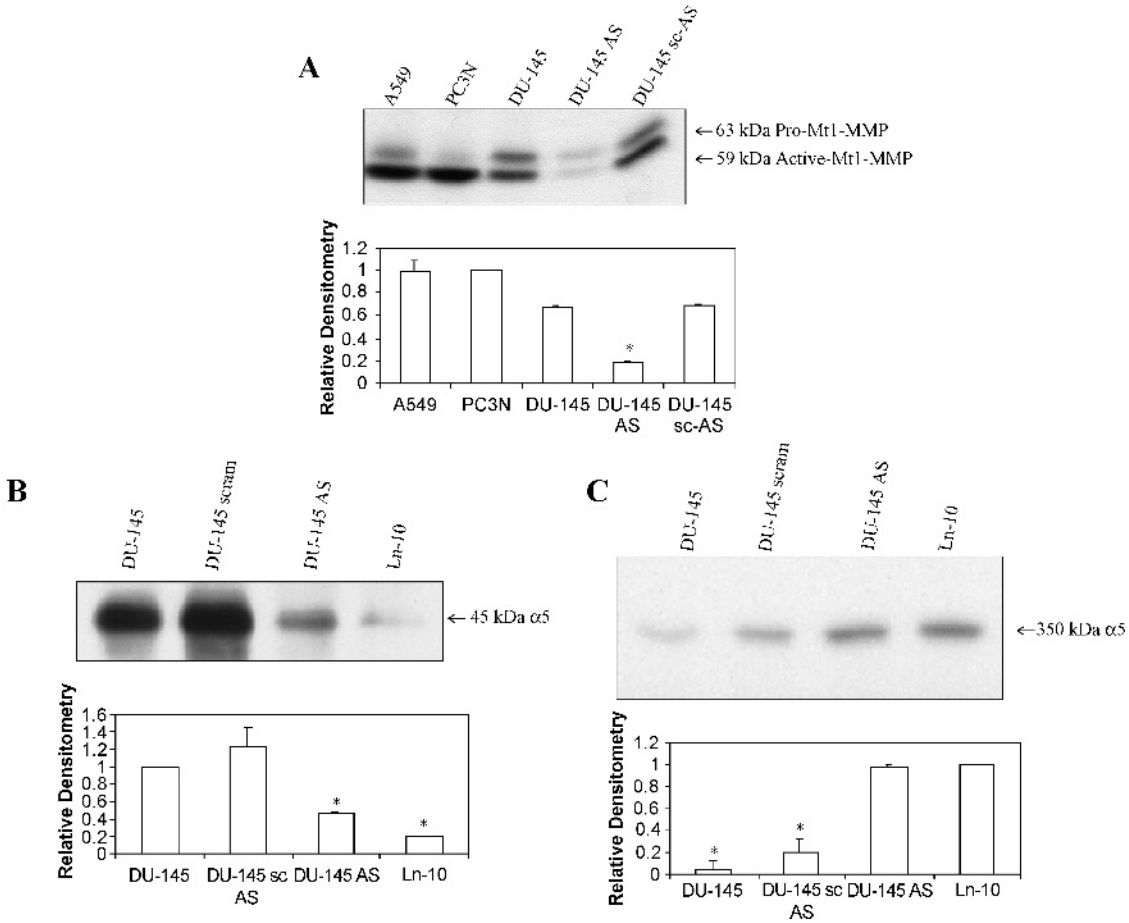

Figure 5. *Prostate cancer cell cleavage of Ln-10. (A) Western blot for MT1-MMP of cell lysates with densitometry analysis. (B) Western blot of total protein in CM for Ln-10 fragments with densitometry analysis. (C) Western blot for Ln-10 of matrix after removing cells with densitometry analysis. Results are representative of three experiments. *P < .006.*

was observed on cleaved Ln-10 in the presence of the MT1-MMP antisense oligonucleotides. This demonstrates that cleavage of Ln-10 allows for increased cell motility regardless of MT1-MMP status. Blocking MT1-MMP inhibits cell motility on intact Ln-10. This indicates a role for MT1-MMP in cell motility on Ln-10–coated surfaces. The antisense-treated prostate cancer cells were still able to migrate on cleaved Ln-10 (although to a lesser extent than untreated cells). This indicates that cleaving Ln-10 was the major contributing factor to the increased motility, but MT1-MMP does play some role in motility in addition to its ability to cleave the Ln-10 substrate.

Prostate Cancer Cells Are Capable of Processing Ln-10

Purified Ln-10 obtained from A549 cells contains some cleaved Ln-10. This indicates that MT1-MMP expressed on the surface of cells can cleave Ln-10, as A549 cells have been previously shown to express MT1-MMP [44]. To confirm that cleavage of Ln-10 could occur at the cellular level, we determined that prostate cancer cell lines expressing high levels of MT1-MMP could cleave intact Ln-10. We seeded an equal number of DU-145 cells treated with antisense oligonucleotides to MT1-MMP or scrambled oligonucleotides as described in previous experiments in SFM on tissue culture plates coated with purified Ln-10. As a control, one well of Ln-10 coated on the plate did not receive any cell, only SFM. MT1-MMP expression by these cells is shown in Figure 5*A*. Also shown is MT1-MMP expression in A549 and PC3N cells. Densitometry analysis demonstrates that DU-145 cells treated with antisense oligonucleotides express lower levels of MT1-MMP compared to untreated DU-145 cells. We found the 45-kDa cleaved fragment of Ln-10 to be released from the ECM into the CM, although to a lesser extent in cells treated with antisense oligos or in coated Ln-10 without the addition of cells (Figure 5*B*). In the matrix removed from the tissue culture plate, full-length Ln-10 α_5 chain was observed, although the amount of full-length Ln-10 remaining was reduced in the untreated DU-145 cells or the scrambled oligonucleotide-treated DU-145 cells, indicating that the Ln-10 is being cleaved by these cells (Figure 5*C*).

Ln-10 Cleavage Occurs in Prostate Tissue

Expression of both Ln-10 and MT1-MMP in prostate cancer has been previously reported [17,33]. Here, we show that MT1-MMP and Ln-10 are expressed in the same area of prostate cancer (Figure 6*A*), suggesting that MT1-MMP is available to cleave Ln-10 *in vivo*. An H&E stain of the tissue

area in Figure 6*A* is included to show tissue morphology (Figure 6*B*). To investigate whether Ln-10 cleavage occurs *in vivo*, we used immunohistochemical analysis of invasive prostate cancer tissue samples with 4C7 antibody to determine whether Ln-10 cleavage could be detected. We observed a discontinuous Ln-10 BL surrounding the cancer (Figure 6*C*), indicative of Ln-10 cleavage occurring *in vivo*. An H&E of this tissue area is also included (Figure 6*D*). Also, we manually microdissected snap-frozen serial sections of prostate tissues (20 μm), separating areas of high cancer concentration or areas of high normal gland concentration from the surrounding stroma. These microdissected samples were analyzed by Western blotting with 15H5 antibody. We found detectable levels of the full-length 350-kDa α_5 chain in both normal and cancer glands, but the 310-kDa cleavage fragment was only detected in cancer samples (Figure 6*E*), suggesting that MT1-MMP expressed in prostate cancer is capable of cleaving the full-length Ln-10 α_5 chain.

Discussion

We have previously shown that MT1-MMP is capable of cleaving human Ln-5 β_3 chain and that this cleavage increased prostate cancer cell migration and invasion [17]. In this study, we have explored a potential mechanism for the invasion of prostate cancer cells through the Ln-10–rich BL surrounding them *in vivo*. We have shown that MT1-MMP is expressed in prostate cancer along with Ln-10, and that MT1-MMP cleaves the α_5 chain of Ln-10 and that this cleavage has effects on both adhesion and migration. MT1-MMP is not expressed in normal prostate but its expression increases in prostate cancer progression. Because prostate cancer cells reform a BL rich in Ln-10, it seemed likely that MT1-MMP would be involved in degrading this BL in order for prostate cancer cells to metastasize. The role of the BL in cancer biology is not completely understood, but loss of BL continuity (due to degradation) has been associated with increasing malignancy [48,49].

The present study shows that incubation of MT1-MMP with Ln-10 generated novel cleavage products, which were identified by mass spectrometry analysis to be derived from the α_5 chain. We showed that this cleavage was specific for MT1-MMP and did not occur with MMP-2 (an MMP that MT1-MMP is known to activate) [50] treatment. We did not find any evidence that either the β_1 or γ_1 chains of Ln-10 were cleaved with MT1-MMP treatment. We found four distinct cleavage products of different apparent molecular weights: 310, 190,

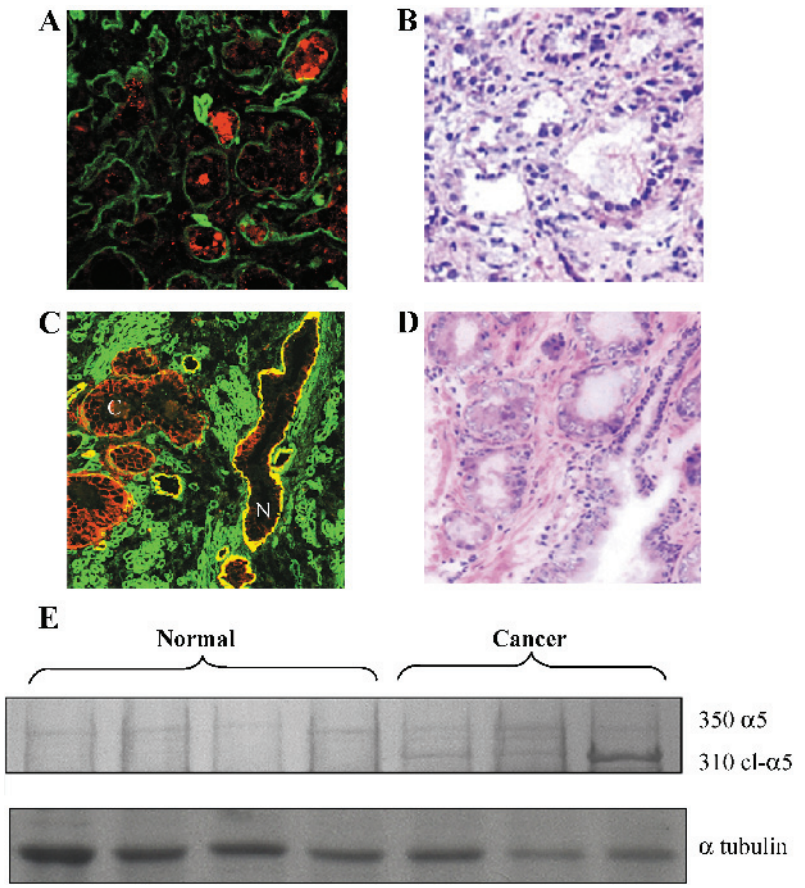

Figure 6. *Cleavage of Ln-10 occurs in human prostate tissue. (A) Immunohistochemistry of area of prostate cancer demonstrating Ln-10 (green) expression surrounding cancer and MT1-MMP (red) expression in prostate cancer. (B) H&E stain of the tissue area shown in (A). (C) Immunohistochemistry of prostate tissue with Ln-10 antibody. Areas of normal (N) and cancer (C) are indicated. Ln-10 is stained green and α_6 integrin is stained red. (D) H&E stain of tissue area shown in (C). (E) Western blot for Ln-10 and α-tubulin. Normal and cancer are indicated, as are M_W and corresponding protein identification.*

160, and 45 kDa. We propose that the 45-kDa product is the N-terminal fragment from the cleavage of the 350-kDa full-length α_5 chain into the 310- and 45-kDa products, based on peptide coverage given by mass spectrometry. We propose that the 190- and 160-kDa products are additional cleavage products of the 310-kDa band based on the mass spectrometry analysis. We expect that the additional cleavages by MT1-MMP give rise to approximately 150, 120, and 30-kDa products that are not present in high-enough concentrations to be detected by Coomassie or silver staining.

The Ln-α_5 chain is the largest of the α chains and is considered the primordial α chain. Ln α chains contain EGF-like domains in their N-terminus, and α_5, being the largest α chain, has the greatest number of EGF-like repeats. Interestingly, the proposed 45-kDa product contains two EGF-like repeats, and the additional processing of the 310-kDa product into 190- and 160-kDa products would yield fragments consisting almost entirely of EGF-like repeats. This provides a potential explanation for increased invasion on MT1-MMP–cleaved Ln-10 if an EGF-like fragment from Ln-10 cleavage can activate the EGF receptor on the cell surface, signaling for migration (Figure 7). Essentially, the Ln-10 fragments could function as matrikines [51], enzymatic fragments of ECM that contain cryptic biologically active sites, also called matricryptins [52]. In fact, an EGF-like fragment from rat Ln-5 cleavage by MT1-MMP has been shown to bind to the EGFR and stimulate downstream MAPK signaling to induce migration [53]. Also, colon cancer cells have been shown to spread on Ln-10 as a result of EGFR stimulation [54], indicating that EGF-like fragments could have similar effects on prostate tumor cells. We have demonstrated that at low molar enzyme/substrate ratios, we are able to detect these laminin cleavage products. Although the exact ratio of Ln-10/MT1-MMP in human tissues in unknown, we expect that due to the localization of MT1-MMP to the invading tumor front, this ratio might approach 1:1 in tissues. Our results show that cleavage is possible at 0.5 mol MT1-MMP/1 mol Ln-10 concentrations, which are likely to occur *in vivo*. The increased intensity of the 160-kDa band correlates to the decreased 190-kDa band. This indicates that the 190-kDa product is sequentially cleaved into the 160-kDa product, which corresponds to the mass spectrometry data of protein coverage of the bands.

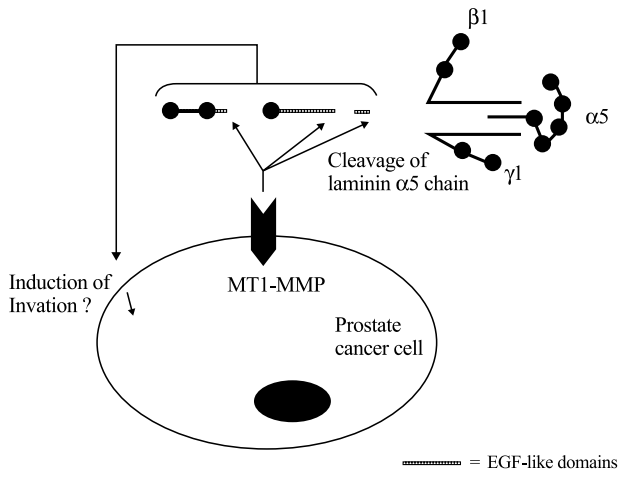

Figure 7. *Schematic model of MT1-MMP–dependent invasion through Ln-10. MT1-MMP expressed on the surface of a prostate cancer cell cleaves Ln-10 in the BL, forming cleavage products. Cleavage of Ln-10 disrupts the BL, allowing the prostate cancer cells to invade the BL. Additionally, the cleavage products may have effects on migration through exposure of cryptic peptides, potentially EGF-like peptides that may bind to the EGF receptor and induce signaling for migration.*

We have also shown that cleavage of Ln-10 by MT1-MMP causes a decrease in adhesion and an increase in migration of prostate cancer cells. The increase in migration on intact Ln-10 was inhibited with MT1-MMP antisense oligonucleotide treatment, suggesting that MT1-MMP cleavage of Ln-10 is responsible for the increased motility. Because some inhibition of migration was seen with MT1-MMP antisense oligonucleotide treatment on cleaved Ln-10, we suggest that although cleavage of Ln-10 is the major cause of both migration and movement on Ln-10, some other effects of MT1-MMP are necessary for this motility. In fact, recent studies have suggested and shown a possible role of MT1-MMP in downstream signaling for cell migration [55–57]. Although proteolysis of the ECM is essential for migration and invasion, excessive proteolysis can degrade ECM and disrupt cell–matrix interactions, actually inhibiting migration. Therefore, although we observed a decrease in adhesion to MT1-MMP–treated Ln-10, cells eventually did adhere, indicating that the strength of adhesion was modified in MT1-MMP–cleaved Ln-10 and not that the Ln-10 was degraded. Indeed, strength of adhesion is one mechanism that controls the speed of cell motility [58]. In this study, increased migration and invasion were correlated with a decrease in adhesion of cells to substrates coated with cleaved Ln-10.

We have demonstrated that this processing of Ln-10 can occur at the cellular level in cells that express high amounts of MT1-MMP on their cell surface. DU-145 cells were shown to cleave Ln-10 in tissue culture, and this cleavage was inhibited with antisense oligonucleotides to MT1-MMP, indicating that endogenous MT1-MMP is responsible for Ln-10 cleavage. Finally, we have demonstrated that Ln-10 cleavage occurs in prostate tissues. In areas of normal glands, full-length α_5 chain was detected, although in areas of cancer, both the full-length α_5 chain and the 310-kDa cleaved α_5 chain fragment were detected. Also detected was a discontinuous Ln-10 BL surrounding prostate cancer, further indicating that Ln-10 cleavage occurs *in vivo*. Although our initial experiments demonstrate Ln-10 cleavage and identify cleavage fragments used in the recombinant catalytic domain of MT1-MMP, our data demonstrating that prostate cancer cells can cleave Ln-10 and that Ln-10 cleavage occurs *in vivo*, along with our linear migration and transmigration assays, verify that Ln-10 cleavage by MT1-MMP is biologically significant.

These data clearly indicate that the Ln-10 α_5 chain cleavage plays an important role in tumor cell migration and invasion. This is a novel and important finding as this cleavage may enhance the invasion of prostate cancer cells *in vivo*. In addition, because Ln-10 is widely expressed, similar effects may be seen in other malignant tissues where

Ln-10 and MT1-MMP are also expressed. These effects of Ln-10 cleavage are probably even more widespread with respect to metastasis because blood vessels are surrounded by Ln-10. Therefore, any cancer that metastasizes through the bloodstream and expresses MT1-MMP can use this mechanism of Ln-10 cleavage to enter the blood vessels and to extravasate from the blood vessels at a distant location to form a metastasis. Because this study was conducted using human MT1-MMP, human Ln-10, and prostate cancer cells, these findings are particularly relevant to human prostate cancer and can lead to new approaches to intervention in preventing the metastasis of prostate cancer.

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