Cancer Cell–Associated MT1-MMP Promotes Blood Vessel Invasion and Distant Metastasis in Triple-Negative Mammary Tumors

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Abstract

Functional roles for the cancer cell–associated membrane type I matrix metalloproteinase (MT1-MMP) during early steps of the metastatic cascade in primary tumors remain unresolved. In an effort to determine its significance, we determined the in vivo effects of RNAi-mediated downregulation in mammary cancer cells on the migration, blood and lymphatic vessel invasion (LVI), and lymph node and lung metastasis. We also correlated the expression of cancer cell MT1-MMP with blood vessel invasion (BVI) in 102 breast cancer biopsies. MT1-MMP downregulation in cancer cells decreased lung metastasis without affecting primary tumor growth. The inhibition of lung metastasis correlated with reduced cancer cell migration and BVI. Furthermore, cancer cell–expressed MT1-MMP upregulated the expression of MT1-MMP in vascular endothelial cells, but did not affect MT1-MMP expression in lymphatic endothelial cells, LVI, or lymph node metastasis. Of clinical importance, we observed that elevated MT1-MMP expression correlated with BVI in biopsies from triple-negative breast cancers (TNBC), which have a poor prognosis and high incidence of distant metastasis, relative to other breast cancer subtypes. Together, our findings established that MT1-MMP activity in breast tumors is essential for BVI, but not LVI, and that MT1-MMP should be further explored as a predictor and therapeutic target of hematogenous metastasis in TNBC patients. Cancer Res; 71(13): 4527–38. ©2011 AACR.

Introduction

During metastatic dissemination, cancer cells activate a complex molecular machinery to migrate through the surrounding extracellular matrix (ECM) and intravasate into blood or lymphatic vessels (1–3). To negotiate barriers to cell migration, cancer cells secrete their own proteolytic enzymes or induce their expression in other cells through the release of cytokines (e.g., endothelial cells, tumor-infiltrating fibroblasts, or leukocytes; ref. 4). In particular, matrix metalloproteinases (MMP) are considered as key players in tumor progression because of their ability to remodel the ECM and cleave/activate membrane bound and matrix molecules, and cytokines that stimulate cancer cell migration and proliferation (5, 6). However, broad inhibition of MMPs for the treatment of advanced cancer has been unsuccessful in the clinic (7, 8). It is now known that MMPs can have both inhibitory and stimulatory effects on tumor progression (9, 10), thus a better understanding of the in vivo functions of specific MMPs in tumors is needed to develop effective therapies.

Membrane type 1 matrix metalloproteinase (MT1-MMP/MMP14), a surface bound MMP, plays a significant role in cancer cell migration and tumor progression. MT1-MMP is essential for the remodeling and invasion of fibrillar collagen gels and reconstituted basement membranes by cancer cells (11–13). In vivo, in the chick chorioallantoic membrane (CAM), breast cancer cells expressing MT1-MMP induced the degradation of the vascular basement membrane and cancer cell intravasation (11, 14). However, in a similar study done in the CAM, the downregulation of MT1-MMP in the invasive cancer cell line HT-1080 did not affect vascular invasion (15). In experimental tumor models, the expression of MT1-MMP in cancer cells or fibroblasts enhances tumor growth (5, 16–19). Cancer or stromal cell MT1-MMP also promotes the formation of experimental and spontaneous lung metastasis (18, 20–23). However, how cancer cell MT1-MMP affects the early steps of the metastatic cascade in primary tumors—such as cancer cell migration, blood vessel invasion (BVI), and lymphatic vessel invasion (LVI)—is unknown. We thus determined the role of MT1-MMP downregulation in cancer cells on migration and invasion in mammary tumors. Our findings show that cancer cell MT1-MMP expression promotes cancer invasion.
cell migration, induces the expression of MT1-MMP in vascular endothelial cells, and leads to vascular basement membrane remodeling, BVI and lung metastasis. The changes in cancer cell MT1-MMP expression did not significantly affect the expression of MT1-MMP in lymphatic endothelial cells, LVI or lymph node metastasis. We also show that cancer cell MT1-MMP expression correlates with BVI in human biopsies from TNBCs.

Materials and Methods

Cell culture

Human MDA-MB-231 (231) and MDA-MB-435S (435S) cell lines were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS in a humidified 5% CO2 incubator.

Cell line transfection

To produce a short-hairpin RNA (shRNA) targeting the MT1-MMP mRNA in position 436 to 456 (GenBank Acc.# NM_004995.2), 2 DNA sequences: 5’-GATCCATGCAGTTTTTCCAAAAAATGCAGAAAAGCTTTTCCAAAAAATGCAGAAGTTTTACCGTGTTCAAGAGACAAGCCGTAAAACTTCTGCATTTTTTGAAA3’ and 5’-AGCTTTTCCAAAAAATGCAGAAGTTTTACCGTGTTCAAGAGACAAGCCGTAAAAACCTTCTGTCATG3’ were annealed and cloned into the pSilencer 3.1-hyg vector (Ambion) according to the manufacturer’s protocol. A scrambled sequence was provided by Ambion (rf: AM5766). It consists of the same pSilencer hygro plasmid with a shRNA sequence that is not found in the human, mouse or rat genome database. MDA-MB-231 and MDA-MB-435S cells were transfected with the shRNA or control vector by electroporation and selected in Hygromycin (Invitrogen). To rescue MT1-MMP expression in shRNA cells (shc), cDNA reexpression was done by using a pEAK12 vector (gift from Dr. Brian Seed, Massachusetts General Hospital). MT1-MMP full-length coding sequence (GenBank Acc.# NM_004995) was amplified by PCR and cloned into the pEAK12 vector. This expression vector contains the EF-1 alpha promoter and the coding sequence of MT1-MMP was cloned between SpeI and HindIII restriction enzyme sites. The vector also contains puromycin-resistant marker and transfected cells were selected with 1 ug/mL puromycin. In parallel, the 231 shRNA and 435 shRNA cells were transfected with the empty pEAK12 vector. The in vitro and in vivo experiments were done with pooled populations of selected cells.

Western blotting

Protein extracts were prepared from tumor cells, cultured in the absence of serum, or homogenized tumor tissue by using a protein extraction buffer (radioimmunoprecipitation assay buffer + protease inhibitor, Complete Mini, Roche Diagnostics GmbH). Thirty micrograms of protein were separated on a 4% to 10% SDS-PAGE gel (Bio-Rad), transferred to a polyvinylidene difluoride membrane and incubated with anti-MT1-MMP (Millipore, ab815) at 4°C overnight. This antibody recognizes the pro, active, and degraded forms of MT1-MMP. The blots were then incubated with the appropriate secondary antibodies and visualized by using the enhanced chemiluminescence kit (Amersham). For loading controls, the blots were stripped and incubated with either anti-actin (Santa Cruz, sc-1616) or anti-glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling, mAb #2118).

Immunocytochemistry

For staining of cell surface MT1-MMP, cells were plated in 8-well slide chambers and fixed with 4% paraformaldehyde for 30 minutes. The cells were then incubated overnight with an MT1-MMP antibody (Millipore, LEM-2/15.8 clone, MAB3328), a monoclonal mouse MT1-MMP antibody that reacts with the catalytic domain of human and murine MT1-MMP. After staining with a anti-mouse Cy3-conjugated secondary antibody and a 4’,6-diamidino-2-phenylindole (DAPI) counterstain, we imaged the fixed cells with a confocal microscope. Confocal images were quantified with a custom Matlab program that determined the percent of high expressing MT1-MMP cells per field with high expression defined as fluorescence intensity greater than 25% of the maximum staining intensity for MT1-MMP.

In vitro invasion assay

Collagen I invasion assays were done as previously described (24). Briefly, collagen I stock solution (Vitrogen, Cohesion Inc) was diluted and adjusted to pH 7.4 with sterile NaOH (1 mol/L). The collagen mixture was poured into 24-well plates. After polymerization at 37°C, 50,000 cells were seeded on top of the collagen gel and cultured in 0.5% FBS DMEM for 72 hours. Seventy hours later, 200 μm stacks, which included the top of the gels and 40 μm steps, were acquired in 3 areas of each well with an inverted brightfield/epifluorescent microscope with a motorized stage (Olympus) and 20× objective. All cells in the stack were counted and the fraction of cells located between 40 and 200 μm was determined. The percentage of cancer cell invasion was determined in 3 independent experiments.

Collagen I degradation

Rat tail collagen I (BD Biosciences) was prepared at a concentration of 3 mg/mL and coated evenly on a 24-well plate then polymerized at 37°C for 1 hour. The uncovered plates were left to air-dry in the cell culture hood for 2 hours. The wells were then washed and dried for 1 hour. Twenty-five thousands cells were added to each well and incubated for 4 hours at 37°C. Control wells were used with no cells added to the collagen but underwent the same conditions. The cells were then treated with 10 μmol/L tissue inhibitor of metalloproteinase (TIMP)-1 (R&D Systems), 10 μmol/L of TIMP-2 (R&D Systems), or vehicle controls and incubated for 5 days at 37°C. To assess the amount of collagen degradation, the medium was removed and the wells were washed 2 times with PBS. The cells or collagen only wells were trypsinized for 2 to 3 minutes at 37°C and removed with PBS washes (2×). The wells were then fixed with 10% paraformaldehyde for 20 minutes, washed 3× with PBS, and then 500 μL of Coomassie Brilliant Blue stain (Bio-Rad) was added to each well for 5 minutes. Finally, the wells were washed 3× with water and air
dried overnight. Images (200×) of the well centers were collected with an Olympus BX40 microscope and a custom written Matlab program processed the images based on Coomassie blue signal density. As expected, the trypsinization did not affect the collagen content in the collagen only wells. The percent collagen was calculated by comparing the Coomassie staining density of collagen in wells with and without (collagen only wells) cells.

Animal experiments and metastasis assays

The Massachusetts General Hospital Subcommittee on Research Animal Care approved all the mice experiments. For each cell line, 3 million cells were injected in the third mammary fat pad of 6 to 8 week old severe combined immunodeficient mice (SCID) females. Tumor sizes were measured every 3 to 4 days and volumes were based on the formula: 1/6 × π × L × W². In another experimental group, to allow the development of detectable metastasis, 231 and 435S tumors were resected 6 and 8 weeks after tumor implantation, respectively. Eight weeks after surgical removal of the primary tumor, mice were sacrificed and a necropsy was done to determine the presence of axillary lymph nodes and distant lung metastases. Macroscopic metastases were counted on the surface of the lung with a stereo microscope. Lung macro-metastasis and micrometastasis were also determined in whole lung sections stained with hematoxylin and eosin.

Mammary fat-pad window and multiphoton laser scanning microscopy

After the injection of 231 cells in the third mammary fat pad, a mammary fat-pad window was implanted 2 weeks later. Two symmetrical titanium frames (custom design) were implanted so as to sandwich the extended double layer of skin. Images were obtained by using a custom built multiphoton laser scanning microscope, constructed around a Bio-RAD MRC600 confocal scan head and a Zeiss inverted fluorescence microscope. The green fluorescent protein (GFP; cancer cells) and second-harmonic generation (SHG; fibrillar collagen) signals were obtained simultaneously using 900 nm excitation laser light, with detection via (i) 435DF30 (SHG) and (ii) a high-pass 475 dichroic filter and 525DF100 (GFP) emission filters (Chroma Technologies). At least 3 regions per tumor were imaged every 10 minutes by using a 20×, 0.5 numerical aperture aqueous long working distance objective lens. Each image stack consisted of 20 images spaced 5 μm apart, covering an area of 3.0 × 10² μm². Cancer cell migration was assessed in the individual zxy images from the time volumes. In general, the cells observed did not migrate between different z slices between 0 and 60 minutes. The difference in centroid position between t = 0 to t = 60 gave a measure of cell speed and only cells migrating 4 μm per hour or more were considered. The speed of migrating cancer cells was averaged for each tumor at each time point. In each image stack, the fraction of migrating cancer cells was determined.

Immunostaining of paraffin and frozen sections

For the detection of MT1-MMP in mice tissues, we used the LEM-2/15.8 clone, a mouse monoclonal MT1-MMP antibody that reacts with the catalytic domain of human and murine MT1-MMP (Millipore, MAB3328). The MT1-MMP immunostaining of human sections was done with Ri So144, a rabbit polyclonal antibody (Applied Genomics Inc.). We confirmed the specificity of LEM-2/15.8 and Ri So144 with MT1-MMP−/− fibroblasts (a kind gift from Dr. Kenn Holmbeck, NIH). Both MT1-MMP antibodies stained wild-type fibroblasts but did not cross-react with MT1-MMP−/− fibroblasts. Furthermore, the preincubation of LEM-2/15.8 or Ri So144 with the recombinant MT1-MMP protein abolished the immunostaining on tumor sections. Immunostaining was also done with antibodies against CD31 (DAKO), keratin 19 (Neomarkers), MECA-32 (BD Bioscience), podoplanin (AngioBio) or collagen IV (Millipore). Five-μm-thick sections were obtained from paraffin-embedded tumor samples. Sections were dewaxed in xylene and rehydrated through graded ethanol. After inhibition of the endogenous peroxidase activity and antigen retrieval, sections were incubated with primary antibodies overnight. The sections were then incubated with the corresponding secondary antibodies and developed in diaminobenzidine (DAKO). BVI and LVI were quantified in peritumoral areas and within 10 cancer cell layers from the tumor surface. Collagen IV immunostaining was used to assess changes in vascular basement membrane morphology, which appeared to have focal changes in collagen IV thickness or interruptions in collagen IV but not MEGA-32 immunostaining. The total number of vessels with thinning or collagen IV interruption was determined in at least 6 tumors per group.

The triple immunostaining for CD31, collagen IV and MT1-MMP was done with frozen sections (20 μm thick) from 4% paraformaldehyde-fixed tumors. The sections were incubated first with Armenian hamster anti-mouse–CD31 (Millipore) and rabbit anti-mouse collagen IV (Millipore) overnight at 4°C. The sections were then incubated for 1.5 hours with anti-Armenian hamster-Cy5 and anti-rabbit–fluorescein isothiocyanate (FITC). Following the first set of secondary antibodies, the sections were blocked for 1 hour with mouse on mouse (M.O.M) solution (Vector Laboratories) to block endogenous mouse IgG and then incubated with mouse anti-human–MT1-MMP (Millipore) overnight at 4°C. Finally, anti-mouse–Cy5 3 was applied for 1.5 hour and the slides were mounted in vectashield with DAPI counterstain (Vector Laboratories). LYE-1 immunostaining of lymphatic vessels (rat anti-mouse–LYVE-1; R&D) combined with collagen IV and MT1-MMP was done as described above for CD31. Stained slides were imaged with a confocal microscope and quantified with custom Matlab programs.

In situ zymography combined with MT1-MMP immunostaining

Cryostat sections (20 μm thick) from fixed (25) (paraformaldehyde 4%) and nonfixed tumors were incubated in dye quenched (DQ) collagen IV (1:30 EnzChek kit Invitrogen) in the dark at room temperature for 1 hour. The sections were then incubated with rat anti-mouse LYE-1 overnight at 4°C followed by anti-rat–Cy5 secondary antibody at room temperature. The sections were then treated with the M.O.M solution followed by the anti–MT1-MMP antibody overnight.
at 4°C. After applying the anti-mouse–Cy3 for 1.5 hours, slides were mounted in vectashield. For CD31 staining the same protocol was followed except that the CD31 and MT1-MMP primary antibodies were incubated simultaneously. To assess whether the degradation of DQ collagen IV was MMP dependent, the sections were treated with EDTA (30 mmol/L) or 1,10-phenanthroline (250 μmol/L). Using a confocal microscope, 15 to 30 μm stacks were acquired by using a 20× objective. For colocalization of DQ collagen IV degradation and MT1-MMP with either CD31 or LYVE-1, custom Matlab programs were used to segment vessels and quantify the overlap between staining.

To determine the location of DQ collagen IV degradation activity, we used an NIH image analysis macro that gave the distance of DQ collagen IV degradation signal from the MECA-32 immunostaining signal of blood vessels: images were autothresholded (with manual verification) and subjected to serial dilation. At each dilation level, the number of overlapping pixels between the red (MECA-32) and green (DQ FITC-collagen degradation) channels was assessed. This procedure allowed quantification of the average distance of the collagen degradation from blood vessels.

MT1-MMP immunostaining in human breast cancer

The Massachusetts General Hospital institutional review board approved this study in accordance to NIH (Bethesda, MD) guidelines. Formalin fixed paraffin-embedded tumor samples from 75 consecutive ER-positive and 73 consecutive ER-negative invasive breast carcinomas diagnosed at the Massachusetts General Hospital (Boston, MA) between 2005 to 2006 were prospectively collected. MT1-MMP expression and tumor BVI were determined by immunostaining (see immunostaining methods). Tumor BVI was defined as CD31-positive vessels containing tumor clumps (keratin-19 positive cells) and LYVE-1 negative endothelia.

Statistical analysis

Unpaired Student’s t tests were done. P values for association between MT-MMP and other variables are from unconditional Fisher exact test, using Berger’s method with 99.9% CI (26). In addition, we present the estimates of ORs with 95% CIs calculated by the method of Miettinen and Nurminen (27).

Results

Downregulation of MT1-MMP in cancer cells

To determine the causal role of MT1-MMP in cancer cell dissemination, an shRNA construct was stably transfected to downregulate the MT1-MMP expression in 2 human metastatic cell lines: the ER-PR-HER2-negative breast carcinoma MDA-MB-231 (231) and MDA-MB-435S (435S). Both 231 and 435S produce lung metastasis when implanted in the mammary fat pad of SCID mice. Western blotting of cell lysates confirmed the downregulation of the MT1-MMP protein and rescue of MT1-MMP expression in shRNA cells (Supplementary Fig. S1A). Furthermore, the downregulation of MT1-MMP was also confirmed by immunofluorescence staining of the cells. The shRNA downregulation of MT1-MMP in 231 and 435S reduced the fraction of cells expressing high levels of surface MT1-MMP by 68 and 75%, respectively (Fig. 1A and B). To ensure the reduction in expression modified the MT1-MMP activity, we determined the collagen I degradation and invasive potential of mock and shRNA cells. shRNA cells degraded significantly less collagen fibers than mock cells (Fig. 1C and D). A similar reduction in collagen degradation could be observed with mock cells in the presence of TIMP-2 (an inhibitor of MT1-MMP) but not TIMP-1 (an MMP inhibitor that does not affect MT1-MMP, Fig. 1D). Cell invasion into collagen gels was also significantly decreased by MT1-MMP downregulation or in the presence of TIMP-2 but not TIMP-1 (Fig. 1E). Taken together, the expression and activity of cancer cell MT1-MMP was specifically and significantly reduced in shRNA cells compared with mock-transfected cells.

Cancer cell MT1-MMP downregulation has no effect on primary tumor growth but reduces the occurrence of spontaneous lung metastasis

To determine the effect of MT1-MMP modulation on tumor growth and spontaneous metastasis, 231 and 435S were grown in the mammary fat pad of SCID mice until they reached ~250 mm³, resected and metastasis development was evaluated 8 weeks later. MT1-MMP protein downregulation was maintained up to 8 weeks after tumor implantation and did not affect primary tumor growth (Supplementary Fig. S1B, Fig. 2A). Conversely, MT1-MMP downregulation significantly decreased the occurrence of lung macrometastasis and micrometastasis in mice with 231 and 435S tumors, respectively (Fig. 2B and C). Furthermore, histologic examination revealed that the number of metastatic lesions per lung was reduced by more than 85% in mice with 231 and 435S tumors (Fig. 2D). The transfection of an MT1-MMP expressing construct in shRNA cells restored the lung metastatic potential in both tumor models suggesting that this was a specific effect of MT1-MMP (Fig. 2C). In addition to lung metastasis, the 231 mammary tumors produced lymph node metastasis but their occurrence was not significantly affected by MT1-MMP downregulation (Fig. 2C).

Cancer cell MT1-MMP downregulation reduces BVI but not LVI

To investigate the role of cancer cell MT1-MMP on metastatic steps in primary tumors, we determined the effect of MT1-MMP downregulation on BVI and LVI (Fig. 3A and B). We quantified BVI and LVI in intratumoral (10 cancer cell layers from the tumor surface) and peritumoral areas. In both tumor types, we found significantly less BVI in shRNA compared with mock tumors (Fig. 3C, Supplementary Fig. S2A and B). Although LVI was more frequent than BVI (20% of LVI vs. 3.2% BVI in mock 231), there was no difference in LVI between...
shRNA and mock tumors in either tumor model (Fig. 3D). Collectively, these data suggest that cancer cell MT1-MMP affects tumor cell intravasation in blood but not lymphatic vessels.

**Cancer cell MT1-MMP expression affects the expression of endothelial-associated MT1-MMP and basement membrane remodeling in blood but not lymphatic vessels**

Because blood vessel—but not lymphatic—invasion was reduced by MT1-MMP downregulation, we used immunofluorescence to determine the relationship between MT1-MMP and the vascular basement membrane. Consistent with our western and immunocytochemistry data there was significantly less overall MT1-MMP staining in shRNA than mock tumors (Supplementary Fig. S3A). Interestingly, we found in shRNA compared with mock tumors significantly less colocalization of MT1-MMP with CD31 and collagen IV immunostaining of blood vessels (Fig. 4A–C). We next assessed whether this endothelial-associated MT1-MMP expression resulted in changes in enzymatic activity by conducting *in situ* zymography with the DQ collagen IV substrate (Fig. 4D). In shRNA tumors, there was less collagen IV degradation associated with both the tumor bulk (Supplementary Fig. S3B) and CD31-positive blood vessels compared with mock tumors (Fig. 4E). We also found a significant decrease in DQ collagen IV degradation in shRNA tumors within 2 μm from the wall of blood vessels (Supplementary Fig. S3C and D). Furthermore, the blood vessel colocalization between MT1-MMP immunostaining and collagen IV degradation was reduced (Fig. 4F). Broad MMP inhibition by EDTA or 1,10-phenanthroline abolished the observed DQ collagen IV degradation activity.
In light of these results, we next determined whether the structural integrity of vascular basement membranes was disrupted by using collagen IV immunohistochemistry (Fig. 4G). At the tumor edge in shRNA tumors, we found fewer vessels with thinning and breaches in basement membrane (Fig. 4H). These findings suggested that the MT1-MMP expression in cancer cells modulates MT1-MMP expression and activity in CD31-positive blood vessels surrounded by collagen IV positive basement membranes.

Interestingly, even if lymphatic vessels had limited collagen IV coverage, we found MT1-MMP expression in Lyve-1–positive endothelial cells—especially in intratumoral vessels—but there was no difference between shRNA and mock tumors (Supplementary Fig. S4A and B). Importantly, in terms of enzymatic activity, we found no difference in DQ collagen IV signal around lymphatic vessels between mock and shRNA tumors (Supplementary Fig. S4C). Altogether, the data from blood and lymphatic vessels suggest that cancer cell–associated MT1-MMP primarily affects blood vessel endothelial MT1-MMP expression, activity and basement membrane remodeling.

Cancer cell MT1-MMP downregulation reduces the number of migrating cancer cells in primary tumors

In addition to degrading basement membrane, cancer cells need to migrate to invade blood vessels. To assess the involvement of MT1-MMP in tumor cell migration in vivo, we developed a chronic mammary fat-pad window. In 231 tumors, the migrating cancer cells exhibited a rounded morphology reminiscent of amoeboid motion (Supplementary Movie S1). The fraction of migrating cancer cells continued to increase significantly with tumor growth in mock

Figure 2. MT1-MMP downregulation does not affect primary tumor growth but reduces lung metastasis. A, mock, shRNA, or shRNA cells transfected with pEAK12-MT1-MMP expressing vector (shc cells) implanted in the mammary fat pad of SCID mice exhibited no differences in primary tumor growth; 231, n = 11 (mock), n = 10 (shRNA), n = 5 (shc cells); 435, n = 10 (mock), n = 10 (shRNA), n = 10 (shc cells). B, lungs from 231 tumor-bearing mice were determined to be negative (left) or positive (right) for lung macrometastasis. C, the percent of mice with lungs positive for macrometastasis (231) or micrometastasis (435S) was significantly reduced in the shRNA groups whereas lymphatic metastases were not significantly reduced. In mice with shc cells and mock mammary tumors, the percentage of lung metastasis was similar (*, P < 0.05, lung mets: 231, n = 18 (shRNA), n = 19 (mock), n = 9 (shc cells); 435, n = 16 (shRNA), n = 18 (mock), n = 5 (shc); lymph node mets: 231, n = 15 (shRNA), n = 14 (mock). D, in histologic sections, the number of metastatic lesions per lung were significantly reduced in shRNA compared with mock tumors (*, P < 0.05, n = 10 (shRNA) and n = 12 (mock)). Error bars represent the SEM.
tumors while we found no increase in migrating cancer cells over time in shRNA tumors (Fig. 5A). The range of migration speed varied between 4 and 37 μm/h in mock, and 4 and 15 μm/h in shRNA tumors. Furthermore, the average migration speed of tumor cells was reduced in the shRNA tumors compared with mock tumors (shRNA = 8.0 ± 0.49 μm/h, *, mock = 12.8 ± 1.37 μm/h, *, P = 0.001, n = 14 tumors for shRNA, n = 13 for mock, ± SEM). To assess whether the increased number of migrating cancer cells was due to the separation of cancer cells from the main tumor mass, we grew 231 cells on agarose to stimulate homotypic aggregation. The 231 shRNA cells produced large compact clumps surrounded by a few isolated cells whereas mock cells were much more dispersed suggesting that cancer cell expression of MT1-MMP reduced cell–cell adhesion (Fig. 5B). These data suggest that MT1-MMP expression can increase the probability of metastasis through an increase number of cancer cells reaching vessels.

**Cancer cell MT1-MMP expression correlates with BVI in TNBC**

We determined if MT1-MMP expression, in human breast cancer, correlated with tumor grade, stage, size, hormone receptor expression, lymph node status, and BVI in a prospectively collected consecutive series of 75 ER-positive and 73 ER-negative human breast tumors (Supplementary Table S1). Of these, 102 patients had analyzable tumor sections and relevant clinical/pathologic data. By immunostaining, we found MT1-MMP expression in cancer cells of 52.9% of breast tumors (Fig. 6A and B). Most tumors had diffuse MT1-MMP expression but in some cases, MT1-MMP expression was restricted to focal areas. In addition to tumor cells, MT1-MMP expression was also found in most myoepithelial structures lining normal breast ducts and in tumor fibroblasts and histiocytes (Fig. 6C and D). In parallel, we determined, for each tumor, the presence of BVI by CD31-Keratin19 and Lyve-1 immunostaining (Fig. 6E and F). BVI was defined as CD31-positive, Lyve-1-negative endothelial-lined vessels that contain tumor cells. We found BVI in 13.7% of cases. Tumor MT1-MMP expression also did not correlate with hormone receptor status, tumor size, grade, stage, and BVI. Lymph node metastasis frequency was higher in patients with primary tumors that were MT1-MMP-positive (23/46) than MT1-MMP-negative (13/43), however, the trend was not significant (P = 0.08). The same correlation analysis was done in tumor subtypes segregated by ER, PR, and HER2 status. In TNBC, MT1-MMP expression was comparable with the overall cohort (present in 48.5% of the cases); however, BVI was significantly more frequent in TNBC than other breast cancer subtypes (25% vs. 7%, P = 0.01). Interestingly, MT1-MMP expression correlated significantly with BVI [P = 0.043, OR: 5.3 (1.06, 28.3)], but not with lymph node metastasis [P = 0.11, OR: 3.5 CI (0.88, 13.3)], tumor size, grade, or stage in the TNBC subtype (Fig. 6G).
Discussion

The ectopic expression of MT1-MMP in cancer cells can stimulate tumor growth (16, 29). In contrast, our results show that MT1-MMP downregulation in cancer cells does not inhibit primary tumor growth, but significantly inhibits the formation of lung metastasis. It has also been shown that the genetic ablation of MT1-MMP in stromal cells of the...
mammary gland induced a slightly faster growth of orthotopic tumors, but also significantly reduced the incidence of lung metastasis (23). Although the downregulation of cancer cell or stromal MT1-MMP expression did not affect primary tumor growth, antibody blockade of MT1-MMP reduces the primary tumor growth and number of lung metastasis in mice with mammary 231 tumors (30). Hence, our findings suggest that cancer cell MT1-MMP plays a critical role in the dissemination of mammary cancer cells to the lungs while it maybe less critical for primary tumor growth.

A hallmark of malignant cancer is the invasion of blood and lymphatic vasculatures. The vascular basement membrane of blood vessels is thought to be a significant barrier to BVI but not LVI (31). In vitro cancer cell MT1-MMP, MT2-MMP, and MT3-MMP were individually sufficient for basement membrane remodeling and cellular transmigration (12). In vivo, the introduction of breast cancer cells, expressing MT1-MMP, to the CAM induced the degradation of the vascular basement membrane and cancer cell intravasation (11, 14). In another study, the downregulation of MT1-MMP in the fibrosarcoma cell line HT-1080 did not affect the intravasation of cancer cells in the CAM (15). Here, we show in an orthotopic model of breast cancer that cancer cell MT1-MMP downregulation affects BVI but not LVI. The limited basement membrane around lymphatic vessels could explain the higher frequency of LVI than BVI, and why LVI is not affected by the modulation of cancer cell MT1-MMP because basement membrane remodeling is not required. The greater BVI in mock than shRNA tumors correlated with the enhanced endothelial MT1-MMP expression and collagen IV degradation activity around blood vessels along with basement membrane interruptions. Although the disruption of the vascular basement membrane by cancer cell MT1-MMP plays a role in BVI, other mechanisms mediated by MT1-MMP degradation may also be involved. MT1-MMP can cleave and release cell surface (CD44, syndecan-1) or matrix molecules (laminin-332 and laminin-511) that increase cancer cell migration (32–35). Cancer cell MT1-MMP can also cleave and release EMMPRIN, which stimulates the expression of other MMPs including MT1-MMP in other cell types (36). We also found that cancer cell MT1-MMP stimulates the migration and most likely the detachment of cancer cells from the main tumor mass, increasing the number of cancer cells that can disseminate via blood vessels. Although MT1-MMP can cleave E-cadherin and promote epithelial to mesenchymal transition (37), we show here in E-cadherin negative cells (MDA-MB-231) that MT1-MMP activity also affects cell–cell adhesion. Thus, the cancer cell MT1-MMP activity enhances the expression of endothelial MT1-MMP and basement membrane remodeling in blood vessels, and the number of migrating cancer cells, promoting BVI in mammary tumors.

In women with breast cancer, the MT1-MMP mRNA, extracted from tumor or stromal cells, is an independent predictive indicator of overall survival (38, 39). Peritumoral lymphovascular invasion correlates with distant metastasis and is also an independent prognostic factor of overall survival in node-negative breast cancer (40, 41). Although the prognostic value of BVI and LVI was evaluated in breast cancer (42), to our knowledge, the assessment of both BVI and cancer cell MT1-MMP expression in different breast cancer subtypes was never done. Here, we found in TNBC significantly more peritumoral BVI compared with other breast cancer subtypes, and MT1-MMP expression in cancer cells correlated with BVI. Patients with TNBC have a poor prognosis, which has been associated with rapid metastatic recurrence at distant sites (43). Also, in breast cancer patients with extensive peritumoral vascular invasion, disease-free survival is significantly shorter for TNBC patients (41). Although our experimental results and findings in clinical samples suggest that MT1-MMP plays a significant role in BVI and distant metastasis in the triple-negative phenotype, other molecules, associated with epithelial-mesenchymal transition and cancer cell dissemination, may also be involved. In comparison with other breast cancer subtypes, the proportion of tumors expressing epidermal growth factor receptor, vimentin, CD151, or low E-cadherin is significantly higher in TNBC (44, 45). Hence, our experimental results and findings in human samples suggest that MT1-MMP expression by cancer cells most likely facilitates BVI and may increase the risk of distant metastasis in patients with TNBC.

In conclusion, cancer cell MT1-MMP is necessary for increased tumor migration, vascular basement remodeling, BVI, and distant metastasis. In addition, in TNBC BVI is
more prevalent than in other breast cancer subtypes and correlates with the expression of MT1-MMP in cancer cells warranting further therapeutic investigation in this breast cancer subtype.

Disclosure of Potential Conflicts of Interest

R.K. Jain received commercial research grants from Dyax, AstraZeneca, MedImmune, and Roche; consultant fees from AstraZeneca, Dyax, Astellas, Genzyme, and Noxxon Pharma; and a speaker honorarium from MPM Capital. R.K. Jain owns stock in SynDevRx. No reagents or funding from these companies were used in these studies. There is no significant financial or other competing interest in the work.

Acknowledgments

The authors thank Julia Kahn, Carolyn Smith, Sylvie Roberge, and Peigen Huang for their outstanding technical assistance. The authors thank Dr. Marek Ancukiewicz for his help with biostatistics. The authors also thank Drs. Ivan Stamenkovic, Dan Duda, Timothy Padera, Elias Perentes, and Aditi Hazra for useful comments on the manuscript.

Grant Support

This work was supported by NIH grants P01-CA80124 and R01-CA115767 and a DOD Breast Cancer Research Program grant (BC-095991) (R.K. Jain); R01-CA98706 (Y. Boucher); T32-CA073479 (N.D. Kirkpatrick); the Swiss National Funding for young scientists n°107382, Fond de Peftectement du CHUV and the bourse de la faculté de Biologie et de Médecine (jeunes chercheurs: J.Y. Perentes); and Japan Society for the Promotion of Science (S. Nagano).

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Received December 21, 2010; revised May 2, 2011; accepted May 5, 2011; published OnlineFirst May 13, 2011.

Figure 6. MT1-MMP immunostaining and BVI in human breast tumors. A and B, representative images of MT1-MMP immunostaining. MT1-MMP expression (brown) was present (A) or absent (B) in the epithelial compartment of breast tumors. C, however, the myoepithelium was always strongly positive (brown) and used as a positive control for the immunostaining in each section. D, in some cases, MT1-MMP expression was also observed in fibroblasts and immune infiltrates (brown arrowheads). Bar for A–D, 20 μm. E, tumor BVI was observed by using CD31 (brown, white arrowheads) to mark blood vessels and Keratin19 (pink, black arrowheads) for cancer cells. F, invaded vessels were confirmed to be blood vessels via negative Lyve-1 (lymphatic) staining. Scale bars for E and F, 100 μm. G, in tumor sections of TNBC patients, the expression of cancer cell MT1-MMP correlated significantly with BVI (P < 0.05, 17 of 35 patients positive for MT1-MMP) but not lymph node metastasis (16 of 32 patients positive for MT1-MMP).
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Cancer Research

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Jean Y. Perentes, Nathaniel D. Kirkpatrick, Satoshi Nagano, et al.

Cancer Res 2011;71:4527-4538. Published OnlineFirst May 13, 2011.

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