Cancer cell associated MT1-MMP promotes blood vessel invasion and distant metastasis in triple-negative mammary tumors

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Cancer cell-derived MT1-MMP promotes blood vessel invasion

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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, 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, lymphatic vessel invasion (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, which have poor prognosis and high incidence of distant metastasis, relative to other breast cancer subtypes. Together, our findings establish 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 triple-negative breast cancer patients.
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) (4). In particular, matrix metalloproteinases (MMPs) are considered 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 performed 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 MTI-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 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 demonstrate that cancer cell-MT1-MMP expression correlates with BVI in human biopsies from triple-negative breast cancers.

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 (Manassas, VA). Cells were cultured in DMEM supplemented with 10% FBS in a humidified 5% CO2 incubator.

Cell line transfection

To produce an shRNA targeting the MT1-MMP mRNA in position 436 to 456 (GenBank Acc.# NM_004995.2), two DNA sequences: 5'-GATCCATGCAGAAGTTTTACGGCTTGTCTCTTGAACAAGCCGTAAAACTTCTGCATTTTTTTGGAAA3' and 5'AGCTTTTCCAAAAATGCAGAAGTTTTACGGCTTGTCTCTTGAACAAGCCGTAAAACTTCTGCATG3' were annealed and cloned into the pSilencer 3.1-H1 hygro 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 an 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, cDNA re-expression was performed using a pEAK12 vector. MT1-MMP full-length coding sequence (GenBank accession #NM_004995) was amplified by PCR and cloned into peak12 vector (gift from Dr Brian Seed, MGH). This expression vector contains the EF-1alpha 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 performed 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 using a protein extraction buffer (RIPA buffer + protease inhibitor, Complete Mini, Roche Diagnostics GmBH). Thirty μg of protein were separated on a 4-10% SDS-PAGE gel (Biorad), transferred to a PVDF 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 using the enhanced chemoluminescence kit (Amersham). For loading controls, the blots were stripped and incubated with either anti-actin (Santa-Cruz, sc-1616) or anti-GAPDH (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 min. 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 dapi counter stain, 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 performed as previously described (24). Briefly, collagen I stock solution (Vitrogen) was diluted and adjusted to pH 7.4 with sterile NaOH (1M). 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 hrs. Seventy hrs 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 20X 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 hr. The uncovered plates were left to air-dry in the cell culture hood for 2 hrs. The wells were then washed and dried for 1 hr. Twenty-five thousands cells were added to each well and incubated for 4 hrs at 37°C. Control wells were used with no cells added to the collagen but underwent the same conditions. The wells were then treated with 10 μM TIMP-1 (R&D Systems), 10 μM 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 2-3 min at 37°C and removed with PBS washes (2X). The wells were then fixed with 10% paraformaldehyde for 20 min, washed 3X with PBS, and then 500 μl of Coomassie Brilliant Blue stain (Bio-Rad) was added to each well for 5 min. Finally, the wells were washed 3X with water and air dried overnight. Images (200X) 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 staining density of collagen to the collagen control with added.
**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 6-8 week old SCID females. Tumor sizes were measured every 3 to 4 days and volumes were based on the formula: \(\frac{1}{6} \times \pi \times L \times l^2\). In another experimental group, to allow the development of detectable metastasis, 231 and 435S tumors were resected at 6 and 8 weeks after tumor implantation, respectively. Eight weeks after surgical removal of the primary tumor, mice were sacrificed and a necropsy was performed to determine the presence of axillary lymph nodes and distant lung metastasis. At 4 weeks the animals were sacrificed and lung metastasis were evaluated and counted. Macroscopic metastases were counted on the surface of the lung with a stereoscope. Lung macrometastasis and micrometastasis were also determined in whole lung sections stained with hematoxylin and eosin.

**Mammary fat pad window and MPLSM**

After the injection of 231 cells in the third mammary fat pad, a mammary fat pad window was implanted two weeks later. Two symmetrical titanium frames (custom design) were implanted so as to sandwich the extended double layer of skin. Images were obtained using a custom built MPLSM, constructed around a BioRAD MRC600 confocal scan head and a Zeiss inverted fluorescence microscope. The GFP (cancer cells) and SHG (fibrillar collagen) signals were obtained simultaneously using 900nm excitation laser light, with detection via 1) 435DF30 (SHG) and 2) a high-pass 475 dichroic filter and 525DF100 (GFP) emission filters (Chroma Technologies, Rockingham VT). At least 3 regions per tumor were imaged every 10 min using a 20X, 0.5 numerical aperture aqueous long working distance objective lens. Each image stack consisted of 20 images spaced 5 \(\mu\)m apart, covering an area of 3.0x10^5 \(\mu\)m^2. Cancer cell migration was assessed in the individual xyz images from the time volumes. In general, the cells observed did not migrate between different z slices between 0 and 60 min. The difference in centroid position between \(t=0\) to \(t=60\) gave a measure of cell speed and only cells migrating...
≥ 4 μm / hr 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 performed with Rf So144, a rabbit polyclonal antibody (Applied Genomics Inc). We confirmed the specificity of LEM-2/15.8 and Rf So144 with MT1-MMP null 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-null fibroblasts. Furthermore the pre-incubation of LEM-2/15.8 or Rf So144 with the recombinant MT1-MMP protein abolished the immunostaining on tumor sections. Immunostaining was also performed 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 peroxydase 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). Blood and lymphatic vessel invasion were quantified in peri-tumoral 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 has focal changes in collagen IV thickness or interruptions in collagen IV but not MECA-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 performed 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 hrs with anti-Armenian hamster-Cy5 and anti-rabbit-FITC. Following the first set of secondary antibodies, the sections were blocked for 1 hr 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-Cy 3 was applied for 1.5 hr and the slides were mounted in vectashield with DAPI counterstain (Vector Laboratories). LYVE-1 immunostaining of lymphatic vessels (rat anti-mouse-LYVE-1; R & D) combined with collagen IV and MT1-MMP was performed 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 non-fixed tumors were incubated in dye quenched (DQ) Collagen IV (1:30; EnzChek kit Invitrogen) in the dark at room temperature for 1 hr. The sections were then incubated with rat anti-mouse LYVE-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-Cy 3 for 1.5 hrs, 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 if the degradation of DQ collagen IV was MMP-dependent, the sections were treated with EDTA (30 mM) or 1,10-phenanthroline (250 μM). Using a confocal microscope, 15-30 μm stacks were acquired using a 20X objective. For co-localization of DQ collagen IV degradation, blood vessels, and MT1-MMP, 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 of were auto-thresholded (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 National Institute of Health (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 2005-2006 were prospectively collected. MT1-MMP expression and tumor blood vessel invasion were determined by immunostaining (see immunostaining methods). Tumor blood vessel invasion 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 performed. P-values for association between MT-MMP and other variables are from unconditional Fisher exact test, using Berger’s method with 99.9% confidence interval (26). In addition, we present the estimates of odds ratios with 95% confidence intervals 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, a short hairpin RNA (shRNA) construct was stably transfected to down-regulate the MT1-MMP expression in two 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 immunodeficient mice, although recent genetic analyses have shown that 435S cells maybe of melanocytic origin (28). We used the same vector with a scrambled
shRNA (mock), and restored MT1-MMP expression in shRNA cell lines with an MT1-MMP overexpressing construct (shc). MT1-MMP mRNA expression (qPCR) was reduced by 51% in 231 shRNA and 55% in 435S shRNA cells (data not shown). Western blotting of cell lysates confirmed the down regulation of the MT1-MMP protein and rescue of MT1-MMP expression in shc cells (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-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 to mock-transfected cells.

**Cancer cell MT1-MMP downregulation has no effect on primary tumor growth or lymph node metastasis 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 (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,C). Furthermore, histological 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 (shc) 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,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 to mock tumors (Fig. 3C, S2A,B). While 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**

Since 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 (Fig. S3A). Interestingly, we found in shRNA compared to mock tumors significantly less co-localization of MT1-MMP with CD31 and collagen IV immunostaining of blood vessels (Fig. 4 A, B, C). We next assessed whether this endothelial associated MT1-MMP expression resulted in changes in enzymatic activity by performing 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
(Fig. S3B) and CD31-positive blood vessels compared to 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 (Fig. S3C, D). Furthermore, the blood vessel co-localization 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 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 suggest 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 (Fig. S4A, B). Importantly, in terms of enzymatic activity, we found no difference in DQ collagen IV signal around lymphatic vessels between mock and shRNA tumors (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
rounded morphology reminiscent of amoeboid motion (Movie S1). The fraction of migrating cancer cells continued to increase significantly with tumor growth in mock 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 / hr in mock, and 4 and 15 μm / hr in shRNA tumors. Furthermore, the average migration speed of tumor cells was reduced in the shRNA tumors compared to mock tumors (shRNA = 8.0 ± 0.49 μm/hr*, mock = 12.8 ± 1.37 μm/hr, *p = 0.001, n = 14 tumors for shRNA, n = 13 for mock, ± SEM). To assess if 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 while mock and shc 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 triple-negative breast cancer

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 (Table S1). Of these, 102 patients had analyzable tumor sections and relevant clinical/pathological data. By immunostaining, we found MT1-MMP expression in cancer cells of 52.9% of breast tumors. Most tumors had diffuse MT1-MMP expression but in some cases, MT1-MMP expression was restricted to focal areas (Fig. 6A). 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,D). In parallel, we determined, for each tumor, the presence of BVI by CD31-Keratin19 and Lyve-1 immunostaining (Fig. 6E,F). BVI was defined as CD31-positive, Lyve-1-negative endothelial-lined vessels that contained 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 -negative (13/43), however the trend was not significant (p=0.08). The same correlation analysis was performed in tumor subtypes segregated by ER, PR and HER2 status. In triple-negative breast cancer (TNBC) MT1-MMP expression was comparable to 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, Odds ratio: 5.3 [1.06, 28.3], but not with lymph node metastasis (p=0.11, OR: 3.5 CI [0.88, 15.3]), 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). While 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, suggests 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 chick chorioallantoic
membrane (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 since 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. While 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, -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. While 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 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). While 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 performed. Here, we found in TNBC significantly more peritumoral BVI compared to 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). While 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 to other breast cancer subtypes, the proportion of tumors expressing EGFR, 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.

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References

Figure legends

Figure 1: Downregulation of MT1-MMP in 231 and 435S cells reduces the in vitro collagen I degradation and invasion. A) Mock 231 cells stained with MT1-MMP antibody (red) show stronger membrane staining than shRNA knockdown 231 cells (blue = DAPI). B) Quantification of the percent of cells highly expressing MT1-MMP protein as shown in Panel A suggests a significant decrease in surface protein expression in shRNA cells (*p<0.01, n = 40 independent fields). C) Following culture of cells on polymerized collagen, images of Coomassie blue stained collagen I illustrate more collagen remaining in wells with shRNA 231 cells. D) Quantification of collagen degradation using Coomassie blue staining showed that the mock 231 cells lose their ability to degrade collagen when blocking with Timp-2 but not with Timp-1, thus suggesting an MT1-MMP directed process. This blockade of collagen I degradation with Timp-2 in the mock cells was similar to the reduced degradation capability of shRNA 231 cells (*p<0.05, n = 8 wells in each group). E) Timp-2 and MT1-MMP downregulation also reduced the invasion of 231 cells in collagen gels (*p<0.01). Error bars represent the standard error of the mean (SEM).

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) 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); 435, n = 10 (mock), n = 10 (shRNA), n = 10 (shc). 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 while lymphatic metastases were not significantly reduced. In mice with shc 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); 435, n = 16 (shRNA), n = 18 (mock), n = 5 (shRNA); lymph
node mets: 231, n= 15 (shRNA), n=14 (mock).  **D)** In histological sections the number of metastatic lesions per lung were significantly reduced in shRNA compared to mock tumors (*p<0.05, n = 10 (shRNA) and n= 12 (mock). Error bars represent the SEM.

**Figure 3:** MT1-MMP downregulation in cancer cells reduces BVI but not LVI.  **A)** Cancer cells are observed in the lumen of blood vessels (MECA-32, brown) and **B)** lymphatic vessels (podoplanin, brown). Bar = 100 μm. **C)** BVI is significantly reduced in 231 and 435S tumors (*p<0.01). The percentage of BVI was dividing the number of invaded blood vessels by the total number of vessels.  **D)** LVI is not affected by MT1-MMP downregulation. Error bars represent the SEM.

**Figure 4:** In shRNA tumors, blood vessel associated MT1-MMP expression and activity decreases, which correlates with basement membrane thinning and interruption.  **A)** We found not only a decrease in MT1-MMP immunostaining in tumor cells when comparing mock to shRNA tumors (red channel, white arrows) but also observed a decrease in MT1-MMP co-localization (yellow channel, white arrowheads) with CD31+ blood vessels (blue) and collagen IV basement membrane staining (green). Bar = 100 μm.  **B)** The MT1-MMP expression associated with blood vessels, quantified by the co-localization of MT1-MMP and CD31 staining, was significantly less in the shRNA group for both tumor and peritumoral regions (*p<0.01, n = 8 for each tumor type).  **C)** The MT1-MMP expression that overlapped with collagen IV basement membrane staining around CD 31+ blood vessels was also significantly reduced in shRNA tumors.  **D)** To assess whether this change in blood vessel associated MT1-MMP resulted in a functional change in enzymatic activity, DQ Collagen IV was applied to frozen tissue sections and we analyzed DQ Collagen fluorescence (green) co-localization with CD31+ blood vessels (blue) and MT1-MMP (red). **E)** DQ Collagen IV signal showed a significant decrease in activity around vessels in shRNA tumors (*p<0.01, n = 8 for each tumor type) that **F)** correlated with a decreased co-localization of CD31, DQ collagen IV, and MT1-MMP expression (*p<0.05, n = 8 for each tumor type).
for each tumor type). **G**) Collagen IV immunostaining (brown) in paraffin sections shows breaches in the vascular basement membrane at the tumor edge (red arrows). Bar = 50 μm. **H**) There was a reduced fraction of blood vessels with thinned or interrupted basement membranes in shRNA tumors (minimum 9 tumors per group; *p<0.05). This fraction was calculated by dividing the number of vessels with compromised basement membranes by the total number of vessels. Error bars represent the SEM.

**Figure 5:** MT1-MMP downregulation reduces the migration of cancer cells in 231 tumors. **A**) Multiphoton laser scanning microscopy of 231 tumors in a mammary fat pad window. Tumor areas were imaged every 10 min for 1 hr. The fraction of migrating cancer cells was quantified and significantly decreased in shRNA tumors on day 25 and 40 (*p<0.01, n = 4 tumors in each group). Error bars represent the SEM. **B**) In vitro images of tumor cell aggregates suggest that MT1-MMP affects cell-cell adhesion in aggregates of 231 cells.

**Figure 6:** MT1-MMP immunostaining and BVI in human breast tumors. **A, 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 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-F = 100 μm. **G**) In tumor sections of triple-negative breast cancer 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).
Figure 1

A

mock

shRNA

B

% Highly expressing cells

221 cells

mock

shRNA

435 cells

mock

shRNA

C

mock

shRNA

D

% Degraded collagen

E

% Invaded cells

mock

mock+Temp1

mock+Temp2

shRNA

mock

mock+Temp1

mock+Temp2

shRNA
Figure 2

A

B

no lung metastases

lunge metastases

C

D

% mice with metastasis

number of metastases per lung

mock

shRNA

shc

mock

shRNA
Figure 6

A

B

C

D

E

F

G

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Cancer cell associated MT1-MMP promotes blood vessel invasion and distant metastasis in triple-negative mammary tumors
Jean Y Perentes, Nathaniel D Kirkpatrick, Satoshi Nagano, et al.

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