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Tissue Inhibitor of Metalloproteinase-2 Regulates Matrix Metalloproteinase-2–Mediated Endothelial Barrier Dysfunction and Breast Cancer Cell Transmigration through Lung Microvascular Endothelial Cells

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Abstract

Matrix metalloproteinases (MMP) have been implicated in multiple stages of cancer metastasis. Tissue inhibitor of metalloproteinase-2 (TIMP-2) plays an important role in regulating MMP-2 activity. By forming a ternary complex with pro-MMP-2 and its activator MMP-14 on the cell surface, TIMP-2 can either initiate or restrain the cleavage and subsequent activation of MMP-2. Our recent work has shown that breast cancer cell adhesion to vascular endothelial cells activates endothelial MMP-2, promoting tumor cell transendothelial migration (TEM_E). However, the mechanism of MMP-2 regulation during TEM_E remains unclear. In the current study, we present evidence that MMP-14 is expressed in both invasive breast cancer cells (MDA-MB-231 and MDA-MB-436) and lung microvascular endothelial cells (HBMVEC-L), whereas TIMP-2 is exclusively expressed and released from the cancer cells. The tumor cell–derived TIMP-2 was further identified as a major determinant of endothelial MMP-2 activity during tumor cell transmigration in the presence of MMP-14. This response was associated with endothelial barrier dysfunction because coculture of MDA-MB-231 or MDA-MB-436 with HBMVEC-L caused a significant decrease in transendothelial electrical resistance concomitantly with endothelial cell-cell junction disruption and tumor cell transmigration. Knockdown of TIMP-2 or inhibition of TIMP-2/MMP-14 attenuated MMP-2–dependent transendothelial electrical resistance response and TEM_E. These findings suggest a novel interactive role of breast cancer cells and vascular endothelial cells in regulating the TIMP-2/MMP-14/MMP-2 pathway during tumor metastasis.

Introduction

Tumor metastasis to vital organs such as the lungs is a leading cause of death in breast cancer patients (1–3). As an essential step in tumor cell invasion and extravasation, transendothelial migration (TEM) across the vascular endothelium is not only dependent on directed tumor cell motility but is also regulated by the local endothelial barrier function. In

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the past decades, tremendous efforts have been devoted to characterize the molecular biology of invasive tumor cells, and an array of cytokine receptors (e.g., CXCR4) and adhesion molecules (e.g., integrins, E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, thrombospondin receptors) have been identified as participants in the endothelial response to tumor cell adhesion and transmigration. However, the precise mechanism of tumor-endothelial cell interactions and the specific role of endothelial barrier properties in controlling the TEM process are still far from clear. Further characterization of tumor cell-mediated endothelial responses may lead to the identification of new therapeutic targets for treatment of breast cancer metastasis.

Matrix metalloproteinase-2 (MMP-2) has long been associated with tumor angiogenesis and metastasis (4–8). Compelling evidence has suggested MMP-2 in tumors, as well as the level of its active form in the circulation, as a sensitive indicator of metastasis in breast cancer (9–12). A high level of active MMP-2 in breast cancer tissues has been correlated with decreased overall survival and recurrence-free survival (12–14). As an essential member of the MMP family of zinc-dependent endopeptidases, MMP-2 is secreted in its latent form (pro-MMP-2) and activated on the cell surface by MMP-14, which is also known as membrane type 1 MMP (15–17). A dual role for tissue inhibitor of metalloproteinase-2 (TIMP-2) has been proposed to explain the regulatory mechanism of MMP-14-induced MMP-2 activation (18). On one hand, TIMP-2 acts as a ligand of MMP-14. The NH₂-terminal domain of TIMP-2 can bind the catalytic site of MMP-14, resulting in a TIMP-2/MMP-14 binary complex (19). On the other hand, the COOH-terminal domain of TIMP-2 serves as a receptor for the COOH-terminal region of MMP-2, thereby preventing it from further interacting with MMP-14 and subsequent MMP-2 activation (19, 20). It has been reported that the COOH-terminal region of TIMP-2 can form a noncovalent complex with latent MMP-2 through binding to the hemopexin-like domain of the zymogen (21). However, if the concentration of TIMP-2 is relatively low and cell surface MMP-14 is not saturated, non-TIMP-2-bound MMP-14 in close proximity can cleave the Asn³⁷-Leu³⁸ bond of a latent MMP-2 in the ternary complex (20, 22, 23), producing an inactive intermediate form of MMP-2 that subsequently undergoes autocatalytic hydrolysis at the Asn⁸⁰-Tyr⁸¹ peptide bond, rendering a fully active form of MMP-2 (24, 25). In addition to TIMP-2, TIMP-4 is capable of binding to MMP-2 as well as MMP-14, preventing the autocatalytic activation process (26, 27). When TIMP-4 is coexpressed with TIMP-2, it inhibits the activation of latent MMP-2 via MMP-14 (26). These findings suggest that the balance between TIMP-2 and TIMP-4 in the extracellular microenvironment is a critical factor in regulating MMP-2 activity.

It is well documented that active MMP-2 degrades fibrillar collagens in the extracellular matrix (ECM; refs. 28–31), thereby facilitating cell invasion during various physiologic or pathologic processes (32–34). MMP-2 has also recently been found to be able to degrade cell-cell adhesion structures, such as N-cadherin and leukocyte cell adhesion molecules (35, 36). MMP-2 activation may therefore lead to reduced cell-cell and/or cell-ECM adhesions. It has been proposed that increased MMP-2 activity contributes to the initial process of metastasis by promoting tumor cell penetration across the basement membrane and invasion into the blood or lymphatic circulation (37, 38). However, little is known about the effect and mechanism of MMP-2-dependent extravasation of tumor cells from the circulation. In

an effort to characterize the effect of MMP-2 in breast cancer cell TEM_E, we have shown that microvascular endothelial cells serve as an important source of MMP-2 production following breast cancer attachment to the endothelial surface (39).

In the present study, we investigated the underlying mechanism of MMP-2 activation during tumor cell transmigration. Our data revealed that MMP-14, a MMP-2 activator, was expressed in both breast cancer cells (MDA-MB-231 and MDA-MB-436) and lung microvascular endothelial cells (HBMVEC-L), whereas TIMP-2 was detected exclusively in tumor cells. We further showed that it was the tumor cell-derived TIMP-2 that activated endothelial MMP-2. This activation process required the presence of active MMP-14. Subsequent assays using the electric cell-substrate impedance sensing system and fluorescence immunocytochemistry showed that TEM_E was coupled with endothelial barrier dysfunction and cell-cell junction disruption; the effects were caused by TIMP-2/MMP-14-induced activation of endothelial MMP-2. Taken together, these findings suggest a cooperative mechanism between metastatic breast cancer cells and microvascular endothelial cells in promoting MMP-2 activation and tumor cell transmigration.

Materials and Methods

Antibodies and reagents

Blocking antibodies against the catalytic domain of MMP-14 (MAB3328/LEM-2) and the NH₂ terminus of MMP-2 (MAB13405) were from Millipore. Human TIMP-2 and TIMP-4 ELISA kits, as well as the polyclonal TIMP-2 (amino acids 27–220) neutralizing antibody, were from R&D Systems. TIMP-4, β -tubulin, VE-cadherin, and normal IgG antibodies, as well as horseradish peroxidase- and TRITC-conjugated secondary antibodies, were from Santa Cruz Biotechnology. Radioimmunoprecipitation assay lysis buffer was from Upstate Cell Signaling Solutions. Protease inhibitor cocktail was from Thermo Scientific. Triton X-100 and phosphatase inhibitor cocktails were purchased from Sigma-Aldrich. Hoechst 33342, calcein-AM, Tris-glycine SDS sample buffer, sample reducing agent, 4–12% Tris-glycine gradient gel, 10% zymogram gelatin gel, zymogram renaturing and developing buffers, and the Colloidal Blue staining kit were purchased from Invitrogen.

Cell culture and siRNA transfection

Breast cancer MDA-MB-231 and MDA-MB-436 cells were purchased from the American Type Culture Collection and were grown in DMEM supplemented with 10% fetal bovine serum. Human lung blood microvascular endothelial cells (HBMVEC-L) were obtained from Lonza and maintained in EGM-2 medium. MDA-MB-231 and MDA-MB-436 cells were grown in six-well tissue culture plates until 80% confluence and then transfected with 1 μ g of TIMP-2 siRNA (a pool of three target-specific 20- to 25-nt siRNAs, Santa Cruz Biotechnology) per well using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Protein expression was examined 48 hours after transfection.

Gelatin zymography

HBMVEC-L cells were seeded (10^5 cells/cm²) on the gelatin-coated 48-well plates (Corning Costar) overnight at 37°C, followed by serum starvation for 12 hours in EBM medium. In

coculture experiments, MDA-MB-231 or MDA-MB-436 cells were added onto the endothelial cells at 10^5 cells/cm². Equal amounts of MDA-MB-231 or MDA-MB-436 cells were seeded into a series of empty wells in tumor cell monoculture experiments. In endothelial monoculture assays, HBMVEC-L cells were refreshed only with EBM medium. At various time points (1, 6, 12, and 24 hours), conditioned media were collected and examined by gelatin zymography for MMP-2 activities. The effect of tumor cell-derived TIMP-2 on MMP-2 activation was examined using MDA-MB-231 and MDA-MB-436 cells transfected with TIMP-2 siRNA or blank vehicles (mock transfection). For functional blocking, cells were treated with neutralizing antibodies against TIMP-2 (5 µg/mL) or MMP-14 (20 µg/mL). Normal IgG (20 µg/mL) was used as an isotype control. At each time point, 20 µL of conditioned media were mixed thoroughly with Tris-glycine sample buffer without reducing agent, and then subjected to zymogram gel electrophoresis. After sequential incubations in renaturing and developing buffers, the zymogram gels were incubated with Colloidal Blue stain buffer and then destained in deionized water. The developed bands were imaged with an Alpha Imager (Alpha Innotech) and quantified using the ImageJ software (NIH).

Western blot analysis

HBMVEC-L cells and breast cancer cells (MDA-MB-231 and MDA-MB-436) were harvested and homogenized in radioimmunoprecipitation assay lysis buffer freshly supplemented with 1% protease and phosphatase inhibitor cocktails. After the insoluble components were pelleted at $14,000 \times g$ for 1 minute, the concentration of proteins in the supernatant was determined using a Bio-Rad detergent-compatible protein assay kit. Equal amounts (20 µg) of proteins were then separated by 4–12% Tris-glycine gradient gel, transferred onto Millipore Immobilon-P membranes, and blotted with appropriate primary antibodies. Following washing and incubation with horseradish peroxidase-conjugated secondary antibodies, the proteins of interest were visualized in Kodak BioMax Film using ECL Plus detection reagent (GE Healthcare). The films were then scanned and quantified using ImageJ software for protein levels.

ELISA assays

Conditioned media from either monoculture or coculture were collected as described above. The TIMP-2 and TIMP-4 levels were measured using ELISAs with the R&D Quantikine kits according to the manufacturer's protocol. Briefly, aliquots of samples and standard controls were added in microplate strips and incubated for 2 hours. After aspiration and four washes, horseradish peroxidase-conjugated TIMP-2 or TIMP-4 antibody was added for 2 hours. This was followed by sequential incubation with substrate solution and stop solution. The absorbance of each sample was determined at 450 nm (wavelength correction at 540 nm) using a microplate reader.

Transendothelial migration assay

HBMVEC-L cells were seeded (10^5 cells/cm²) on gelatin-coated Transwell inserts (Corning Costar, 6.5-mm diameter, 8-µm pore size) overnight to get a confluent monolayer in the upper chamber. The luminal (apical) and abluminal (subendothelial) compartments were therefore generated in the upper and bottom chambers, respectively (40–43). After serum

starvation in EBM medium, 500 μ L of EBM medium containing 10% fetal bovine serum were added to the bottom chamber and 200 μ L of EBM containing calcein-stained breast cancer cells (MDA-MB-231 or MDA-MB-436) with or without TIMP-2 siRNA transfection were added (10^5 cells/cm²) to the upper chamber, and cells were incubated for 12 hours. The mock-transfected cells were used as controls. In functional blockage experiments, MDA-MB-231 or MDA-MB-436 cells were added to the upper chamber and incubated in the presence of an antibody against TIMP-2 (5 μ g/mL), MMP-14 (20 μ g/mL), or MMP-2 (5 μ g/mL). The cells cocultured in the presence of normal IgG were used as controls. After incubation, the Transwell membranes were fixed with 3.7% formaldehyde for 10 minutes, followed by washing with PBS three times. The cells adhering to the upper side of the membrane were removed with a cotton swab, and transmigrated cells at the bottom side of the membrane were visualized using an Axiovert Zeiss 200M fluorescent microscope and counted from six random fields.

Transendothelial electrical resistance

The transendothelial electrical resistance (TER) was examined using the electric cell-substrate impedance sensing system. Briefly, HBMVEC-L cells were seeded on gelatin-coated gold electrode arrays (Applied Biophysics) and starved in EBM medium as previously described. With 1-V, 4,000-Hz alternating current signal supplied through a 1-M Ω resistor to a constant-current source, and culture media serving as the electrolyte, the electrical impedance was recorded with ECMS 1.0 software (CET). As the electrical impedance of a cell monolayer to a current flow is predominantly determined by the tightness of cell-cell and cell-matrix adhesions, decreased TER represents increased current flow that primarily results from disruption of the endothelial barrier integrity (44–46). After addition of MDA-MB-231 cells, 3.7% formaldehyde-fixed MDA-MB-231 cells, or blank vehicles (EBM only), TER was monitored continuously for 12 hours. In separate groups, the TER was measured using MDA-MB-231 or MDA-MB-436 cells transfected with TIMP-2 siRNA or in the presence of a blocking antibody against TIMP-2 (5 μ g/mL), MMP-14 (20 μ g/mL), or MMP-2 (5 μ g/mL), as well as an isotype control antibody (20 μ g/mL). The TER values from multiple wells were normalized to baseline, averaged, and expressed as a fractional change in TER as an indicator of endothelial barrier function.

Immunofluorescence microscopy

HBMVEC-L cells were seeded on gelatin-coated Lab-Tek chamber slides (Nalge Nunc). After serum starvation, endothelial monolayers were cocultured with calcein-AM-stained MDA-MB-231 cells with or without TIMP-2 siRNA transfection in the presence of a blocking antibody against TIMP-2 (5 μ g/mL), MMP-14 (20 μ g/mL), or MMP-2 (5 μ g/mL). Twelve hours after coculture, the cells were fixed with 3.7% formaldehyde for 10 minutes, washed three times with PBS, and then permeabilized with 0.1% Triton X-100 for 5 minutes at room temperature. After blocking with 1% bovine serum albumin in PBS for 30 minutes, the cells were incubated sequentially with VE-cadherin antibody (4 μ g/mL, 1 hour), TRITC-conjugated secondary antibody (4 μ g/mL, 1 hour), and Hoechst 33342 (1:500 dilution, 10 minutes). Each incubation step was followed by three washings with PBS. The images were then captured using an Axiovert Zeiss 200M fluorescent microscope.

Results

Tumor cells activate endothelial MMP-2

Human lung microvascular endothelial cells (HBMVEC-L) and breast cancer cells (MDA-MB-231 and MDA-MB-436) were grown as monoculture or coculture for various time courses (1, 6, 12, and 24 hours). Serum-free conditioned media were collected and analyzed for the presence and activity of MMP-2 using gelatin zymography. The latent form of MMP-2 (pro-MMP-2) was detected in the conditioned media of HBMVEC-L and HBMVEC-L cocultured with MDA-MB-231 or MDA-MB-436, but not the cancer cells alone (Fig. 1A). Active MMP-2 was not detected in either HBMVEC-L or breast cancer cell monoculture (Fig. 1A). However, when HBMVEC-L cells were cocultured with MDA-MB-231, this latent MMP-2 was cleaved and activated, with a plateau reached at 12 hours (Fig. 1A and B). Similar results were also observed in the conditioned media of HBMVEC-L cocultured with MDA-MB-436 (Fig. 1A and C). These findings are in line with our previous study (39) suggesting that tumor cell-originated factors are responsible for the activation of MMP-2 released primarily from endothelial cells.

Tumor cell-derived TIMP-2 induces MMP-2 activation

Recent evidence indicates that the activity of MMP-2 is tightly regulated through the actions of TIMP-2, TIMP-4, and membrane-associated MMP-14. As shown in Fig. 2A, MMP-14 is highly expressed in both HBMVEC-L cells and MDA-MB-231 cells. Likewise, a moderate level of MMP-14 is detected in MDA-MB-436 cells. In contrast, TIMP-2 and TIMP-4 were found expressed primarily in MDA-MB-231 and MDA-MB-436 cells (Fig. 2A), and only negligible levels of TIMP-2 and TIMP-4 were detected in HBMVEC-L cells (Fig. 2A). To further investigate if these tissue inhibitors of MMP were actually released by these breast cancer cells during the monoculture or coculture process, aliquots of conditioned media were subjected to ELISA assays. It was found that the level of TIMP-2 was continuously increased in the conditioned media over the culturing time (Fig. 2B and C). There is no statistically significant difference in the TIMP-2 level between the monoculture and the coculture conditions over a 12-hour culturing period. Intriguingly, TIMP-4 was not detected in the conditioned media of either culture condition (Fig. 2B and C), although it was detected in breast cancer cells (Fig. 2A).

There is some evidence that TIMP-2 plays a crucial role in the regulation of MMP-2 activation. At a relatively low concentration, TIMP-2 binds and presents a latent MMP-2 to its membrane-associated activator MMP-14, which cleaves the MMP-2, thus initiating its activation process. However, at high levels, it blocks latent MMP-2 from interacting with MMP-14, thereby inhibiting MMP-2 activation. In this study, we detected MMP-14 expression in both breast cancer cells and lung microvascular endothelial cells. To characterize the role of tumor cell-derived TIMP-2 in the activation of endothelial cell MMP-2, MDA-MB-231 or MDA-MB-436 cells were transfected with TIMP-2 siRNA followed by coculturing with HBMVEC-L. As shown in Fig. 3A, compared with mock transfection, the levels of TIMP-2 were substantially reduced in both MDA-MB-231 and MDA-MB-436 cells, as well as in the conditioned media at 48 hours post-transfection. In agreement with this, gelatin zymography showed that the level of active MMP-2 was

significantly reduced in conditioned media of coculture with TIMP-2-knockdown MDA-MB-231 or MDA-MB-436 cells (Fig. 3B). These findings suggest that tumor cell-derived TIMP-2 is essential for activating MMP-2 from HBMVEC-L cells. To confirm this result and further investigate the role of MMP-14 in this process, coculture assays were conducted in the presence of a blocking antibody against MMP-14 or TIMP-2 (47–51), with normal IgG used as an isotype control. The results from the subsequent gelatin zymography study substantiated that TIMP-2/MMP-14 mediated the activation of latent MMP-2 from HBMVEC-L cells (Fig. 3B).

TIMP-2 in MMP-14/MMP-2-mediated breast cancer cell TEM_E

Our previous work has shown a critical role for MMP-2 in breast cancer cell transmigration across the endothelial barrier (39). The current study further shows that tumor cell-derived TIMP-2 is required for activating MMP-2 in the presence of functional MMP-14. To clarify the underlying mechanisms of MMP-2-mediated tumor cell TEM_E, we investigated the potential role of TIMP-2 and MMP-14 in this process. As shown in Fig. 4A and B, in comparison with the control tumor cells, TIMP-2-knockdown MDA-MB-231 and MDA-MB-436 cells exhibited significantly lower transmigration activity through the HBMVEC-L monolayers. Moreover, in agreement with previous studies that MMP-2 activation is blocked by TIMP-2 at significantly high concentrations (250–1,250 ng/mL; ref. 52), our data showed that TIMP-2 at 500 ng/mL inhibited transmigration (Supplementary Fig. S1). The crucial role of tumor-derived TIMP-2 in TEM_E was further supported by the data from the subsequent experiments with TIMP-2, MMP-14, or MMP-2 blocking antibodies (Fig. 4C), which showed that the presence of functionally active MMP-14 was required for the TIMP-2-induced tumor cell TEM_E. These findings suggest for the first time that the tumor-derived TIMP-2 and MMP-14 mediate MMP-2 activation and tumor cell transmigration across the endothelial barrier.

Tumor cell-derived TIMP-2 induces endothelial barrier dysfunction

The process of transendothelial migration is determined by directed tumor cell motility and microvascular endothelial barrier function. To test the role of tumor cell-derived TIMP-2 as well as MMP-4 and MMP-2 in microvascular endothelial barrier function, we first examined the TER dynamics of HBMVEC-L monolayers in response to MDA-MB-231 cell stimulation. As shown in Fig. 5A and B, MDA-MB-231 cells reduce the TER of HBMVEC-L monolayers in a concentration (cell number)- and time-dependent manner, which is reversely correlated with the level of active MMP-2 detected in coculture using gelatin zymography assays. Moreover, in contrast to live MDA-MB-231 cells, formaldehyde-fixed MDA-MB-231 cells did not cause significant decreases in TER (Fig. 5A and B), indicating that the tumor cell-released factors, rather than the physical effects of cell-cell contact, are the primary mediators of endothelial barrier dysfunction.

In the following TER assays with TIMP-2-knockdown MDA-MB-231 and MDA-MB-436 cells, we found that TIMP-2 was required for tumor cell-induced endothelial barrier dysfunction (Fig. 6A). Antibody blockage assays additionally showed the critical role of MMP-14 and MMP-2 in tumor cell-induced endothelial barrier dysfunction (Fig. 6B). Supporting these findings, immunofluorescence microscopy showed that the HBMVEC-L

monolayer was extensively disrupted following a 12-hour coculture with MDA-MB-231 cells (Fig. 6C). Large intercellular gaps were frequently observed in the HBMVEC-L monolayer, with tumor cells occupying the wide-open interendothelial space (Fig. 6C). In addition, VE-cadherin was sequestered from the periphery of HBMVEC-L cells at the areas losing intercellular contact (Fig. 6C). In the presence of a blocking antibody against TIMP-2, MMP-14, or MMP-2, the endothelial barrier damage was considerably reduced, although some small interendothelial gaps were still observed (Fig. 6D). Collectively, the data show the critical roles of TIMP-2, MMP-14, and MMP-2 in breast cancer cell-induced endothelial barrier dysfunction.

Discussion

Tumor cell extravasation is a critical step in metastasis. It was originally proposed as a passive process driven primarily by mechanical factors (53). However, numerous *in vivo* and *ex vivo* studies show that tumor cell TEM_E is an active process involving the interaction between tumor cells and local microvascular endothelium (54–56). It is characterized by orchestrated signaling events involving adhesion molecules and cytokines, such as sLe^x/E-selectin, integrins, mucins, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, stromal cell-derived factor-1/CXC chemokine receptor-4, thrombospondin and its receptors, etc. (57–63). Moreover, increased activity of extracellular proteolytic enzymes has been found to correlate with poor prognosis of breast cancer in patients. Our previous work has shown a critical role of endothelial-originated MMP-2 in promoting breast cancer cell TEM_E (39). Knockdown of constitutive MMP-2 in endothelial cells diminished MMP-2 production and activation in cocultured tumor/endothelial cells (39). Selective inhibition of MMP-2 activity in endothelial cells, but not MDA-MB-231 cells, significantly attenuated tumor cell transendothelial migration (39). In this study, we provide further mechanistic evidence that TIMP-2 expressed in breast cancer cells activates endothelial MMP-2 in the presence of active MMP-14; this response causes endothelial barrier dysfunction and promotes tumor cell transmigration across endothelial monolayers on tumor cell adhesion. These findings suggest a novel mechanism underlying the interaction between cancer cells and microvascular endothelium during tumor cell extravasation. Malignant breast cancer cells exhibit a preferential pattern of metastases and tend to metastasize to specific organs, such as the lungs, liver, and brain (1, 2, 64, 65). Given that both MDA-MB-231 and MDA-MB-436 cells are highly invasive breast cancer cells originated from lung metastases (66, 67), our study focusing on the interaction between these cells and lung microvascular endothelial cells is of clinical relevance to organ-specific breast cancer metastasis.

Accumulating evidence indicates that TIMP-2 levels in tumors correlate with progress in breast cancer. Consistent with the generally accepted role as a tumor suppressor, overexpression of TIMP-2 was shown to inhibit tumor invasion and metastasis (68–70). On the contrary, Ree et al. found that TIMP-2 mRNA levels were considerably higher in breast cancer patients who developed remote metastasis than in those who were free of metastasis (71). It was further found that a high protein level of TIMP-2 in breast cancer tissues correlated with decreased overall survival and recurrence-free survival (72). These findings suggest that TIMP-2 levels may be tightly regulated at different stages of breast carcinogenesis and its effects vary depending on the microenvironment of the tumor. For

prognosis evaluation, TIMP-2 should be considered together with other factors, such as local levels of TIMP-4, MMP-14, and latent MMP-2. Supporting this is the current data that both MDA-MB-231 and MDA-MB-436 produced a low level of TIMP-2; a similar concentration of TIMP-2 has been shown to activate MMP-2 in other studies (52). Furthermore, although both TIMP-2 and TIMP-4 were detected in the tumor cells, TIMP-4 was not detected in culture media of the tumor cells when they were monocultured or cocultured with endothelial cells, suggesting that TIMP-4 was not released from tumor cells. Given that TIMP-4 inhibits TIMP-2/MMP-14-mediated MMP-2 activation (26), selective release of TIMP-2 (but not TIMP-4) may represent a critical mechanism by which tumor cells facilitate their TEM_E. Decreased production of TIMP-2 to around 2 ng/mL, a level comparable to that in the serum of healthy subjects (data not shown), substantially reduced the level of active MMP-2. On the other hand, consistent with the negligible level of active MMP-2 found in human blood (data not shown), active MMP-2 was not detected in conditioned media of HBMVEC-L monoculture regardless of the presence of MMP-14. Therefore, complex mechanisms involving multiple factors, such as TIMP-2, TIMP-4, and MMP-14, seem to contribute to MMP-2 activation and function.

Tumor cell TEM_E is an orchestrated process of numerous signaling events in both cancer cells and endothelial cells, characterized by tumor cell invasive motility and endothelial barrier dysfunction. Previous work showed that the heterotypic interaction between breast cancer cells and endothelial cells induces promigratory signaling in tumor cells, resulting in increased cell invasive motility (73–76). Currently, many theories tend to consider tumor cell TEM_E as a process similar to leukocyte extravasation during inflammation; the sequence of events includes leukocyte adhesion to the endothelium, endothelial cell retraction and junction opening, followed by leukocyte transmigration. Therefore, the barrier function of the vascular endothelium is an important determinant of transmigration and extravasation of cells from the circulation. The integrity of the endothelial barrier is maintained by an equilibrium between actomyosin-mediated centripetal tension and cell-cell/ECM adhesive forces (77, 78). An array of inflammatory cells and substances have been identified as mediators of protein leakage and leukocyte diapedesis by altering endothelial cell-cell and cell-matrix adhesive barrier structures (77, 79–82). This study suggests that similar mechanisms may be involved in cancer cell invasion and extravasation across the vascular endothelium. Using TER as an indicator of cell-cell/substrate adhesive barrier properties (44, 45, 83–86), we show that TIMP-2/MMP-14-mediated activation of MMP-2 causes lung microvascular endothelial barrier dysfunction coupled with enhanced tumor cell TEM_E. Notably, although the TER response and TEM_E were significantly attenuated with TIMP-2 siRNA transfection and by neutralizing the activity of the TIMP-2/MMP-14/MMP-2 complex, they were not completely blocked. This indicates that tumor cell-derived TIMP-2 is only partially responsible for endothelial barrier dysfunction and tumor cell TEM_E. As other factors, such as adhesion receptors and cytokines, have been implicated in tumor cell adhesion and transendothelial migration (40, 57, 59, 60), a more dramatic reduction in TEM_E is expected by targeting multiple related pathways.

Based on the current data and our previous study (39), we hypothesize that MMP-2 functions as a potent metalloproteinase causing structural or conformational changes in endothelial cell-cell junctions and/or endothelial cell-matrix focal adhesions; both are

critical components of the endothelial barrier that restricts tumor cell transmigration. In particular, activated MMP-2 may induce ectodomain shedding or degradation of transmembrane molecules that form cell-cell adherens junctions (e.g., VE-cadherin), leading to opening of intercellular gaps and facilitating tumor cell transmigration. In support of this theory, previous studies have shown that MMP-2 is capable of degrading cell-cell adhesion molecules, such as N-cadherin (35, 36). Consistently, our immunofluorescence microscopic study showed TIMP-2/MMP-2-induced junction disruption in human lung microvascular endothelial monolayers during coculture with breast cancer cells; this was accompanied by sequestration of VE-cadherin from the endothelial cells. Alternatively, MMP-2 may cause endothelial barrier dysfunction by affecting endothelial focal adhesion to the basement membrane. In this study, the barrier functional assays were done in endothelial monolayers grown on matrix protein-coated surfaces that resemble the basement membrane. Further, endothelial cells in culture can synthesize and deposit type IV collagen, a major constituent of the matrix underlying the vascular endothelium (87–90).

Using formaldehyde-fixed MDA-MB-231 cells as a control, we found that cell contact-induced mechanical stress did not significantly alter endothelial barrier function. Similar to the result of the TEM_E assay, selective inhibition of MMP-2 and MMP-14 also exhibited a protective effect on endothelial barrier function, indicating the TIMP-2/MMP-14/MMP-2 pathway as a potential target for preventing breast cancer extravasation during metastasis. With regard to the therapeutic potential of manipulating this pathway, several MMP-2 inhibitors have been developed and tested in clinical trials. For example, batimastat (BB-94), one of the early generations of peptidomimetic inhibitors, was shown to halt breast cancer metastasis in various animal models (18, 91–93). Its phase III trial was, however, terminated due to severe adverse reactions in local tissues. Subsequently, marimastat (BB-2516), a batimastat derivative that can be delivered orally, has been developed and tested (94). Although there is clinical data supporting the use of marimastat in advanced cancer (95), a randomized phase III study in patients with metastatic breast cancer who had responding or stable disease after chemotherapy failed to show evidence of clinical benefit (96, 97). Other metalloproteinase inhibitors that have been tested as anticancer therapies include BMS-275291, BAY 12-9566, CGS27023A, and FYK-1388, etc. (97–99). Nevertheless, the ineffectiveness or serious side effects limit their usage in further clinical application. The results of this study suggest that targeting the TIMP-2/MMP-14 pathway specific to the microvascular endothelial barrier of metastatic organs may serve as a potential alternative means for blockage of TEM_E signaling from tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Breast cancer cells (MDA-MB-231 and MDA-MB-436) activate human lung microvascular endothelial cells (HBMVEC-L) MMP-2. A, zymographic analysis of conditioned media collected from HBMVEC-L, MDA-MB-231, or MDA-MB-436 cell monoculture and coculture at various time points. HBMVEC-L cells produce latent MMP-2 that can be activated when cocultured with breast cancer MDA-MB-231 or MDA-MB-436 cells. B, quantitative analysis of active MMP-2 in conditioned media of HBMVEC-L/MDA-MB-231 coculture. C, quantitative analysis of active MMP-2 in conditioned media of HBMVEC-L/MDA-MB-436 coculture. Data were normalized by the levels of active MMP-2 at 24 h and presented as mean $\pm$ SE ($n = 3$). *, $P < 0.01$.

FIGURE 2.

Expression of MMP-14, TIMP-4, and TIMP-2 in lung microvascular endothelial cells and breast cancer cells (MDA-MB-231 and MDA-MB-436). A, Western blot analysis showing the protein levels of MMP-14, TIMP-4, and TIMP-2 in HBMVEC-L, MDA-MB-231, and MDA-MB-436 cells. Tubulin is used as a loading control. B, ELISA assays for TIMP-2 and TIMP-4 levels in conditioned media from MDA-MB-231 monoculture or MDA-MB-231/HBMVEC-L coculture. C, TIMP-2 and TIMP-4 levels in conditioned media of MDA-MB-436 monoculture or MDA-MB-436/HBMVEC-L coculture. Data presented as mean $\pm$ SE ($n = 3$). NS, not statistically significant; #, $P < 0.05$.

FIGURE 3.

Tumor cell-derived TIMP-2 and MMP-14 are required for MMP-2 activation. A, top, TIMP-2 levels in MDA-MB-231 and MDA-MB-436 cells 48 h after transfection of TIMP-2 siRNA or mock transfection. Bottom, TIMP-2 levels in conditioned media (12 h) of MDA-MB-231 and MDA-MB-436 cells transfected with TIMP-2 siRNA. Mock transfectants were used as controls. B, top, representative zymography showing the levels of latent and active MMP-2 in conditioned media of HBMVEC-L cocultured with MDA-MB-231 cells that were transfected with TIMP-2 siRNA or in the presence of a blocking antibody against TIMP-2 or MMP-14. Mock transfectants were used as controls for TIMP-2-knockdown

cells. Normal IgG is used as an isotype control for antibody blockage assays. Middle, quantitative analysis of active MMP-2 in HBMVEC-L/MDA-MB-231 coculture conditioned media. For TIMP-2-knockdown MDA-MB-231 cells, data were normalized to the levels of active MMP-2 in coculture with mock transfectants. For the antibody blockage assays, data were normalized to the levels of active MMP-2 in the presence of control antibodies. Bottom, normalized levels of active MMP-2 in HBMVEC-L/MDA-MB-436 coculture conditioned media. Data presented as mean $\pm$ SE ($n = 3$). *, $P < 0.01$.

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FIGURE 4.

TIMP-2 promotes transmigration of breast cancer cells (MDA-MB-231 and MDA-MB-436) through HBMVEC-L monolayers. A, representative images of transigrated MDA-MB-231 cells: a, mock-transfected MDA-MB-231 cells; b, TIMP-2-knockdown MDA-MB-231 cells. MDA-MB-231 cells were identified by the fluorescence of cell-loaded calcein-AM. B, quantitative analysis of transigrated MDA-MB-231 and MDA-MB-436 cells (number/mm²) that are transfected with mock or TIMP-2 siRNA. Untreated MDA-MB-231 and MDA-MB-436 cells were used as controls. C, quantitative analysis of transigrated MDA-MB-231 and MDA-MB-436 cells (number/mm²) in the presence of isotype control antibody

or anti-TIMP-2, anti-MMP-2, or anti-MMP-14 antibody. Data presented as mean $\pm$ SE ($n = 6$). #, $P < 0.05$; *, $P < 0.01$. Bar, 50 μm .

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FIGURE 5.

TER indicative of barrier function is reduced in HBMVEC-L monolayers during coculture with MDA-MB-231. A, TER dynamics in the HBMVEC-L monolayers cultured with serum-free EBM medium, MDA-MB-231 cells (10^3 or 10^5 cells/cm²), and formaldehyde-fixed MDA-MB-231 cells (10^5 cells/cm²). B, concentration-dependent effects of MDA-MB-231 cells on the TER of HBMVEC-L monolayer after 12 h of coculture. The data are presented as mean $\pm$ SE ($n = 6$). *, $P < 0.01$.

FIGURE 6.

TIMP-2 induces endothelial barrier dysfunction in HBMVEC-L cell monolayers. A, effects of knocking down TIMP-2 in MDA-MB-231 and MDA-MB-436 cells on the TER of HBMVEC-L monolayers after 12 h of coculture. B, effects of neutralizing TIMP-2, MMP-2, and MMP-4 on MDA-MB-231- and MDA-MB-436-induced TER decreases after 12 h of coculture. Normal IgG was used as isotype control. C, representative image of HBMVEC-L monolayer (red) after a 12-h coincubation with MDA-MB-231 cells (green) in the presence of normal IgG. Insets show higher magnifications of the designated area under individual channel, where HBMVEC-L cells were identified by VE-cadherin (red), MDA-MB-231

cells were identified by cell-loaded calcein-AM (green), and nuclei by Hoechst 33342 (blue). Note the MDA-MB-231-occupied intercellular gaps, where VE-cadherin was sequestered from the periphery of HBMVEC-L (white arrows). D, representative images of HBMVEC-L monolayers (red) after a 12-h coincubation with MDA-MB-231 cells (green) in the presence of a blocking antibody against TIMP-2, MMP-14, or MMP-2. Insets show higher magnifications of the designated areas in anti-TIMP-2. Compared with the images in C, interendothelial gaps were less frequent and more VE-cadherin (red) was localized to the cell periphery; there were fewer MDA-MB-231 cells (green cells indicated by white arrow) transmigrating to endothelial tricellular corner. Data presented as mean $\pm$ SE ($n = 6$). #, $P < 0.05$; *, $P < 0.01$. Bar, 50 μ m.