EGFR Activation and Signaling in Cancer Cells Are Enhanced by the Membrane-Bound Metalloprotease MT4-MMP

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Abstract

MT4-MMP (MMP-17) is a glycosylphosphatidyl inositol–anchored matrix metalloprotease expressed on the surface of cancer cells that promotes tumor growth and metastasis. In this report, we identify MT4-MMP as an important driver of cancer cell proliferation through CDK4 activation and retinoblastoma protein inactivation. We also determine a functional link between MT4-MMP and the growth factor receptor EGFR. Mechanistic experiments revealed direct association of MT4-MMP and its positive effects on EGFR phosphorylation in response to TGFβ and EGF in cancer cells. Notably, the effects of MT4-MMP on proliferation and EGFR activation did not rely on metalloprotease activity. Clinically, MT4-MMP and EGFR expressions were correlated in human triple-negative breast cancer specimens. Altogether, our results identify MT4-MMP as a positive modifier of EGFR outside-in signaling that acts to cooperatively drive cancer cell proliferation.

Introduction

Tumor growth relies on cancer cell properties dysregulation associated with an intense host tissue remodeling. Mitogenic signals consist mainly of growth factors and extracellular matrix (ECM) components acting through cell surface receptors (1). EGFR is a receptor tyrosine kinase (RTK) whose activation by ligand binding (such as EGF, amphiregulin, TGFβ) contributes to cancer cell proliferation, invasion, and metastasis. EGFR activation and signaling are leading causes of malignancy in many types of cancer such as breast, lung, metastatic colorectal, head and neck, ovarian, and brain cancers (2–7). Notably, in breast cancers, EGFR is expressed by triple-negative breast cancers (TNBC) or basal-like breast cancers, which are highly aggressive cancers with a high rate of early-occurring lung and brain metastasis.

Several proteinases are able to control the bioavailability and activity of growth factors (8, 9). These effects rely on the release of active molecules from the ECM, the cleavage of growth factors binding proteins, or the shedding of receptor ligands from the cell surface (9–12). Among proteinases that modulate growth factor/growth factor receptor activities or bioavailability are the zinc-binding endopeptidase family including matrix metalloproteinases (MMP) and A disintegrin and metalloproteinases (ADAM; refs. 13–15). For instances, MMP9 and MMP2 are responsible for the release of VEGF sequestered into the ECM and the activation of TGFβ (16, 17), whereas ADAM10 and TACE (ADAM17) shed several EGFR ligands from the cell surface such as TGFβ, amphiregulin, and proHB-EGF (18). The transmembrane MT1-MMP has been reported to induce intracellular signaling through Src (19–21) and MAPK cascade (12, 22, 23). In addition, MT1-MMP has been identified as a modulator of tyrosine kinase receptors including receptor of platelet-derived growth factor (24) and fibroblast growth factor receptor 2 (25). Two other MT-MMPs (MT4- and MT6-MMP) are linked to the cell membrane through a glycosylphosphatidyl inositol (GPI) anchor (26).

In sharp contrast with the known role of MT1-MMP–mediated signaling, a putative outside-in cell signaling through the GPI-anchored MT-MMPs (MT4- and MT6-MMPs) is unknown. Although, most MMPs are produced by stromal cells, MT4-MMP (MMP17) is produced by tumor cells in human breast cancer samples (27). MT4-MMP emerged recently as a key intrinsic feature of breast cancer cells that stimulates tumor growth and metastasis into the lung (27–29). This impact on metastatic dissemination is related to changes in blood vasculature characterized by pericyte detachment, vessel enlargement, and destabilization (28). The aim of the present study is to determine whether this GPI-anchored cell surface proteinase could contribute to an outside-in signaling involved in tumor aggressiveness.

Herein, by using in vivo xenografts and in vitro 3D multicellular spheroids embedded in Matrigel, we show that MT4-MMP...
promotes tumor cell proliferation by inducing EGFR activation and signaling. Together these results not only identify an unexpected role of MT4-MMP in cancer proliferation, but also establish a functional link between MT4-MMP and EGFR.

Materials and Methods

Cell culture and plasmids

Human breast cancer (MDA-MB-231, ZR-75-1 and BT549) cells, epidermoid cancer A431 cells, Hela cells, and monkey epithelial COS-1 cells were purchased from ATCC. MDA-MB-435 cells were kindly provided by Dr. Christine Gilles from the GIGA at University of Liège, Liège, Belgium. All cell lines described above were authenticated within 1 year before being used in experiments. Cell line authentication for interspecies contamination was performed by Leibniz-Institute DSMZ, GmbH. Cells were grown in DMEM supplemented with 10% FBS, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a 5% CO2 humid atmosphere. All culture reagents were purchased from Invitrogen. Cell transfection and plasmid construction are described in Supplementary Experimental Procedures.

In vivo tumorigenicity

For subcutaneous (s.c.) injection of breast cancer cells, subconfluent MDA-MB-231 cells were collected in serum-free medium (5 × 10⁶ cells/mL) and mixed with an equal volume of cold Matrigel according to previous report (30). Cell suspension (10⁶ cells/400 µL) was injected subcutaneously into RAG-1 immunodeficient mice in both flanks (n = 6). All assays were repeated at least three times. For orthotopic injection of cells in the mammary fat pad (MFP), 8 weeks old (n = 6) RAG1−/− female mice were anesthetized and 50 µL of cell suspension (10⁶ cells) was injected in the mammary gland after a small incision in the skin (0.5 cm). Tumor volumes were estimated every 3 to 4 days as described (length × width² × 0.4). All animal procedures were performed according to the Federation of European Laboratory Animal Sciences Associations (FELASA) within the accredited GIGA animal facility (University of Liège).

RT-PCR and Western blot analyses

RNA and protein extractions, as well as RT-PCR and Western blot analyses are described in Supplementary Experimental Procedures.

Coimmunoprecipitation and immunofluorescence assay

Coimmunoprecipitation of EGFR with MT4-MMP in COS-1 cells transfected with FLAG-tagged MT4-MMP and their colocalization in MDA-MB-231, BT549, and A431 cells transfected with MT4-MMP cDNA or Hela, MDA-MB-435, and ZR-75-1 cells endogenously expressing MT4-MMP are described in Supplementary Experimental Procedures.

IHC

Proliferating cells were visualized on tumor sections by using antibodies raised against Ki-67 and vimentin as described in Supplementary Experimental Procedures.

In vitro three-dimensional proliferation assay and DNA quantification

The three-dimensional (3D) culture model and quantification of cell proliferation methods are described in Supplementary Experimental Procedures.

Statistical analysis

Differences between experimental groups were assessed using Mann–Whitney test. P < 0.05 (*) were considered as significant. Statistical analyses were carried out using the Prism 4.0 software (GraphPad).

Supplementary information

Supplementary Information includes two figures and Supplementary Experimental Procedures.

Results

MT4-MMP promotes tumor cell proliferation in vivo

MDA-MB-231 cells overexpressing MT4-MMP or control cells were subcutaneously injected into RAG1−/− mice as previously described (27, 29). In accordance with our previous reports, MT4-MMP expression resulted in increased tumor growth as indicated by the higher volumes of MT4-MMP-expressing tumors (MT4 tumors) than those of control tumors (CTR tumors; Fig. 1A). A tumor-promoting effect of MT4-MMP was also observed when cells were orthotopically injected in the MFP of female RAG1−/− mice (Fig. 1A). To assess the proliferation index in vivo, subcutaneous and MFP tumors were double-immunostained with human Ki-67 for proliferating cells and human vimentin (Fig. 1B). A computerized method based on image binarization was used to determine the density of proliferating cancer cells (Ki-67 and human vimentin positive; Fig. 1B). A significant enhancement of the proliferation index was observed in MT4-MMP-expressing tumors, in both heterotopic (subcutaneous) and orthotopic (MFP) sites.

MT4-MMP mitogenic effect is associated with increased Rb inactivation in vivo

The increased proliferation index observed in vivo led us to analyze the phosphorylation status of retinoblastoma protein (Rb), a signaling molecule involved in cancer cell proliferation (Fig. 2). Inactivation of Rb upon hyperphosphorylation is known to stimulate cell proliferation through the transcription of early genes required at the G1–S-phase transition. A significant increase in Rb phosphorylation at S807/S811 was found in MT4-MMP expressing subcutaneous tumors, as compared with control tumors. Rb hyperphosphorylation at S807/S811 appeared specific for MT4-MMP-expressing tumors because other serine residues such as S780, S795, and Thr 821 were not affected (data not shown). Rb inactivation is known to be dependent on a concurrent increased expression or activation of the cyclin/CDK/E2F pathway or on downregulation of endogenous cyclin inhibitors. The immunoblot analysis of these putative regulators (Fig. 2) revealed a significant increase in the amounts of cyclin D1, cyclin D3, cylin E, and CDK4 in...
MT4-MMP promotes tumor cell proliferation in vitro in a 3D culture model

The cell proliferation rate in two-dimensional (2D) cultures on plastic dishes was not affected by MT4-MMP expression (Fig. 3A). In accordance with our previous study (27), this result confirms that MT4-MMP tumor-promoting effect is microenvironment dependent. This observation prompted us to embed cells in a 3D matrix (Matrigel) to mimic the in vivo situation of tumor microenvironment. After 7 days, MDA-MB-231 cells formed spheroids that were larger upon MT4-MMP expression (Fig. 3B). In these culture conditions, a significant increase in the proliferation rate of MT4-MMP–expressing cells was evidenced by DNA quantification (Fig. 3B). Remarkably, the increase in cell proliferation induced by MT4-MMP production was inhibited upon treatment with CDK4 inhibitor PD0332991, confirming the key involvement of the Rb pathway in vitro (Fig. 3C). To extend our study to other cell types, breast carcinoma BT549 and epidermoid A431 cell lines expressing MT4-MMP were generated by a stable transfection with MT4-MMP cDNA and spheroids were induced by 3D culture (Fig. 3D and E). Again, a MT4-MMP–mediated mitogenic effect was observed with BT549 and A431 cells. Together, these observations highlight the suitability of this 3D culture system to investigate the outside-in signaling pathway activated by MT4-MMP and resulting in enhanced tumor cell proliferation.

MT4-MMP exerts its mitogenic effect through EGFR signaling

A pharmacologic approach using small-molecule inhibitors was next applied to provide further insights into the mechanisms underlying MT4-MMP action (Fig. 4). Neither the inhibitors of the MAPK/P38 kinase pathway (SB203580), MAPK/JNK pathway (SP600125), adenylate cyclase pathway (MDL-12) nor the protein kinase C pathway (GF109203x), affected the MT4-MMP–mediated mitogenic effect (Fig. 4A). In contrast, U0126, a MAPK/MEK1/2 inhibitor, slightly reduced the proliferative phenotype in MT4-MMP–expressing cells without affecting the proliferative rate in control cells (Fig. 4A). The PI3K inhibitor LY294002 induced a strong inhibition of cell proliferation in MT4-MMP–expressing cells. Similarly, genistein, a broad spectrum tyrosine kinase (TK) inhibitor (TKI), significantly inhibited MT4-MMP–mediated mitogenic effect (Fig. 4B). These data pointed to a functional interplay between MT4-MMP and TKs, which results in downstream MAPK and PI3K activation.

The implication of receptor or nonreceptor tyrosine kinases was then explored by treating cells with inhibitors of EGFR including AG1478 and erlotinib, its analogue used in clinic (Fig. 4B), Src inhibitor (PP2 or 4-amino-5-(4-chlorophenyl)-7-(4-buty1)pyrazol[3,4-d]pyrimidine), or insulin-like growth factor receptor inhibitor (AG538; Supplementary Fig. S1A and S1B). Only the inhibition of EGFR signaling with TKI (AG1478 and erlotinib) led to a strong reduction of the MT4-MMP–mediated mitogenic effect (Fig. 4B). Erlotinib antiproliferative effect on MDA-MB-231 cells expressing or not MT4-MMP was more efficient than AG1478. Structurally, erlotinib and AG1478 share the same structural quinazolone backbone but AG1478 lacks the hydrophylique side chains.
that may confer different properties to erlotinib (33). Erlo-
tinib inhibited also the MT4-MMP–mediated proliferation in
BT549 cells and has a slight effect on control cells (Fig. 4C).
Altogether, these data demonstrate that MT4-MMP exerts
its mitogenic effect through EGFR signaling.

MT4-MMP promotes EGFR ligand expression and EGFR
phosphorylation

MT4-MMP could exert its mitogenic effect by releasing or
activating growth factors embedded in the matrix. However,
this mitogenic effect was still observed when cells were
embedded in Matrigel depleted with growth factors (Fig.
5A). Unexpectedly, a mutated inactive form (MT4-E249A) of
the enzyme expressed by MDA-MB-231 cells promoted cell
proliferation in a similar extent than the wild-type active form
(Fig. 5B). Thus, MT4-MMP is acting on cell proliferation
independently on its proteolytic activity. Although MT4-MMP
effect does not require matrix remodeling, it is dependent on
 cell–matrix interactions as assessed by the use of focal adhe-
sion kinase (FAK) inhibitor (Fig. 5C).

Figure 2. MT4-MMP–dependent cell proliferation is associated with Rb phosphorylation and cyclin-dependent kinase in tumors. Tumors collected at day 21 were analyzed for production of cell proliferation regulator proteins: Rb and its phosphorylated form at [S807-811], cyclins (A, D1, D2, D3, and E), cyclin-dependent kinases (CDK2, CDK4, and CDK6), and inhibitors (P15, P21 and P27). MT4-MMP tumors showed hyperphosphorylation of Rb and higher levels of cyclin D1, D3, E, CDK4, and P27. Representative Western blot analyses are shown for three different tumors out of four (n = 4) and densitometry quantifications (right) are those from four different tumors. Actin was used as a loading control except for pRb [S807-811], where total Rb is used. *, P < 0.05.
We next hypothesized that MT4-MMP can regulate EGFR ligand expression. RT-PCR analyses revealed enhanced mRNA levels of EGFR ligands in MT4-MMP-expressing xenografts (Fig. 5D) and cells embedded in 3D matrix (Fig. 5E). No modulation of ligand production was seen in 2D culture (Fig. 5F). The anti-EGFR TKI reduced this modulation of EGF mRNA levels observed in 3D cultures (Fig. 5G), suggesting the implication of an EGFR signaling loop that regulates growth factor production in the presence of MT4-MMP and matrix compounds. In 3D cultures, the blockade of ligand binding to the receptor with a neutralizing EGFR antibody reduced the proliferation of cells expressing or not MT4-MMP (Supplementary Fig. S2A). This indicates that the binding of ligand to EGFR is important for cell proliferation, but is independent to MT4-MMP status. However, anti-EGFR TKI AG1478 and erlotinib (Fig. 4B and C) abrogated the MT4-MMP effect more efficiently than the blocking antibody.

Dose-dependent and time course EGFR ligand–dependent EGFR phosphorylation was performed in 2D culture by comparing control and MT4-MMP–expressing MDA-MB-231 cells treated or not with EGF and TGFβ (Fig. 6A and B). MT4-MMP increases the ligand-induced EGFR activation in a dose-dependent manner for both EGF and TGFβ. For a single concentration of ligand, MT4-MMP cells displayed a rapid response after 5 minutes of incubation with ligands. The EGFR activation was sustained for 40 minutes and decreased after 60 minutes of incubation.

The analysis of EGFR phosphorylation in 2D cultures was next extended to different cancer cell lines (MDA-MB-231, BT-549, and A431 cells), transfected or not with MT4-MMP (Fig. 6C). Importantly, EGFR phosphorylation in response to both growth factors was significantly enhanced in cells expressing MT4-MMP as compared with their corresponding control cells. Similar results were obtained in COS-1 cells known to produce high amounts of EGFR receptor and transiently transfected with active and inactive (MT4-E249A) MT4-MMP cDNAs (Fig. 6D). It is worth mentioning that similar increase of EGFR phosphorylation induced by MT4-MMP expression was detected when cells were embedded in 3D matrix (Supplementary Fig. S2B). Notably, erlotinib and anti-EGFR blocking antibody blocked EGFR phosphorylation at a similar extent (Supplementary Fig. S2B). However, the phosphorylation of the key downstream EGFR signaling molecule ERK1/2 was inhibited by erlotinib but not by neutralizing blocking antibody. Altogether, these findings support the increased EGFR activation upon ligand stimulation in the presence of MT4-MMP that is not dependent on its proteolytic activity.

**MT4-MMP interacts with EGFR in cancer cells**

COS-1 cells transiently transfected with FLAG-tagged MT4-MMP (MT4-FLAG) and whole-cell extracts were subjected to immunoprecipitation with an anti-FLAG antibody to pull down MT4-MMP before immunodetection with an anti-EGFR antibody (Fig. 6E). EGFR was coimmunoprecipitated with
Figure 4. MT4-MMP–dependent mitogenic effect is dependent on tyrosine kinase activity and EGFR signaling. Cells expressing MT4-MMP (MT4) and control (CTR) cells were incubated in 3D Matrigel with or without inhibitors. A, MDA-MB-231 cell proliferation in the presence or absence of MEK1/2 (U0126), JNK (SP600125), PI3K (Ly-294002), adenylate cyclase (MDL-12), and PKC (GF109203x) inhibitors. B, MDA-MB-231 cell proliferation with or without genistein, EGFR (AG1478 and erlotinib) inhibitors. C, BT549 cell proliferation with or without erlotinib. Cell proliferation was assessed after 7 days of incubation. Results are expressed as DNA content (µg/mL). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 5. MT4-MMP promotes EGFR ligand expression and EGFR phosphorylation in tumors. A, MT4-MMP promotes cell proliferation growth factor-depleted Matrigel. B, cell proliferation in 3D culture of MDA-MB-231 cells transfected with active (MT4), inactive (MT4-E249A) forms of MT4-MMP, or control vector (CTR). C, cell proliferation of MDA-MB-231 cells expressing MT4-MMP (MT4) and control (CTR) cells incubated in Matrigel with or without FAK inhibitor PF573228. D--F, RT-PCR analysis of EGFR ligands in MT4-MMP-expressing tumors (MT4), control (CTR) tumors in vivo (D), or in vitro in 3D culture (E) and in 2D culture (F). Graphs correspond to the densitometric quantification of RT-PCR analysis, showing a significant increase of EGFR ligand mRNA in MT4-MMP tumors and spheroids. G, RT-PCR analysis of EGF expression in cells expressing (MT4) or not (CTR) MT4-MMP incubated in 3D matrix and in the presence or not of erlotinib. *, P < 0.05; **, P < 0.01.
MT4-MMP in cells transfected with MT4-MMP cDNA, whereas a slight nonspecific signal was detected in cells transfected with the control vector. Furthermore, MT4-MMP communoprecipitated with EGFR when immunoprecipitation was conducted with an anti-EGFR antibody prior to immunodetection for FLAG-tag (Fig. 6F). No MT4-MMP was detected in control cells. This reverse immunoprecipitation further supports our finding that MT4-MMP physically interacts with EGFR. The inactive form of MT4-MMP (MT4-E249A) was also found to communoprecipitate with EGFR in COS-1 cells (Fig. 6E and F), demonstrating that the formation of a complex between EGFR and MT4-MMP is not dependent on its proteolytic activity.

To determine whether MT4-MMP interacts with EGFR in cancer cells, MDA-MB-231 cells expressing Flag-tagged MT4-MMP were communostained with anti-FLAG and anti-EGFR antibodies (Fig. 7). Immunofluorescence analysis performed on stable transfectants of MDA-MB-231 cells expressing FLAG-tagged MT4-MMP revealed the presence of MT4-MMP and EGFR as yellow spots, at the cell surface and intracellularly (Fig. 7A). The inactive form of MT4-MMP was also found to colocalize with EGFR in MDA-MB-231 cells (Fig. 7A). MT4-MMP and EGFR were also colocalized in BT549 and A431 cells transfected with MT4-MMP (Fig. 7B). Similar observation was done, although to a lesser extend in cells endogenously expressing MT4-MMP (Hela, MDA-MB-435, and ZR-75-1; Fig. 7C).

MDA-MB-231 cells being issued from TNBC, we extended our study to human samples of TNBC. MT4-MMP and EGFR stainings were found to be superimposed on the same tissue areas in 17 of 20 (85%) samples (Fig. 7D). The three remaining samples were either double negative for MT4-MMP and EGFR or positive for a single marker. In sharp contrast, no MT4-MMP was found in normal breast tissues.

Discussion

The implication of the GPI-anchored MT4-MMP in breast cancer progression is supported by clinical data (27, 34, 35) and experimental observations (27–29). Until now, MT4-MMP functions have been ascribed to its capacity to regulate tumor vascularization. Herein, we describe a novel mechanism by which MT4-MMP promotes breast cancer cell proliferation in vivo and in vitro by promoting an outside-in signaling through the EGFR pathway in a nonproteolytic manner. Our data highlight a central role for MT4-MMP in cancer cell proliferation and identify this enzyme as a partner of EGFR increasing tumor cell sensitivity to EGFR ligands. This mitogenic effect of MT4-MMP is supported by data generated using different cell lines.

The proliferative advantage conferred to tumors by MT4-MMP expression correlated with a strong inactivation of the tumor suppressor Rb protein that plays a pivotal role in the negative control of the cell cycle. The increase in Rb phosphorylation was associated with enhanced expression of cyclins D1, D3, E, and CDK4 in MT4-MMP tumors. Rb hypophosphorylation represses gene transcription required for G1 to S phase transition, by binding to the promoter of targeted genes as a complex formed with E2F. Interestingly, E2F expression was increased in MT4-MMP tumors, supporting the implication of the pRB pathway in MT4-MMP–mediated mitogenic effects in vivo. In line with this finding, the inhibition of CDK4 in the 3D in vitro culture model abolished the proliferation of MT4-MMP–expressing cells but not that of control cells. These data provide evidence for a novel oncogenic function of MT4-MMP by suppressing Rb activity, thereby promoting cancer cell proliferation.

Through a pharmacologic approach, we identify tyrosine kinases as targets of MT4-MMP–mediated effect and more specifically EGFR as a potential molecular partner. Accordingly, EGFR stimulates cell proliferation through Rb signaling pathway (36–38). Aberrant EGFR signaling is a major feature of breast cancers, particularly in highly aggressive TNBC where the EGFR pathway appears as a potential therapeutic target (39). Notably, both MT4-MMP and EGFR were immunodetected in human TNBC samples supporting the clinical significance of our finding. We found a significant increase in EGFR ligand (TGFα, EGF, and amphiregulin) expression, at the mRNA levels, in MT4-MMP tumors, and in 3D cultures. Interestingly, EGF mRNA expression was partially reduced by anti-EGFR TKI. It is likely that the increased EGFR phosphorylation induced by the presence of MT4-MMP results in an EGFR signaling loop (40–42) that can reinforce the mitogenic effect of MT4-MMP. In addition, in these conditions, the anti-EGFR TKIs that inhibit EGFR phosphorylation abolished the proliferation of MT4-MMP–expressing cells. In sharp contrast, EGFR neutralizing antibody that blocks EGFR ligand binding only partially inhibited the proliferation of MT4-MMP–expressing cells and has similar effect on control cells. This difference observed between TKIs and neutralizing antibody could be explained by different capacity to induce sustained inhibition of EGFR downstream signaling molecule. Indeed, ERK1/2 activation was inhibited by erlotinib but not by the antibody. Our data are in agreement with the report of Carrasco-Garcia and colleagues (33), in which they have compared the antiproliferative effects of small-molecule inhibitors of EGFR (AG1478, erlotinib) and cetuximab (the clinically used anti-EGFR antibody) on glioblastoma cell proliferation. This study demonstrated that EGFR TKIs are able to sustain the inactivation of ERK1/2, whereas cetuximab did not. It is worth mentioning that TKI can also inhibit other members of the EGFR family such as erbB3 and erbB4, whereas cetuximab only inactivates EGFR (erbB1). Thus, the differential effects of anti-EGFR drugs used herein might be related to the heterodimerization of EGFR with other members of the EGFR family such as erbB3 and erbB4 that could be affected by the presence of MT4-MMP. Altogether, our data provide clear evidence that MT4-MMP controls EGFR phosphorylation and signaling.

Evidence for an interaction between EGFR and MT4-MMP was provided by immunoprecipitation and immunofluorescence experiments where both MT4-MMP and EGFR were found in immunoprecipitates obtained with anti-EGFR or MT4-MMP antibodies and colocalize at the cell surface and in the cytoplasm of breast cancer cells transfected or not with MT4-MMP. Because of the protein overexpression upon cell transfection, this colocalization was stronger in cells.
MT4-MMP/EGFR Axis Triggers Tumor Growth

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transfected with MT4-MMP than in cells endogenously producing MT4-MMP. Mechanistically, beside the capacity of MT4-MMP to interact with EGFR, we provide evidence that MT4-MMP is an activating partner for EGFR. Indeed, EGFR phosphorylation was observed in the presence of MT4-MMP upon stimulation with EGFR ligands in four different cell lines. Furthermore, these effects are not dependent on the catalytic function of MT4-MMP as demonstrated by the use of the mutated inactive form of the enzyme.

Our findings clearly establish a novel functional link between MT4-MMP and EGFR and give new insight into how a GPI-anchored MMP contributes to EGFR signaling in breast cancers. A proliferative advantage attributed to cancer cells by membrane-associated MMP expression has been previously reported. Indeed, MT1-MMP overexpression in cancer cells promotes tumor cell proliferation in 3D collagen matrix and tumor growth in vivo (43, 44). In addition, MT1-MMP expression in breast cancer confers protection against type I collagen-mediated apoptosis (45). In contrast with MT1-MMP, MT4-MMP endows cells with a proliferative advantage in Matrigel, but not in a collagen gel. Indeed, cell proliferation was not affected by MT4-MMP expression when cells were cultured in 3D type I collagen matrix or were first allowed to form spheroids in methyl cellulose and then embedded in type I collagen matrix (data not shown). The failure of MT4-MMP to induce proliferation when cells are mixed in 3D collagen matrix might be ascribed to its inability to cleave type I collagen in contrast with MT1-MMP (26).

Alternatively, these observations combined with the absence of a mitogenic effect of MT4-MMP in 2D cultures (27, 29) suggest that MT4-MMP proliferating phenotype is dependent on matrix-induced cell aggregation, which could affect TK-mediated signal transduction. In addition, it is worth mentioning that 3D culture conditions can affect the homodimerization or heterodimerization of EGFR family member and downstream signaling pathway (46). Thus 3D matrix conformation could influence cell–cell interactions (47) and modulate MT4-MMP–EGFR interactions. FAK and EGFR are constitutively associated in promoting cancer cell survival (48, 49) and inhibition of FAK was shown to inhibit EGF-stimulated motility (50). In the context of our study, FAK inhibition in 3D matrix abolished the MT4-MMP/EGFR-mediated mitogenic effect. These data emphasize the importance of cell–matrix interaction in integrating proliferation signals for EGFR, a process that is not possible in 2D culture model.

In addition, similar mitogenic effect of MT4-MMP observed in complete Matrigel as well as in growth factor-depleted Matrigel demonstrates that this effect does not rely on the release of growth factors sequestered in the matrix, but rather suggests that MT4-MMP can directly affect cell proliferation by enhancing the transduction of mitogenic signals through EGFR activation. Surprisingly, this novel function of MT4-MMP is independent of its catalytic activity as assessed by the use of a mutant proteolytically inactive form. These data point to unprecedented noncatalytic function of MT4-MMP on cell proliferation. This differs from its angiogenic effect requiring its proteolytic activity (29). Indeed, we previously demonstrated that the inactive MT4-MMP E249A form failed to induce an early angiogenic switch, leading to tumor growth. This is in line with data reported with MT1-MMP, which induces intracellular signaling and promotes cancer cell migration in a nonproteolytic manner (22, 23). These novel findings shed light on the catalytic-dependent effect of MT4-MMP during the angiogenic switch (29) and the nonproteolytic mitogenic effect leading to tumor growth and metastatic dissemination.

Altogether, our data establish an unexpected link between MT4-MMP and EGFR pathway and highlight the importance of the GPI-anchored MMP in breast cancer etiopathology. Although the targeted anti-EGFR strategy has shown considerable clinical promise in colorectal and lung cancers, minimal activity of anti-EGFR drugs is seen in TNBCs that are very aggressive cancers. In addition, most patients do not respond or become resistant to targeted strategies. Our data demonstrate a novel interplay between a membrane-associated proteinase (MT4-MMP) and EGFR produced by TNBC cells. These findings open new opportunities for designing novel therapies targeting the nonproteolytic functions of MT4-MMP. The combination of such anti-MT4-MMP agents and anti-EGFR drugs might improve the treatment of EGFR-dependent cancers and/or to overcome acquired resistance to anti-EGFR strategies used currently in clinics.

Figure 6. EGFR phosphorylation is enhanced in the presence of MT4-MMP. A and B, ligand-dependent EGFR phosphorylation in MDA-MB-231 cells expressing or not MT4-MMP cultured in 2D. Cells were serum-starved for 1 hour and incubated for 40 minutes with increasing concentrations of EGF (left) or at different incubation times with TGFα (10 ng/mL; right; B). EGFR phosphorylation status was analyzed in total cell extracts by Western blot analysis using antibodies against phospho-EGFR (Tyr 1068) and total EGFR. Membranes were stripped and reincubated with total EGFR and actin for loading control. C, MDA-MB-231, BT549, A431 cells expressing or not MT4-MMP were serum-starved for 1 hour and incubated with or without TGFα (10 ng/mL) or EGF (20 ng/mL) for 40 minutes, and EGFR phosphorylation status was analyzed in total cell extracts by Western blot analysis using antibodies against phospho-EGFR (Tyr 1068) and total EGFR. D, COS-1 cells transiently transfected with MT4-MMP (MT4) or its inactive form (MT4-E249A) cDNA or control vector (CTR) were serum-starved for 1 hour and incubated with TGFα (10 ng/mL) or EGF (20 ng/mL) for 20 minutes and EGFR phosphorylation was analyzed by Western blot analysis for phospho-EGFR (Tyr 1171) and total EGFR. Cell incubation with EGFR ligands induced a more pronounced EGFR phosphorylation in the presence of MT4-MMP or its inactive form MT4-E249A. Graphs correspond to the ratio of phospho-EGFR/total EGFR after densitometry quantification using Quantity-One software.
Figure 7. MT4-MMP is detected with EGFR in cancer cells and in human TNBC tumors. Detection of MT4-MMP and EGFR in breast cancer cells by immunofluorescence and confocal microscopy. A, Flag-tagged MT4-MMP or its inert form MT4-249A–expressing MDA-MB-231 cells were cultured on cover slips and stained with anti-FLAG (green) and rabbit anti-EGFR antibodies (red). B and C, BT549 and A431 cells transfected with MT4-MMP cDNA (B) or Hela, MDA-MB-435, and ZR-75-1 cells expressing MT4-MMP endogenously (C) were stained with MT4-MMP (green) or EGFR (red). Colocalization of MT4-MMP (left) and EGFR (middle) is shown as yellow spots (arrows in merged images, right). Data are those of three independent experiments. D, IHC staining of MT4-MMP and EGFR on human breast samples. MT4-MMP was not immunodetected in normal breast tissues (top). Both EGFR and MT4-MMP were expressed by cancer cells in the same areas in serial sections of TNBC (left and right, respectively). Two representative samples are shown with a higher magnification of the area corresponding to the dashed square. Scale bar, 100 μm.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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