MT1-MMP Controls Tumor-induced Angiogenesis through the Release of Semaphorin 4D*

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The semaphorins are a family of proteins originally identified as regulators of axon growth that recently have been implicated in blood vessel development. Theplexins are high affinity receptors for the semaphorins and are responsible for initiation of signaling upon ligation. Emerging evidence indicates that many human cancers overexpress Semaphorin 4D, which promotes neovascularization upon stimulating its receptor, Plexin-B1, on endothelial cells. However, to exert its pro-angiogenic functions, Semaphorin 4D must be processed and released from its membrane bound form to act in a paracrine manner on endothelial cells. Here we show that Semaphorin 4D is a novel target for the membrane-tethered collagenase membrane type 1-matrix metalloproteinase. We demonstrate that this metalloproteinase, which is not expressed in normal or immortal but non-tumorigenic epithelial cell lines, was present in several head and neck squamous cell carcinoma cell lines and was required for processing and release of Semaphorin 4D into its soluble form from these cells, thereby inducing endothelial cell chemotaxis in vitro and blood vessel growth in vivo. These results suggest that the proteolytic cleavage of Semaphorin 4D may provide a novel molecular mechanism by which membrane type 1-matrix metalloproteinase controls tumor-induced angiogenesis.

The semaphorins represent a large family of phylogenetically conserved molecules, both membrane-bound and secreted, originally identified by their ability to provide attractive and repulsive axon guidance cues during axon growth (1). Semaphorins have been grouped into eight classes based upon their species of origin and sequence similarity: Classes 1 and 2 are found in invertebrates, Classes 3–7 in vertebrates, and Class V is encoded by some non-neurotropic DNA viruses (2). The plexins and the neuropilins are the two main families of receptors for the semaphorins, and they can homodimerize or heterodimerize upon ligand binding depending on whether the semaphorin is soluble or membrane bound (3, 4). Neuropilins and their homologues have been shown to complex with Plexin-B1 (5) and Plexin-D1 (6) and, in certain conditions, serve as co-receptors with A family plexins for class 3 semaphorins (4, 7). It is the plexins, however, that initiate the signaling cascades upon binding of a semaphorin ligand. There have been nine plexins identified in humans so far, most of which have been shown to regulate neuronal cell growth and contact and nerve fasciculation (8–10). They are grouped into four families, A through D, based upon sequence homology. While sharing homology in their extracellular region with the scatter factor receptors such as RON and c-Met, the intracellular region of the plexins contain a unique domain called the Sex-Plex domain that is highly conserved within and across species.

The semaphorins and plexins recently have been implicated in a host of responses including regulation of cell migration (11), immune responses (12), tumor progression (13), and tissue organization during development (14–16). In addition, the functions of proteins involved in the transmission of axonal guidance cues have been expanded to include regulation of blood vessel growth and endothelial precursor cell homing during vessel development (17, 18). We and others (19, 20) have observed that Plexin-B1 is highly expressed in endothelial cells and promotes migration and tubulogenesis when bound by its ligand, Semaphorin 4D (Sema4D). Surprisingly, while exploring the nature of the molecules expressed in head and neck squamous cell carcinomas (HNSCC), we have recently observed that Sema4D is highly expressed in HNSCC as well as in some of the most prevalent solid tumors, including breast, prostate, and colon carcinomas (21). These findings suggest that the class IV semaphorins may regulate angiogenesis in vivo and raise the possibility that Sema4D could play a role in tumor-induced angiogenesis. However, to exert its pro-angiogenic functions, Sema4D, a membrane bound protein, must be processed and released into a soluble form to act in a paracrine manner on endothelial cells.

Semaphorin 4D is known to be expressed on the surface of cells as a homodimer, but it has also been shown to be shed into the surrounding environment through proteolytic cleavage, the mechanism for which has only recently been investigated (22). The protease responsible for Semaphorin 4D cleavage is likely a matrix metalloproteinase (MMP), a group of zinc-dependent enzymes that hydrolyze numerous components of the extracellular matrix, because shedding could be partly inhibited by the MMP inhibitors EDTA and EGTA (22). Up-regulation of MMPs in cancer cells has been linked to acquisition of an inva-

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‡ The abbreviations used are: Sema4D, semaphorin 4D; HNSCC, head and neck squamous cell carcinoma(s); MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-MMP; MEF, mouse embryonic fibroblast; TIMP, tissue inhibitor of metalloproteinase; shRNA, short hairpin RNA.
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sive phenotype, with cells acquiring the ability to digest extracellular matrix substrates and invade underlying tissue and metastasize (23). Indeed, we have found HNSCC secrete a soluble form of Sema4D that acts through Plexin-B1 on the surface of endothelial cells to enhance tumor growth and survival by promoting angiogenesis (21).

In this study, we use general and specific inhibitors of MMPs and knock-out mouse embryonic fibroblasts (MEFs) to demonstrate that Sema4D is a novel target for membrane type 1-MMP (MT1-MMP, also called MMP14), a member of a family of metalloproteinases that are tethered to the cell membrane and confer peri-cellular proteolytic activity but also participate in the processing of membrane bound receptors and proteins (24). We found that MT1-MMP, while not expressed in non-tumorigenic epithelial cell lines, was present in several head and neck squamous cell carcinoma cell lines. MT1-MMP was required for processing and release of Sema4D into its soluble form from these cells, thereby inducing endothelial cell chemotaxis in vitro and tumor-induced angiogenesis in vivo. These results suggest that the proteolytic cleavage of Sema4D by which MT1-MMP controls tumor-induced angiogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—HNSCC cell lines and MT1-MMP and MMP-2 wild-type and knock-out MEFs were grown in Dulbecco’s modified Eagle’s medium (Sigma). The human T cell line, Jurkat, was grown in RPMI (Sigma). Porcine aorta endothelial cells were cultured in HAM F-12 media (Sigma). All media were supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin/amphotericin B (Sigma). Cells were treated with GM6001 (Chemicon, Temecula, CA), TIMP-1 (Sigma), or TIMP-2 (Sigma) where indicated.

Immunoblot Analysis—Analysis of whole cell extracts was performed as described previously (25). For transmembrane proteins, cells were processed as previously described (26), with minor modifications. Briefly, cells were lysed in buffer (50 mm Tris-HCl, 150 mm NaCl, 5 mm EDTA, 1 mm CaCl₂, 1 mm MgCl₂, 1.5% precondensed Triton X-114) supplemented with protease and phosphatase inhibitors for 15 min at 4 °C, and centrifuged at 16,000 × g for 15 min at 4 °C. The supernatant was removed and incubated at 37 °C for 2 min, after which it separated into an upper (aqueous) layer, which was discarded, and a lower (detergent) phase that was quantified and loaded onto the gel. For analysis of conditioned media, 4.5 ml of serum free media was placed on cells growing in 10 cm dishes and left overnight to concentrate molecules released to the media. Sample buffer was added directly to the media after collection and a transfer was done onto a polyvinylidene difluoride membrane (Osmonics, GE Water Technologies, Trevose, PA, 8 µm pore size) coated with 10 µg/ml fibronectin (Invitrogen). After 7 h, the chamber was disassembled and the membrane stained with Diff-Quick Stain (Diff-Quick, Dade Behring, Deerfield, IL), placed on a glass slide, and scanned. Densitometric quantitation was performed with NIH image software.

Lentivirus Infections—The short hairpin RNA (shRNA) sequences for human MT1-MMP were obtained from Cold Spring Harbor Laboratory’s RNAi library (RNAi Codex) (28, 29). Oligonucleotides based upon the following sequence, identified as shRNA2, worked best to knock down MT1-MMP: 5′-TGCTGTTCAGTAGCCGCAGCTTTCAACTCTGGAGTAAATAGTGAAAGCCACAGATGTATATCATCCAGATGTTGAGGCTTTTGCTACTGCTGGAA-3′. shRNA1, used as a control, was generated by the following oligonucleotides: 5′-TGCTGTTCAGTAGCCGCAGCTTTCAACTCTGGAGTAAAATAGTGAAAGCCACAGATGTATATCATCCAGATGTTGAGGCTTTTGCTACTGCTGGAA-3′. Oligonucleotides were digested with XhoI/EcoRI, and cloned into pSHAG MAGIC2 (29), an entry vector for the Gateway cloning system (Invitrogen). In the case of wild-type MT1-MMP, the sequence was cloned into pSHAG MAGIC2 at Sall/XhoI. For catalytically inactive MT1-MMP, the E240A mutation was generated using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) prior to cloning into pSHAG MAGIC2. An LR reaction was performed to transfer the inserts into pWPI GW, a Gateway-compatible CS CG-based retroviral destination vector. Virus was prepared as reported previously (30) using 293T cells as the packaging cells. Target cells were infected with viral supernatants for 24 h at 37 °C in the presence of 8 µg/ml of polybrene (hexadimethrine bromide, Sigma).

Transfection—Where indicated, cells were transfected prior to immunoblotting, migration assays, or angiogenesis assays with pcDNA 3.1/GS/Sema4D or pSecTag2B Sema4D, or the appropriate control vectors, using Lipofectamine Plus (Invitrogen), supplemented with CombiMag transfection agent (Oz Biosciences, Marseille, France), to increase transfection efficiency.

In Vivo Cultrex Assay—A DIVAA assay (Trevigen, Gaithersburg, MD) was performed as described previously (31), with modifications. Briefly, angioreactors were filled with 18 µl of Cultrex-reconstituted basement membrane substrate (Trevigen) containing 37.5 ng of vascular endothelial growth factor and 12.5 ng of basic fibroblast growth factor (positive control), phosphate-buffered saline (negative control), or 1 × 10⁶ HN12 cells in serum-free Dulbecco’s modified Eagle’s medium, treated as indicated prior to the assay. These were implanted subcutaneously into 6-week-old nude mice (Jackson Laboratory, Bar Harbor, ME). Nine days after implantation, the mice (Invitrogen) were loaded with 25 µg of protein and run as a standard immunoblot (see above). The gels were soaked in renaturing buffer for 30 min, incubated overnight in developing buffer, and stained with Coomassie Blue.

Migration Assays—Serum free medium containing the indicated cell type or chemottractant was placed in the bottom well of a Boyden chamber, while serum-free medium containing endothelial cells was added to the top. The two chambers were separated by a polyvinylpyrrolidone filter membrane (Osmonics, GE Water Technologies, Trevose, PA, 8 µm pore size) coated with 10 µg/ml fibronectin (Invitrogen). After 7 h, the chamber was disassembled and the membrane stained with Diff-Quick Stain (Diff-Quick, Dade Behring, Deerfield, IL), placed on a glass slide, and scanned. Densitometric quantitation was performed with NIH image software.
were sacrificed and the angioreactors removed, photographed, and processed with fluorescein isothiocyanate-labeled Griffonia lectin, an endothelial cell-selective reagent (32, 33), to quantify invasion of endothelial cells into the angioreactors. Fluorescence was determined in a plate reader as mean relative fluorescence units for triplicate assays.

RESULTS

We previously have shown that HNSCC cells secrete a soluble form of Sema4D that acts through Plexin-B1 on the surface of endothelial cells to enhance angiogenesis in mouse tumor xenografts (21). Therefore, in search of the proteolytic activity responsible for Sema4D release, we used a representative HNSCC cell line, HN12, which exhibits Sema4D-mediated angiogenesis and focused our attention on metalloproteinases based upon their potential role in Sema4D proteolysis in HNSCC cell line, HN12, which exhibits Sema4D-mediated angiogenesis (35–37). To address the possible role of MT1-MMP in Sema4D release, we used a representative metalloproteinase inhibitor (34) (Fig. 1A), showing that a metalloproteinase activity was required for Sema4D release. Tissue inhibitor of metalloproteinases (TIMP)-1, which inhibits soluble matrix metalloproteinases was unable to block Sema4D release (Fig. 1B). In contrast, TIMP-2, which inhibits both soluble and membrane-type MMPs, completely inhibited Sema4D release (Fig. 1C). This pattern of proteinase inhibitor sensitivity, where Sema4D release is inhibited by TIMP-2 but not TIMP-1, suggested that Sema4D release could be mediated by the MT-MMPs.

Of the six human MT-MMPs, MT1-MMP is most frequently overexpressed in tumors and associated with tumor progression and angiogenesis (35–37). To address the possible role of MT1-MMP in Sema4D release, MEFS from MT1-MMP knock-out mice (38) and wild-type littermates were transfected with a full-length, membrane-bound form of Sema4D. Equal levels of Sema4D were seen in total cell lysates from wild-type and knock-out MEFS transfected with the Sema4D construct (Fig. 2A). However, MT1-MMP knock-out cells failed to release Sema4D into conditioned medium (Fig. 2A). MT1-MMP knock-out MEFS transfected with a Sema4D plasmid engineered to produce a secreted form of Sema4D (39) released Sema4D into conditioned medium (Fig. 2B), indicating that the cellular machinery involved in the processing and secretion of soluble proteins remained intact.

Although zymograms still showed significant secretion of pro-MMP-2 (gelatinase A) in MT1-MMP knock-out MEFS as well as the wild-type cells (Fig. 2A, lower right, lower band), MT1-MMP is known to play an important role in MMP-2 activation (40). Thus, we challenged whether our results were due to a decrease of pro-MMP-2 activation by MT1-MMP by transfecting Sema4D into MMP-2 knock-out MEFS. Both wild-type and MMP-2 knock-out MEFS exhibited equal levels of Sema4D in cell lysates and released equal levels of Sema4D in the conditioned media, establishing that MMP-2 activity is not necessary for Sema4D release (Fig. 2C).

To determine whether the catalytic activity of MT1-MMP was required for Sema4D release, we generated lentiviruses expressing either wild-type MT1-MMP or a catalytically inactive MT1-MMP containing an active site point mutation (E240A) (41) and infected wild-type and MT1-MMP knock-out MEFS transfected with full-length Sema4D. Re-expression of MT1-MMP in knock-out MEFS by infection with lentiviruses coding for wild-type MT1-MMP and its E240A mutant was confirmed by Western blot analysis of membrane extracts (Fig. 3A, lanes 3 and 4, respectively). Immunoblots for Sema4D demonstrated its expression in cells transfected with the full-length Sema4D (Fig. 3B, lysate, upper panel, lanes 4, 5, 8, and 9) and in media conditioned by control infected wild-type MEFS (Fig. 3B, lane 4, CM, upper panel) but in MT1-MMP knock-out MEFS only when infected with lentivirus coding for wild-type MT1-MMP (Fig. 3B, lane 8, CM, upper panel) or when transfected with pSecTag2B Sema4D, which codes for a secretory form of Sema4D (Fig. 3B, lane 11, CM, upper panel). Secretion of soluble Sema4D into the conditioned media could not be rescued in MT1-MMP knock-out MEFS infected with the catalytically inactive MT1-MMP E240A mutant (lane 9, CM, upper panel). In addition, wild-type MEFS infected with the E240A mutant secreted much lower levels of Sema4D (Fig. 3B, lane 5, CM, upper panel), suggesting that the inactive mutant may compete with the endogenous enzyme. Zymograms (Fig. 3B, CM, lower panel) supported these observations, as MMP-2 activity was
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FIGURE 3. MT1-MMP is necessary for induction of Sema4D-dependent angiogenesis. A, membrane extracts of wild-type (MT1-MMP MEF: wt) and knock-out (KO) MEFs, infected with control lentivirus (C) or lentivirus coding for wild-type or the MT1-MMP E240A mutant. EGFR levels (lane 2) decreased in all cells that lack functional MT1-MMP (lanes 3, 7, 9, 10, and 11) or where the MT1-MMP E240A mutant is exerting a potential dominant negative effect over the endogenous enzyme (lanes 3 and 5).

To determine the biological significance of MT1-MMP-induced Sema4D release, we used full-length Sema4D-transfected wild-type and MT1-MMP knock-out MEFs as the chemotactic agents for endothelial cells in in vitro migration assays. While both wild-type and knock-out MEF controls exhibited some chemotactic effects upon endothelial cells (Fig. 3C, MT1-MMP: wt and KO, respectively), wells containing wild-type MEFs transfected with Sema4D (Fig. 3C, wt and S4D) induced a robust endothelial cell migration, while the MT1-MMP knock-out MEFs transfected with Sema4D failed to do so (Fig. 3C, KO and S4D). We next performed an in vivo angiogenesis assays in nude mice. Sema4D-transfected wild-type and MT1-MMP knock-out MEFs infected with lentiviruses transducing either the wild-type MT1-MMP or the E240A mutant were mixed with reconstituted basement membrane material and placed in an open-ended silicone tube angioreactor, which was implanted subcutaneously into nude mice (31). After 9 days, reactors containing wild-type and knock-out MEFs infected with control lentivirus demonstrated an angiogenic response at levels only slightly higher than that seen for negative controls (Fig. 3D, lanes 4 and 6), whereas MT1-MMP wild-type MEFs expressing wild-type Sema4D were able to induce blood vessel growth comparable with that seen in vascular endothelial growth factor and fibroblast growth factor filled control angioreactors (Fig. 3D, lane 7). In contrast, MT1-MMP knock-out MEFs expressing the wild-type form of Sema4D failed to do so (Fig. 3D, lane 5) but reacquired the ability to induce blood vessel ingrowth when infected with lentivirus transducing wild-type MT1-MMP (Fig. 3D, lane 10) but not the E240A catalytic site mutant (Fig. 3D, lane 9). The pro-angiogenic phenotype could also be rescued in knock-out MEFs by transfecting the secreted form of Sema4D, indicating that loss of Sema4D release is sufficient to abrogate the pro-angiogenic response in these cells (Fig. 3D, Sec S4D, lane 8) and that a catalytically active form of MT1-MMP is necessary for cells to release Sema4D and to promote angiogenesis in vivo.

We next determined whether tumor cells employ this paracrine MT1-MMP/Sema4D pathway to promote angiogenesis, as Sema4D is highly expressed in HNSCC cell lines compared with normal or immortalized human oral keratinocytes (21). Interestingly, HNSCC cells also overexpress MT1-MMP, whereas the immortal but non-tumorigenic keratinocyte cell lines HaCaT and HeLa do not (Fig. 4A). To examine the role of MT1-MMP in Sema4D release from HNSCC cells, we reduced the expression of endogenous MT1-MMP by generating lentiviruses expressing shRNAs for MT1-MMP (28). As shown in Fig. 4B, we identified an shRNA construct that caused a marked reduction in MT1-MMP protein levels (shRNA2, lane 5) and MMP-2 activation (Fig. 4C, lower panel, lane 4), which, when expressed in HN12 cells, markedly reduced Sema4D release into the conditioned medium (Fig. 4C, upper panel, lane 4).

To determine the significance of MT1-MMP-mediated Sema4D release for tumor-induced angiogenesis, we used HN12 cells infected with lentiviruses expressing the functional MT1-MMP shRNA (Fig. 4A, shRNA2, lane 5) or a control shRNA (Fig. 4A, shRNA1, lane 4) in vivo angiogenesis assays in nude mice (31). The results showed significant blood vessel growth into angioreactors containing HN12 cells infected with control virus (Fig. 4D, upper panel, HN12, reactor 3), similar to that seen for positive controls (Fig. 4D, upper panel, VEGF/FGF (vascular endothelial growth factor/fibroblast growth factor), reactor 2) but only slight growth into reactors containing HN12
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MT1-MMP is a member of a MMP subfamily that consists of six enzymes, MT (1–6)-MMP, all of which share peptide sequence homology (43). Like other MMPs, MT1-MMP is synthesized as a zymogen that requires proteolytic processing to generate its active, membrane-tethered form. Once activated, MT1-MMP has been shown to be crucial for pericellular collagenolysis and subsequently is considered a key enzyme that contributes to tumor cell invasion and metastasis through direct extracellular matrix degradation (43). MT1-MMP is also known to act on other proteinases and MMPs, proteinase inhibitors, growth factors and their receptors, chemokines and cytokines, and cell adhesion molecules, thereby influencing cell migration, proliferation, and apoptosis by shedding cell surface molecules or by producing biologically functional fragments from extracellular matrix components (37, 44). Consistent with its role in the migration and invasion of malignant cells, MT1-MMP is frequently overexpressed in aggressive, metastatic neoplasms, such as observed in cancers of the lung, colon, liver, breast, brain, head and neck, ovary, and uterine cervix (45, 46).

In addition to its other numerous protumorigenic effects, we demonstrate that MT1-MMP is necessary to generate soluble Sema4D. We focused on this MMP based upon the loss of Sema4D shedding following the administration of various MMP inhibitors. We show that MT1-MMP-mediated Sema4D shedding can induce endothelial cell chemotaxis in vitro and that HNSCC cell lines express this enzyme while non-invasive...
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FIGURE 5. A model for MT1-MMP-induced paracrine stimulation of Plexin-B1-dependent angiogenesis. MT1-MMP expressed on the surface of tumor cells cleaves membrane bound Sema4D, which can then bind Plexin-B1 on endothelial cells of the tumor stroma initiating an angiogenic response.

epithelial cell lines do not. Expression of MT1-MMP in HNSCC cells appears to be important for tumor-induced angiogenesis, as knocking down MT1-MMP expression with lentiviruses expressing MT1-MMP shRNAs greatly reduced the response in an in vivo angiogenesis assay, while re-introduction of MT1-MMP into knock-out MEFs restored the angiogenic phenotype.

MT1-MMP expression in some cells is able to induce activation of the extracellular signal-regulated protein kinase (ERK) cascade and gene transcription under the control of the serum response element (47). Therefore, we generated a catalytically dead form of MT1-MMP and demonstrated that it is the catalytic activity of the enzyme that is, in fact, necessary for processing and shedding of Sema4D. Our catalytically inactive mutant was also able to compete with endogenous protein to reduce, although not completely block, Sema4D cleavage when introduced into wild-type MEFs through lentiviral gene transfer. Taken together, these results strongly suggest that MT1-MMP acts upon Sema4D on the cell surface to cleave and release this protein, which then diffuses out and is able to exert its pro-angiogenic effects at a distance. However, we cannot rule out the possibility that an intermediate protein or proteins exist in this pathway, and it is the activation of the intermediates that then go on to process Sema4D from the cell surface.

Tumor progression and metastasis in large part depend upon the acquisition of an angiogenic capacity. Fast growing tumors become hypoxic because the tumor cells overwhelm the ability of the vasculature to meet their high metabolic demands. Paradoxically, it is the hypoxic environment generated in a tumor as it rapidly outgrows its blood supply that eventually leads to the switch from an avascular to a neovascular phenotype (48), a process that turns on the production of membrane bound pro-angiogenic proteins and soluble survival factors that induce proliferation and migration in surrounding endothelial cells (49). Recent reports have identified MT1-MMP as a hypoxia-induced gene product (50–52). Interestingly, we have found evidence that Sema4D might be up-regulated in some HNSCC cell lines upon exposure to hypoxic conditions as well. These observations raise the possibility of the existence of a pro-angiogenic mechanism whereby tumor cells concomitantly increase expression of MT1-MMP and Sema4D as the neoplasms acquire a neovascular phenotype. Sema4D is cleaved from the surface of the tumor cells in a MT1-MMP-dependent manner, thereby facilitating angiogenesis by acting as a chemotactic agent for Plexin-B1 expressing endothelial cells (Fig. 5). These possibilities are under current investigation. Our present findings indicate that the MT1-MMP-dependent proteolytic cleavage of Sema4D may play a critical role in tumor-induced angiogenesis and therefore may represent new fronts of attack in the anti-angiogenic therapy of cancer.

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