Membrane Type 1-Matrix Metalloproteinase Is Regulated by Chemokines Monocyte-Chemoattractant Protein-1/CCL2 and Interleukin-8/CXCL8 in Endothelial Cells during Angiogenesis*

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We have investigated the putative role and regulation of membrane type 1-matrix metalloproteinase (MT1-MMP) in angiogenesis induced by inflammatory factors of the chemokine family. The absence of MT1-MMP from null mice or derived mouse lung endothelial cells or the blockade of its activity with inhibitory antibodies resulted in the specific decrease of in vivo and in vitro angiogenesis induced by CCL2 but not CXCL12. Similarly, CXCL2- and CXCL8-induced tube formation by human endothelial cells (ECs) was highly dependent on MT1-MMP activity. CCL2 and CXCL8 significantly increased MT1-MMP surface expression, clustering, activity, and function in human ECs. Investigation of the signaling pathways involved in chemokine-induced MT1-MMP activity in ECs revealed that CCL2 and CXCL8 induced cortical actin polymerization and sustained activation of phosphatidylinositol 3-kinase (PI3K). Heterozygous MT1-MMP mice or derived mouse lung endothelial cells or the blockade of its activity with inhibitory antibodies resulted in the specific decrease of in vivo and in vitro angiogenesis induced by CCL2 but not CXCL12. Similarly, CXCL2- and CXCL8-induced tube formation by human endothelial cells (ECs) was highly dependent on MT1-MMP activity. CCL2 and CXCL8 significantly increased MT1-MMP surface expression, clustering, activity, and function in human ECs. Investigation of the signaling pathways involved in chemokine-induced MT1-MMP activity in ECs revealed that CCL2 and CXCL8 induced cortical actin polymerization and sustained activation of phosphatidylinositol 3-kinase (PI3K). Heterozygous MT1-MMP mice or derived mouse lung endothelial cells or the blockade of its activity with inhibitory antibodies resulted in the specific decrease of in vivo and in vitro angiogenesis induced by CCL2 but not CXCL12. 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2 B. G. Galvez, unpublished observations.

pared fibrinogen, C3 ADP-ribosylating exoenzyme, and pertussis toxin (Ptx) were from Calbiochem-Novabiochem, and type I collagen was from ICN Biomedicals (Costa Mesa, CA). Latrunculin A (LAT) and jasplakinolide (JPK) were from Molecular Probes (Eugene, OR). Caveolin-1 small interference RNA was synthesized and used as described (17).

Human EC Culture—Human ECs from umbilical vein were isolated and cultured up to the third passage as described (13). ECs were seeded on dishes coated with 1% GEL and changed to HE-SFM serum-free medium previously from Invitrogen before performing the functional assays.

MT1-MMP Is Required for Tube Formation by Human ECs

MT1-MMP expression was monitored in MLECs from different genotypes by Western blot.

Flux Cytometry and Analysis of Receptor Internalization—Subconfluent ECs were grown on GEL and treated with or without the indicated chemokines for 6 h. Cell surface staining and receptor internalization were analyzed by flow cytometry as described (16). Briefly, receptor internalization was assessed by incubating ECs with the primary mAb at RT for 20 min, exhaustively washing with phosphate-buffered saline, and transferring to 37 °C for 6 h to allow labeled-receptor internalization. Half of the cells were then fixed and permeabilized for 10 min at 4 °C with lysis buffer from BD Biosciences, and all cells were processed for flow cytometry. The fluorescence of permeabilized and non-permeabilized samples (MFI) was detected on a linear scale for optimizing quantitation and analyzed at 0 and 6 h. Internalization percentages at 6 h were estimated with the following equation.

\[ \text{Internalization} \% = \left( \frac{\text{MFI in permeabilized cells} - \text{MFI in non-permeabilized cells}}{\text{MFI in permeabilized cells}} \right) \times 100 \] (Eq. 1)

Immunofluorescence Microscopy—Immunofluorescence staining with anti-MT1-MMP LEM-2/63 mAb was performed after fixation in subconfluent ECs on GEL-coated coverslips incubated with or without different chemokines for 6 h as described (13). To visualize polymerized F-actin, cells were incubated with fluorescein isothiocyanate-phalloidin after stimulation with chemokines and fixation. Cells were examined under a Leica DMR photomicroscope with a 63× oil immersion objective and photographed with a Leica CCD camera.

Rac Small GTPase Activity Assays—Glutathione S-transferase-p21-activated kinase, which is recognized by active Rac, was prepared as described (20). ECs grown at subconfluence and stimulated with CCL2, CXCL12, or sphingosine 1-phosphate for 15 min or 6 h were lysed, and pull-down experiments were performed as described (20).

Zymography and Western Blotting—Subconfluent ECs were grown on GEL, incubated with or without different chemokines in serum-free medium for 6 h. Cell lysates were analyzed by fibrinogen or gelatin zymography or by Western blot with anti-MT1-MMP LEM-2/63 mAb or a mixture of anti-phospho-AKT Ser-473 and Thr-308 Abs as described (13). Anti-MT1-MMP dimer formation by EC membrane was extracted in the hydrophobic fraction of 1% Triton X-114 lysates and resolved on 10% SDS-PAGE under strict non-reducing conditions or under reducing conditions.

Reverse Transcription-PCR—Subconfluent ECs were cultured on GEL in HE-SFM medium, and after 6 h of stimulation with CCL2, CXCL5, CXCL12, or CXCL9, RNA was extracted with RNAzol (Ambion) and cDNA was synthesized from 1 μg of total RNA in the presence of 1 μg of random hexamers and reverse transcriptase. Quantitative PCR of MT1-MMP or actin (as a housekeeping gene control) was performed in a LightCycler using a SYBR Green kit (Roche Diagnostics) and two specific primer sets for MT1-MMP (5'-TCAGACCTTTGGACAGGG-3' and 5'-TTCAATGAGATTGTTGGGAAAATGCTC-3') and for actin (5'-TCAGAGAGCCTATGTGG-3' and 5'-TCTTTTGATGGTCACGGACC-3').

RESULTS

MT1-MMP Is Required for CCL2- but Not CXCL12-induced Marine Angiogenesis in Vivo and in Vitro—Angiogenesis was induced in Balb/c mice by subcutaneous injection of Matrigel premixed with angiogenic factors and blocking or control antibodies as described (Fig. 1). CCL2-induced hemoglobin accumulation was significantly inhibited by the presence of the anti-MT1-MMP LEM-2/63 mAb, which recognizes the murine protease, whereas CXCL12- or VEGF-induced angiogenesis was unaffected; CXCL9 did not alter constitutive angiogenesis (Fig. 1A). In agreement with this, tube formation was induced by the chemokines CCL2 and CXCL12, but only those capillary-like tubes generated by CCL2 were sensitive to inhibition by the anti-MT1-MMP mAb, as demonstrated by staining Matrigel sections with hematoxylin alone or together with anti-CD31 mAb (Fig. 1B).2

Angiogenesis was next analyzed in MT1-MMP-deficient mice (15). CCL2-induced angiogenesis was significantly inhibited in MT1-MMP null mice compared with wt or het mice, whereas CXCL12-induced angiogenesis developed similarly in wt, het, and null mice (Fig. 2A). Staining of frozen Matrigel sections with hematoxylin alone or with anti-CD31 mAb also revealed that no capillary-like tubes were formed in the presence of CCL2 in MT1-MMP null mice, in contrast to wt mice (Fig. 2B). Similarly, cord formation induced by CCL2 was abrogated in MT1-MMP null MLECs and diminished in heter MLECs, whereas the absence of MT1-MMP did not have any effect on CXCL12-induced cord formation (Fig. 2C).

MT1-MMP Is Required for Tube Formation by Human ECs Induced by CCL2 and CXCL8, and Its Expression and Internalization Are Regulated by These Chemokines—We next showed by flow cytometry that the receptors for CCL2, CXCL8,
CXCL12, and CXCL9 were expressed at the surface of human ECs. The MFI values for the corresponding receptors were, respectively: CCR2 = 25; CXCR1 = 22 and CXCR2 = 27; CXCR4 = 28; CXCR3 = 31. The negative value was 12. In addition, the anti-MT1-MMP LEM-2/15 mAb significantly inhibited cord formation in human ECs, by 70% in ECs stimulated with 10 nM CCL2 and in the presence or absence of 10 μg/ml anti-MT1-MMP LEM-263 mAb. Sections were stained with hematoxylin or immunostained with anti-mouse CD31 mAb as indicated. In the latter case, the L and arrowhead mark tube lumina and the surrounding endothelial monolayer, respectively. Original magnification 450x and 1350x.

We next showed that both CCL2 and CXCL8 increase MT1-MMP surface expression in human ECs stimulated for 6 h, in contrast to CXCL12 or CXCL9 (Fig. 3C). However, no changes were observed in the amount of total MT1-MMP protein, as analyzed by Western blot of whole cell lysates, nor in mRNA levels assessed by quantitative reverse transcription-PCR (Fig. 3D and E). Because MT1-MMP levels at the EC surface can be regulated by internalization (16), we next evaluated this. MT1-MMP internalization, close to 100% in untreated subconfluent ECs, was significantly decreased in ECs stimulated for 6 h with CCL2 or CXCL8 but not CXCL12 or CXCL9 (Fig. 3F).

MT1-MMP Subcellular Localization, Activity, and Function Are Modulated by CCL2 and CXCL8 in Human ECs—The effect of the distinct chemokines in modulating the subcellular localization of MT1-MMP was then investigated. The number of MT1-MMP clusters present at motility-associated membrane protrusions of subconfluent ECs was significantly increased upon stimulation for 6 h with CCL2 or CXCL8 (2–3-fold) and with CXCL12 (1.5-fold); no changes were observed with CXCL9 (Fig. 4A).

Because both the amount and clustering of MT1-MMP at the cell surface were up-regulated by CCL2 and CXCL8, we measured MT1-MMP activity under these conditions. MT1-MMP activity was significantly up-regulated by CCL2 or CXCL8 but not by CXCL12 or CXCL9 in subconfluent ECs, as assessed by fibrinogen zymography (Fig. 4B, top). To measure MT1-MMP activity in an independent manner, MMP-2 processing was estimated by gelatin zymography. As shown in Fig. 4B, bottom, the percentage of active MMP-2 was significantly increased in ECs stimulated with CCL2 or CXCL8, in contrast to CXCL12. These findings correlated with endothelial migration assays, in

**Fig. 1. Inhibition of MT1-MMP impairs CCL2- but not CXCL12-induced angiogenesis in mice**—A, Matrigel was mixed with 50 ng/ml VEGF or 100 nM CCL2, CXCL12, or CXCL9 or vehicle and with 10 μg/ml anti-MT1-MMP LEM-2/15 mAb or 10 μg/ml isotype-matched control mAb. Matrigel preparations were then injected into Balb/c mice. Hemoglobin (Hb) quantification was performed on plugs removed after 7 days. The arithmetic means and S.D. obtained from nine mice for each condition in three independent experiments are shown. *, p < 0.02. B, Matrigel sections were frozen from control plugs or plugs containing 100 nM CCL2 and in the presence or absence of 10 μg/ml anti-MT1-MMP LEM-263 mAb. Sections were stained with hematoxylin or immunostained with anti-mouse CD31 mAb as indicated. In the latter case, the L and arrowhead mark tube lumina and the surrounding endothelial monolayer, respectively. Original magnification 450x and 1350x.

**Fig. 2. The absence of MT1-MMP inhibits CCL2- but not CXCL12-induced angiogenesis in mice and MLECs**—A, Matrigel was mixed with 50 ng/ml VEGF or 100 nM CCL2 or CXCL12 and injected into 1-week old MT1-MMP wt (+/+), het (+/-), or null (-/-) C57BL/6 mice. Hemoglobin (Hb) quantification was performed on plugs removed after 5 days. The arithmetic means and S.D. of five mice for CCL2, four mice for CXCL12 and VEGF, and three mice for Matrigel alone are shown. *, p < 0.01. B, frozen Matrigel sections from control plugs or plugs containing 100 nM CCL2 in wt or null mice were stained with hematoxylin or immunostained with anti-mouse CD31 mAb as indicated. In the latter case, the L and arrowhead mark the tube lumina and surrounding endothelial monolayer, respectively. Original magnification 450x and 1350x. C, MLECs from MT1-MMP wt (+/+), het (+/-), or null (-/-) mice were left untreated or were treated with 10 ng/ml VEGF or 100 nM CXCL2 or CXCL12 and seeded onto Matrigel. Formation of cords was quantitated after 6 h as described under “Experimental Procedures.” *, p < 0.04; +, p < 0.02.
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Role of MT1-MMP in chemokine-induced cord formation by human ECs. Regulation of its expression and internalization. A, human ECs (HUVEC) were left untreated or were treated with 10 nM CCL2, CXCL8, CXCL12, or CXCL9 in the presence or absence of 10 μg/ml anti-MT1-MMP LEM-2/15 mAb or control isotype-matched mAb and seeded on Matrigel. Formation of cords was quantitated after 6 h as described under “Experimental Procedures.” *, p < 0.02; **, p < 0.03; +, p < 0.05. B, A dose response of CCL2 and CXCL12 was performed in the absence or presence of 10 μg/ml anti-MT1-MMP LEM-2/15 mAb in human ECs on Matrigel. Formation of cords was quantitated after 6 h as described under “Experimental Procedures.” The mean of two independent experiments is shown. C, the expression of MT1-MMP at the cell surface was analyzed by flow cytometry in subconfluent ECs on GEL untreated or treated with 10 nM CCL2, CXCL8, CXCL12, or CXCL9 for 6 h. X63 was included as a negative control. MFI is also indicated. A representative experiment of four conducted is shown. D, MT1-MMP total protein was assessed by Western blot analysis of cell lysates from subconfluent ECs on GEL untreated or treated with 10 nM CCL2, CXCL8, CXCL12, or CXCL9 for 6 h. Vascular endothelial (VE)-cadherin expression was used as loading control. One of five representative experiments is shown. E, MT1-MMP mRNA levels in human ECs on GEL untreated or treated with 10 nM CCL2, CXCL8, CXCL12, or CXCL9 for 6 h were analyzed by quantitative reverse transcription-PCR. The arithmetic mean and S.D. of the relative units of MT1-MMP mRNA obtained in three independent experiments are shown. F, internalization of MT1-MMP was quantitated by flow cytometry in subconfluent human ECs on GEL untreated or treated with 10 nM CCL2, CXCL8, CXCL12, or CXCL9 for 6 h. The arithmetic mean and S.D. of the internalization percentages of three independent experiments are shown. *, p < 0.03.
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MMP-2 nor tissue inhibitor of metalloproteinase-2 seemed to be present at the 120 kDa band. To explore whether signaling pathways involved in the CCL2-induced up-regulation of MT1-MMP activity (Fig. 5, A and B) were also implicated in MT1-MMP dimerization, signaling inhibitors were tested. Ptx, WMN, CytD, and LAT interfered with CCL2-induced MT1-MMP dimerization at the EC surface, demonstrating that PI3K activity and actin polymerization are required for this process (Fig. 6B) and suggesting that dimerization is related to MT1-MMP proteolytic activity. No effect on dimerization was observed with JPK, EDTA, or C3 (Fig. 6B).

DISCUSSION

In this report we show a preferential involvement of MT1-MMP in the angiogenic response induced by CCL2 and CXCL8, but not by CXCL12, that might be relevant to certain inflammatory diseases. In addition, we characterize the signaling pathways involved in the up-regulation of MT1-MMP activity by CCL2 in ECs, which include activation of PI3K and Rac, and actin polymerization. Finally, we show that MT1-MMP dimerization in primary ECs is regulated by CCL2-induced signaling.

Angiogenesis can be induced in chronic inflammatory disease by the prolonged exposure of ECs to cytokines and chemokines, but the molecular mechanisms underlying this response have not been elucidated yet (27–29). In this regard, CXCL12 and CXCL8 have been shown to favor angiogenesis by increasing EC expression of VEGF and MMP, respectively (9, 30). MT1-MMP is implicated in endothelial migration, invasion, and in vitro formation of capillary-like tubes (13); we therefore investigated its putative role in chemokine-induced angiogenesis. Remarkably, the absence of MT1-MMP in null mice or MLECs derived from them and the blockade of MT1-MMP activity with inhibitory antibodies all resulted in the specific decrease of in vivo and in vitro angiogenesis induced by CCL2 but not CXCL12. In this regard, a defective angiogenic response to fibroblast growth factor-2 in MT1-MMP null mice has been reported (15). We also report a preferential involvement of MT1-MMP in CCL2- and CXCL8- versus CXCL12-induced angiogenesis in human ECs. Our data show that CCL2 and CXCL8, but not CXCL12, increased MT1-MMP surface expression in ECs similar to other angiogenic factors and inflammatory cytokines (31, 32), in this case by partially inhibiting MT1-MMP internalization. In addition, CCL2 and CXCL8 increased MT1-MMP clustering at endothelial membrane protrusions, and this correlated with an increase in MT1-MMP activity. In contrast to previous reports linking MT1-MMP internalization with its activity (16, 33, 34), a partial inhibition of its internalization by CCL2 and CXCL8 resulted in an increase of its clustering and activity, suggesting that MT1-MMP internalization is not the primary regulatory event. MT1-MMP and/or MMP-2 might be important during the angiogenic response induced by CCL2, since both proteolytic activities are up-regulated, as shown by fibronectin and gelatin zymographies.

Occupancy of G protein-coupled receptors by chemokines results in mobilization of intracellular calcium as well activation of PI3K and its effectors (protein kinase C, AKT, Ras, mitogen-activated protein kinase, and small GTPases) and the JAK/STAT (signal transducers and activators of transcription) pathway (35). So far, only CCL2-induced activation of mitogen-activated protein kinase has been shown in ECs (36). Herein, we describe that although CCL2, CXCL8, and CXCL12 quickly mobilize calcium in human ECs, after 6 h only CCL2 and CXCL8 induce the activation of PI3K and Rac and the polymerization of cortical actin. However, CXCL12 did induce Rac activation in ECs at shorter times, as reported in leukocytes (23). Moreover, CXCL12 can also regulate GTPases and MT1-MMP activity in melanoma cells (37), pointing to cell type-specific events. Distinct signal responses to CCL2 and CXCL8
versus CXCL12 in ECs might be due to differences in the maintenance of signaling related to down-modulation or compartmentalization of the different chemokine receptors. In this regard CCR2 is internalized through caveolae in brain ECs (38). CXCL12 might also trigger alternative signaling pathways in ECs, leading to the expression of other genes relevant to angiogenesis such as VEGF, as reported (30). Our data would suggest that inflammatory signals such as CCL2 or CXCL8 might trigger distinct molecular pathways during angiogenesis compared with homeostatic signals such as CXCL12.

Because CCL2-induced MT1-MMP activity required actin polymerization, we analyzed F-actin content in subconfluent human ECs on GEL untreated or treated with 10 nM CCL2, CXCL8, CXCL12, or CXCL9 for 6 h with anti-MT1-MMP LEM-2/15 mAb under either strict non-reducing or reducing conditions; 120- and/or 60-kDa bands were observed (top and bottom, respectively). Densitometric analysis of the ratio MT1-MMP dimers/total MT1-MMP and the single MT1-MMP band are also shown (top and bottom, respectively). A representative experiment of three conducted is shown. βME, β-mercaptoethanol. B, MT1-MMP dimers were analyzed in membrane-enriched fractions from subconfluent human ECs on GEL untreated or treated for 6 h with 10 nM CCL2 alone or in the presence of 1 mM EDTA, 100 ng/ml Ptx, 10 nM WMN, 1 μM CytD, 100 nM LAT, 50 nM JPK, or 10 μg/ml C3. The ratio of MT1-MMP dimers/total MT1-MMP upon different treatment is also shown.

**FIG. 5.** Signaling pathways involved in CCL2-induced MT1-MMP activity in ECs. A, MT1-MMP activity was assessed by fibrinogen zymography of cell lysates from subconfluent human ECs untreated or treated for 6 h with 10 nM CCL2 alone or with 1 mM EDTA, 100 ng/ml Ptx, 10 μM WMN, 1 μM CytD, 100 nM LAT, 50 nM JPK, or 10 μg/ml C3. A representative experiment of three conducted is shown. The arithmetic means and S.D. of the specific activity (active MT1-MMP/total MT1-MMP) calculated by densitometric analysis of three independent experiments are also represented. ***, p < 0.01; **, p < 0.03; +, p < 0.05. B, MT1-MMP activity was assessed by gelatin zymography of cell lysates from subconfluent human ECs untreated or treated for 6 h with 10 nM CCL2 alone or with 1 mM EDTA, 100 ng/ml Ptx, 10 μM WMN, or 1 μM CytD. A representative of two experiments is shown. The arithmetic means and S.D. of the percentage of active MMP-2 (active MMP-

**FIG. 6.** CCL2 and CXCL8 enhance the formation of MT1-MMP dimers at the EC surface in a manner dependent on PI3K activity and actin polymerization. A, MT1-MMP dimers were analyzed in membrane-enriched fractions from subconfluent human ECs on GEL untreated or treated with 10 nM CCL2, CXCL8, CXCL12, or CXCL9 for 6 h with anti-MT1-MMP LEM-2/15 mAb under either strict non-reducing or reducing conditions; 120- and/or 60-kDa bands were observed (top and bottom, respectively). A representative experiment of three conducted is shown. Bar, 20 μm. Mean fluorescence intensity of the F-actin content quantitated by flow cytometry is also included (n = 3). CCL2 significantly increased F-actin content (p < 0.02). E, pull-down assays with glutathione S-transferase-p21-activated kinase were performed on lysates of subconfluent ECs grown on GEL untreated or treated with 10 nM CCL2 or CXCL12 or 1 μM sphingosine 1-phosphate for 15 min or 6 h. 1:10 of total lysates was loaded to estimate total Rac amount. A representative experiment of three conducted is shown.

2/total MMP-2 × 100) calculated by densitometric analysis of two in dependent experiments are also represented. ***, p < 0.01; **, p < 0.03; +, p < 0.05. C, lysates from subconfluent human ECs on GEL untreated or treated for 6 h with 10 nM CCL2, CXCL8, CXCL12, or CXCL9, or WMN were analyzed by Western blot with anti-phospho-AKT Abs. Vascular endothelial (VE)-cadherin expression was used as a loading control. A representative experiment of three conducted is shown. D, subconfluent human ECs on GEL were untreated or treated with 10 nM CCL2 or CXCL12 for 6 h, fixed, and stained with fluorescein isothiocyanate-phalloidin. Differential interference contrast (DIC) images are also shown. Bar, 20 μm. Mean fluorescence intensity of the F-actin content quantitated by flow cytometry is also included (n = 3). CCL2 significantly increased F-actin content (p < 0.02). E, pull-down assays with glutathione S-transferase-p21-activated kinase were performed on lysates of subconfluent ECs grown on GEL untreated or treated with 10 nM CCL2 or CXCL12 or 1 μM sphingosine 1-phosphate for 15 min or 6 h. 1:10 of total lysates was loaded to estimate total Rac amount. A representative experiment of three conducted is shown.
polymerization, which can modulate lateral mobility of membrane receptors (24–26), we examined the putative oligomerization of MT1-MMP in ECs. Interestingly, CCL2 and CXCL8 significantly increased the amount of MT1-MMP dimers. MT1-MMP oligomerization in transformed cells can be mediated by the hemopexin and/or cytoplasmic domains and, in accordance with our data, can be modulated by Rac GTPase (39–41). Here, we provide the first demonstration of a physiological mechanism for the regulation of MT1-MMP dimer formation by specific angiogenic chemokines in primary ECs. Signaling events required for MT1-MMP dimer regulation have also been analyzed. The requirement of CCL2-enhanced MT1-MMP dimerization on the activation of PI3K is in agreement with its role in chemokine-induced lateral mobility of β3-integrins (42). Moreover, actin polymerization is also required for dimer formation. Thus, activation of PI3K by CCL2 might induce sustained actin polymerization, which can modulate lateral mobility of MT1-MMP in ECs. Interestingly, CCL2 and CXCL8 over, actin polymerization is also required for dimer formation.

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