Membrane-Type Serine Protease-1/Matriptase Induces Interleukin-6 and -8 in Endothelial Cells by Activation of Protease-Activated Receptor-2
Potential Implications in Atherosclerosis

Isabell Seitz, Sibylle Hess, Henk Schulz, Robert Eckl, Gabriele Busch, Hans Peter Montens, Richard Brandl, Stefan Seidl, Albert Schömig, Ilka Ott

Objective—The serine protease MT-SP1/matriptase plays an important role in cell migration and matrix degradation. Hepatocyte growth factor (HGF), urokinase-type plasminogen activator (uPA), and protease-activated receptor 2 (PAR-2) have been identified as in vitro substrates of MT-SP1/matriptase. Because PAR-2 is expressed in endothelial cells and contributes to inflammatory processes, we sought to investigate the effects of MT-SP1/matriptase on endothelial cytokine expression and analyzed MT-SP1/matriptase expression in vascular cells and atherosclerotic lesions.

Methods and Results—In endothelial cells, recombinant MT-SP1/matriptase dose-dependently induced interleukin (IL)-8 and IL-6 mRNA and protein expression dependent on its proteolytic activity. MT-SP1/matriptase time-dependently induced phosphorylation of p38 MAPK and p42/44 MAPK. Inhibitor experiments revealed that p38 MAPK and PKCα were necessary for IL-8 induction. PAR-2 downregulation abolished and PAR-2 overexpression augmented MT-SP1/matriptase-induced IL-8 expression as evidence for PAR-2 signaling. In human atherectomies, MT-SP1/matriptase was expressed in blood cells adherent to the endothelium. Concordantly, basal MT-SP1/matriptase expression was detected in isolated monocytes. Coincubation of monocytes and endothelial cells resulted in an increased IL-8 release, which was reduced after downregulation of endothelial PAR-2 and monocyteic MT-SP1/matriptase.

Conclusion—MT-SP1/matriptase induces release of proinflammatory cytokines in endothelial cells through activation of PAR-2. MT-SP1/matriptase is expressed in monocytes, thus, interaction of monocyteic MT-SP1/matriptase with endothelial PAR-2 may contribute to atherosclerosis. (Arterioscler Thromb Vasc Biol. 2007;27:769-775.)

Key Words: pathophysiology • growth factors • endothelium

Membrane-type serine protease (MT-SP1/matriptase) is a trypsin-like, multi-domain serine protease expressed primarily in epithelial cells. Its importance in the biology of surface-lining epithelial cells became apparent in MT-SP1/matriptase knockout mice presenting with a severe deficient epidermal barrier function as well as abnormal hair follicle development and disturbed thymic homeostasis. Moreover, MT-SP1/matriptase is up-regulated in different malignant tissues and may be expressed in microvascular endothelial cells. Besides its N-terminal transmembrane signal anchor, MT-SP1/matriptase contains two putative regulatory modules: 2 tandem repeats of a CUB domain (C1r/s, Uegf, Bone morphogenetic protein-1) and 4 tandem repeats of a low density lipoprotein (LDL) receptor domain. The C-terminal serine protease domain consists of a catalytic triad comprising His-57, Asp-102, and Ser-195 according to chymotrypsin numbering. In addition to the membrane-anchored form of MT-SP1/matriptase, a soluble form of the protease has been identified lacking the N-terminal 172 amino acids. Shedding from the extracellular surface or alternative splicing may be the mechanisms leading to the truncated form of MT-SP1/matriptase isolated from human milk. Cleavage within its activation motif generates the 2-chain active protease from a single-chainzymogen. The activation of MT-SP1/matriptase requires its cognate Kunitz-type inhibitor hepatocyte growth factor activator inhibitor (HAI-1), its noncatalytic domains as well as its serine protease domain. Three macromolecular substrates of MT-SP1/matriptase have been identified: urokinase-type plasminogen activator (uPA), hepatocyte growth factor (HGF), and protease-activated receptor-2 (PAR-2). Both HGF and uPA have been proposed to regulate matrix degradation, cell proliferation,
survival, and motility. Because various cancer cells express MT-SP1/matriptase, tumor progression may be regulated through activation of HGF, uPA, or by extracellular matrix degradation.

PAR-2, a heptahelical G protein–coupled receptor, has been identified in various cell types including vascular endothelial cells. PAR-2 is activated by trypsin, mast-cell derived tryptase, tissue factor/factor VIIa complex, and factor Xa. Proteolytic cleavage of PAR-2 at its N terminus unmasks new amino-terminal residues serving as a tethering ligand, which irreversibly activates the maternal receptor. Subsequently, cleaved receptors undergo lysosomal degradation. PAR-2 is upregulated by cytokines, vascular injury, and in atherosclerosis and contributes to inflammatory responses. Considering the potent trypsin-like activity of MT-SP1/matriptase comprising residues 596 to 855 was expressed in E. coli as His-tagged serine protease domain of MT-SP1/matriptase (V615-V855). MT-SP1/matriptase C122S/S195A was compared with MT-SP1/matriptase C122S.

Results

Expression, Renaturation, and Purification of MT-SP1/matriptase

The serine protease domain of MT-SP1/matriptase comprising residues 596 to 855 was expressed in E. coli as His-tagged protein and purified from inclusion bodies. The N-terminal His-tag including the prodomain was cleaved autocatalytically during refolding as previously described leading to the catalytic domain of MT-SP1/matriptase.

Figure 1. MT-SP1/matriptase induces IL-8 mRNA and release in EC. A, HUVECs were treated with increasing concentrations of MT-SP1/matriptase C122S for 16 hours. IL-8 release in the supernatants is shown (mean ± SEM, n = 3); asterisks indicate a probability value <0.05. B, Before treatment with MT-SP1/matriptase C122S HUVECs were incubated with 5 μg/mL Actinomycin D (AcID). Total RNA was analyzed by quantitative RT-PCR. Values were normalized on GAPDH and are shown as x-fold untreated cells. Shown is the mean ± SEM of at least 3 independent experiments. Asterisks indicate P < 0.05 vs unstimulated cells. C, Proteolytic activity of MT-SP1/matriptase C122S and active site mutant MT-SP1 C122S/S195A after incubation with 25 μmol/L synthetic trypsin substrate Boc-Gln-Ala-Arg-AMC. Shown is the mean ± SEM of the relative fluorescence units (RFU) of 3 independent experiments. D, HUVECs were treated with MT-SP1/matriptase C122S (20 nmol/L) or proteolytically inactive MT-SP1/matriptase C122S/S195A (20 nmol/L) and IL-8 release was measured. Each column represents the mean ± SEM of 3 independent experiments. The asterisk indicates P < 0.05.

MT-SP1/Matriptase Induces IL-8 and IL-6 mRNA Expression and Release Dependent on its Catalytic Activity

HUVECs showed a dose-dependent increase of IL-8 release after treatment with MT-SP1/matriptase C122S (Figure 1A). Because preincubation with actinomycin D resulted in abrogation of MT-SP1/matriptase C122S–induced IL-8 mRNA expression, de novo synthesis of IL-8 is required (Figure 1B). To assess the relevance of the MT-SP1/matriptase active site a proteolytically inactive mutant MT-SP1/matriptase C122S/S195A was compared with MT-SP1/matriptase C122S. In an enzymatic assay using the trypsin substrate Boc-Gln-Ala-Arg-AMC MT-SP1/matriptase C122S/S195A showed no catalytic activity.
activity in contrast to MT-SP1/matriptase C122S (Figure 1C) and did not alter cytokine release, whereas MT-SP1 C122S significantly enhanced IL-8 release (Figure 1D). The proinflammatory effect of MT-SP1/matriptase C122S was confirmed in human coronary arterial endothelial cells (data not shown). In the presence of serum MT-SP1/matriptase C122S induced a 1.5-fold increase in IL-8 expression as compared with an approximately 3-fold increase under serum free conditions (data not shown). Hence, secondary effects by other MT-SP1/matriptase substrates can be excluded. In addition to IL-8, the increased protein secretion and mRNA expression of IL-6 was observed after treatment of HUVECs with MT-SP1/matriptase C122S (supplemental Figure IA and IB). This was elicited only by proteolytically active MT-SP1/matriptase C122S, but not by the inactive mutant C122S/S195A (supplemental Figure IB).

MT-SP1/Matriptase Induces IL-8 Through Activation of PAR-2

Downregulation of PAR-2 using siRNA (siPAR-2) decreased PAR-2 mRNA levels whereas transfection with nonsilencing negative control siRNA (siC) did not alter PAR-2 mRNA compared with nontransfected cells (ctr) (Figure 2A). IL-8 mRNA expression significantly increased after MT-SP1/matriptase C122S treatment of cells transfected with siC (Figure 2C). In contrast, downregulation of PAR-2 abolished MT-SP1/matriptase C122S-induced IL-8 transcription (Figure 2C).

Because of its trypsin-like activity, MT-SP1/matriptase may not only activate PAR-2 but also PAR-1. Hence, we analyzed the role of PAR-1 using specific siRNA (siPAR-1). PAR-1 expression was downregulated as compared with nontreated control (ctr) (Figure 2B). Gene silencing of PAR-1, however, did not affect MT-SP1/matriptase C122S-induced IL-8 transcription (Figure 2C). Similarly, the use of inhibitory antibodies against PAR-1 did not abolish the proinflammatory MT-SP1/matriptase C122S effect (supplemental Figure II). Moreover, flow cytometry analysis using activation-dependent (SPAN12) and independent (WEDE15) antibodies revealed that MT-SP1 C122S did not activate PAR-1 (supplemental Figure III). Thus, PAR-2 exclusively mediates MT-SP1/matriptase-induced IL-8 upregulation in HUVECs.

Overexpression of human PAR-2 and PAR-1 in HUVECs confirmed the importance of PAR-2-mediated IL-8 induction (Figure 2D).

MT-SP1/Matriptase Activates p38 MAPK, p42/44 MAPK, and NF-κB

To determine whether MT-SP1/matriptase-induced signaling pathways via PAR-2 involve the activation of mitogen-activated protein kinases (MAPK), we analyzed p38 MAPK and p42/44 MAPK phosphorylation. MT-SP1/matriptase C122S time-dependently induced activation of p38 MAPK (Figure 3A) and p42/44 MAPK (Figure 3B) with a maximum at 5 minutes. Luciferase reporter gene assays revealed that MT-SP1/matriptase C122S activates NF-κB, which was dependent on p38 MAPK and p42/44 MAPK, because specific pharmacological inhibitors against both MAPK suppressed NF-κB activation (Figure 3C). Transfection with pTAL-Luc control did not elicit reporter gene expression (data not shown). However, only p38 MAPK was essential for induction of IL-8 expression after MT-SP1/matriptase C122S-
treatment of HUVECs (Figure 3D). Similar results were found for the induction of IL-6 (data not shown). NF-κB activation inhibitor was included in the luciferase reporter gene assay before to assure efficacy (Figure 3C).

**MT-SP1/Matriptase-Induced Cytokine Expression Requires PKCa**

Because PAR-2 has not only been associated with p38 MAPK and p42/44 MAPK activation but also with activation of PKCa, the implication of PKCa in activation of p38 MAPK, p42/44 MAPK, and NF-κB and in cytokine induction in response to MT-SP1/matriptase was assessed. A decrease in MT-SP1/matriptase C122S-induced IL-8 (Figure 4D) and IL-6 (data not shown) mRNA synthesis was observed after inhibition of PKCa. Experiments analyzing the involvement of PKCa in p38 MAPK and p42/44 MAPK activation demonstrated that inhibition of PKCa did not alter MAPK phosphorylation (Figure 4A and B). Furthermore, NF-κB activation was not impaired by inhibition of PKCa (Figure 4C). Therefore, MT-SP1/matriptase C122S-

![Figure 3](https://example.com/figure3.png)

**Figure 3.** MT-SP1/matriptase-induced activation of p38 MAPK, p42/44 MAPK, and NF-κB in HUVECs. Requirement of p38 MAPK for enhancement of IL-8 expression. A and B, HUVECs were incubated with 20 nmol/L MT-SP1/matriptase C122S for the indicated time periods (0 to 30 minutes). Cell extracts were subjected to Western blot analysis for p38 MAPK (A) and p42/44 MAPK (B) phosphorylation and for total p38 MAPK (A) and p42/44 MAPK (B) protein. Shown is 1 representative Western blot of 3. C, HUVECs were transfected with pNF-κB-Luc and subsequently treated with inhibitors of p38 MAPK (SB 220025, 20 μmol/L), p42/44 MAPK (PD 98059, 20 μmol/L), or NF-κB activation (1 μmol/L) and stimulated with 20 nmol/L MT-SP1/matriptase overnight. Lysates were subjected to luciferase assays. Each column represents the mean±SEM of 3 experiments, displayed as x-fold untreated. Asterisk indicates a probability value <0.05. D, HUVECs were treated with inhibitors of p38 MAPK (SB 220025, 20 μmol/L), p42/44 MAPK (PD 98059, 20 μmol/L), or NF-κB activation (1 μmol/L) and stimulated with 20 nmol/L MT-SP1/matriptase C122S for 2 hours. Total RNA was analyzed by quantitative RT-PCR, Values are displayed as x-fold untreated cells. Shown are means±SEM of 3 independent experiments. Asterisks indicate P<0.05 vs MT-SP1/matriptase C122S-treated control cells.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Requirement of PKCa for induction of IL-8 expression. A and B, HUVECs were treated with an inhibitor of PKCa (Safingol, 20 μmol/L) and stimulated with 20 nmol/L MT-SP1/matriptase C122S. Cell extracts were subjected to Western blot analysis for p38 MAPK (A) and p42/44 MAPK phosphorylation (B) and for total p38 MAPK (A) and p42/44 MAPK protein (B). Shown is 1 representative Western blot of 6. C, HUVECs were transfected with pNF-κB-Luc, treated with an inhibitor of PKCa (Safingol, 20 μmol/L), and stimulated with 20 nmol/L MT-SP1/matriptase C122S overnight. Cell lysates were subjected to the luciferase assay. Each column represents the mean±SEM of 3 experiments, displayed as x-fold untreated. D, HUVECs were treated with a PKCa-inhibitor (Safingol, 20 μmol/L) and stimulated with 20 nmol/L MT-SP1/matriptase C122S for 2 hours. Total RNA was analyzed by qRT-PCR. Values are displayed as x-fold untreated cells. Shown are means±SEM of 5 independent experiments. Asterisks indicate P<0.05 vs MT-SP1/matriptase C122S-treated control cells.
induced IL-8 gene expression via PAR-2 requires 2 independent pathways, involving p38 MAPK and PKCa.

MT-SP1/Matriptase Expression in Human Atherectomies

By the use of an external standard curve absolute MT-SP1/matriptase mRNA copies in vascular cells and atherosclerotic lesions were determined and subsequently normalized on GAPDH. MT-SP1/matriptase mRNA levels were significantly increased in atherectomy samples as compared with control vessels (Figure 5A, $P=0.002$). A significant correlation of MT-SP1/matriptase with IL-8 mRNA expression ($R^2=0.76, P=0.0002$) provides further evidence for a role of MT-SP1/matriptase in proinflammatory changes during atherosclerosis. The high variance of MT-SP1/matriptase expression in atherectomy samples is likely attributable to varying compositions of the collected specimens. Immunohistochemical analysis revealed that MT-SP1/matriptase was highly expressed in blood cells attached to the endothelium (Figure 5B; supplemental Figures I and II), whereas healthy vessels mainly expressed MT-SP1/matriptase only in the adventitia (data not shown). The hypothesis that monocytic MT-SP1/matriptase may induce IL-8 in HUVECs via PAR-2 was confirmed by coincubation of HUVECs and MonoMac-6 (MM6) cells. Gene silencing of PAR-2 in HUVECs and of MT-SP1/matriptase in MM6 decreased IL-8 release after 8 hours coincubation in contrast to negative control siRNA (siC)-transfected cells (Figure 5C).

Expression of MT-SP1/Matriptase in Peripheral Blood Monocytic Cells

Mononuclear cells (MNCs) expressed MT-SP1/matriptase mRNA, whereas endothelial cells (HUVECs) and smooth muscle cells (SMCs) showed no basal expression under culture conditions (supplemental Figure IVA). In mononuclear cells MT-SP1/matriptase was exclusively expressed in CD14+ monocytes (supplemental Figure IVB I-II). Isotype controls mouse IgG and mouse y1-PE did not show fluorescence (data not shown).

Discussion

Major findings of this study are: (1) Dependent on its proteolytic activity, MT-SP1/matriptase stimulates de novo synthesis of the proinflammatory cytokines IL-8 and IL-6 through activation of PAR-2 in endothelial cells. MT-SP1/matriptase-induced IL-8 expression is dependent on activation of p38 MAPK and PKCa. (2) In atherosclerotic lesions, enhanced mRNA and protein expression of MT-SP1/matriptase was found as compared with nondiseased vessels. Immunohistochemistry showed a strong staining for MT-SP1/matriptase in blood cells attached to the endothelium. (3) Only monocytes, but not lymphocytes, endothelial or smooth muscle cells, expressed MT-SP1/matriptase under culture conditions. Concordantly, monocytic MT-SP1/matriptase and endothelial PAR-2 were crucial for increased IL-8 release in a coincubation model.

MT-SP1/matriptase may play a role in tumor invasion and metastasis by altering migratory responses and by matrix degradation. In this study, we identify a proinflammatory role of MT-SP1/matriptase and provide evidence for potential implications in atherosclerosis. MT-SP1/matriptase dose-dependently induced IL-8 and IL-6 release in human endothelial cells, dependent on its catalytic activity. The increased IL-8 secretion was attributable to upregulation of IL-8 gene transcription. Using RNA interference, we specifically knocked down PAR-2, a known in vitro substrate for MT-SP1/matriptase, to analyze its role as mediator of MT-SP1/matriptase-induced cytokine release. Applying this technique, we identified PAR-2 as the mediator of MT-SP1/matriptase-induced IL-8 expression and established an efficient tool to analyze PAR-2–mediated signaling pathways.

Phosphorylation of p38 MAPK and p42/44 MAPK occurred after stimulation with MT-SP1/matriptase, even...
Although only p38 MAPK activation was necessary for IL-8 induction. These results are in line with a previous study in human peripheral blood eosinophils that identified activation of p38 MAPK as mechanism for an increased IL-8 release after activation of PAR-2 by tryptase.\textsuperscript{32} In contrast, we did not find dependence on p42/44 MAPK activation. Furthermore, activation of PKC\& was required for MT-SP1/matriptase-induced IL-8 expression, independent of p38 MAPK. Although MT-SP1/matriptase activated NF-\kappaB as shown by reporter gene assays, NF-\kappaB activation was not necessary for IL-8 gene expression. NF-\kappaB activation after PAR-2 activation was demonstrated in several cell lines including microvascular endothelial cells, coronary artery smooth muscle cells, and keratinocytes. However, there was no direct evidence for NF-\kappaB-mediated cytokine expression, even though intercellular adhesion molecule-1 (ICAM-1) upregulation was shown to depend on NF-\kappaB activation.\textsuperscript{33,34}

Optimal IL-8 expression may require the activation of several transcription factors (NF-\kappaB, AP-1, and nuclear factor for IL-6/NF-IL-6), which may explain the lack of effect of NF-\kappaB inhibition on MT-SP1/matriptase-induced IL-8 expression.\textsuperscript{35}

Recent studies identified proinflammatory effects of PAR-2 activation in neutrophils.\textsuperscript{36} Moreover, in vivo studies in PAR-2-deficient mice highlight the critical roles of PAR-2 in skin progression and joint inflammation as well as sepsis.\textsuperscript{37,38} In vascular disease, PAR-2-derived peptide agonists reduce vascular tone and increase blood flow via nitric oxide–dependent and –independent actions of the endothelium.\textsuperscript{39} In addition, enhanced expression of PAR-2 was found in human coronary atherosclerosis.\textsuperscript{40} Yet, the role of PAR-2 in atherosclerosis and the underlying mechanisms have not been elucidated. This study reveals a novel physiological PAR-2 agonist abundantly expressed in monocytes, which may enhance local inflammatory responses in the endothelial inner layer of the vessel wall. Compared with IL-1B, which induces IL-8 mRNA in endothelial cells up to 70-fold (data not shown), MT-SP1/matriptase may not be the main proinflammatory stimulus but rather contribute to amplification and perpetuation of the inflammatory reaction. Investigation of MT-SP1/matriptase expression in different vascular cell types revealed that MT-SP1/matriptase is expressed in mononuclear leukocytes but not in smooth muscle and endothelial cells. Immunohistochemistry confirmed expression of MT-SP1/matriptase mainly in blood cells adhering to the endothelium of the atherosclerotic vessel wall. The biological importance of monocytic MT-SP1/matriptase for the induction of inflammatory mediators in the endothelium via PAR-2 was demonstrated in a coincubation experiment. Interaction of monocytes with the endothelium plays a major role in the progression of atherosclerosis. Considering the fact that PAR-2 agonists stimulate P-selectin expression in endothelial cells and increase neutrophil adherence to the endothelium, one might assume that PAR-2 contributes to both adherence and inflammation in progression of atherosclerosis.\textsuperscript{40} Furthermore, previous studies have shown increased PAR-2 expression in atherosclerotic coronary arteries.\textsuperscript{24} Thus, PAR-2 may represent a possible therapeutic target to modify responses to vascular injury.

Because we were interested in the biological effects of MT-SP1/matriptase, we expressed soluble human MT-SP1/matriptase to exclusively analyze the effects of the serine protease domain in endothelial cells. Experiments were performed in a serum-free environment to exclude interference with the other known substrates uPA and HGF.\textsuperscript{3,15} Under these conditions, we identified PAR-2 as the mediator of proinflammatory cytokine release in response to MT-SP1/matriptase. Secondary effects of MT-SP1/matriptase, eg, via uPA with subsequent PAR-1 activation by plasmin,\textsuperscript{41} are unlikely because of the reduced induction of IL-8 in the presence of serum providing additional MT-SP1/matriptase substrates. After adhesion of monocytes to the endothelium in atherosclerosis, activation of PAR-2 by MT-SP1/matriptase may contribute to local inflammatory changes. This mechanism of cytokine induction will presumably not constitute an initial event in inflammation but may perpetuate the inflammatory process within the atherosclerotic vessel wall. In summary, in addition to its role in cancer invasion, metastasis, and oncogenic transformation or as activator of uPA and HGF, we identified a novel proinflammatory role of MT-SP1/matriptase via activation of PAR-2 in endothelial cells.

Acknowledgments

We thank Mrs B. Campbell, A. Stobbe, and C. Bauer for invaluable technical assistance.

Sources of Funding

The study was supported by grants from the Sander Stiftung and Deutsche Herzstiftung.

Disclosures

None.

References


Membrane-Type Serine Protease-1/Matriptase Induces Interleukin-6 and -8 in Endothelial Cells by Activation of Protease-Activated Receptor-2: Potential Implications in Atherosclerosis

Isabell Seitz, Sibylle Hess, Henk Schulz, Robert Eckl, Gabriele Busch, Hans Peter Montens, Richard Brandl, Stefan Seidl, Albert Schömig and Ilka Ott

Arterioscler Thromb Vasc Biol. 2007;27:769-775; originally published online January 25, 2007;
doi: 10.1161/01.ATV.0000258862.61067.14
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/27/4/769

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/02/13/01.ATV.0000258862.61067.14.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
MATERIALS AND METHODS

Substances

Transcription inhibitor actinomycin D was from Sigma (Taufkirchen, Germany). p38 MAPK inhibitor SB 220025, p42/44 MAPK inhibitor PD 98059, PKCα-inhibitor Safingol and NF-κB activation inhibitor 6-amino-4-(4-phenoxyphenylethylamino)quinazoline were from Calbiochem (Schwalbach, Germany). Boc-Gln-Ala-Arg-AMC, a highly reactive substrate for trypsin, was from Bachem (Weil am Rhein, Germany) and was used to determine the enzymatic activity of MT-SP1/matriptase. PAR-1 antibodies SPAN12 and WEDE15 were from Beckman Coulter (Krefeld, Germany), PAR-1 antibody ATAP2 was from Santa Cruz (Heidelberg, Germany).

Expression and Purification of MT-SP1/matriptase

MT-SP1/matriptase encoding DNA was amplified from a human liver cDNA library (Ambion, Austin, USA) using MT-SP1/matriptase 5’- and 3’-UTR specific primers GCGGCCCTCGGGGACCATGGG and CGATGGGTGGCCCCGCAGGTG. The 2627 bp PCR fragment served as template to amplify the DNA encoding residues 596-855 of MT-SP1/matriptase by nested PCR with primers CCCATGGGCTCAGATGAAAAAGACTGC and CCGGCCCTATACCCAGGTG. The resulting 791 bp fragment was first cloned into TOPO TA vector pCR2.1 (Invitrogen, Basel, Switzerland), digested with NdeI/BamHI and cloned into pDS-6HisNdeI NdeI, BamHI. Insertion into pDS-6HisNdeI resulted in a 5’-end coding for an N-terminal tag of additional 14 residues including six consecutive histidines. The point mutants MT-SP1/matriptase C122S and C122S/S195A were constructed using a modified quick-change protocol (Stratagene, La Jolla, USA). The mutant C122S was generated because following activation the pro-peptide remained attached to the catalytic
domain via a disulfide bridge otherwise. All sequences were verified using the dye terminator cycle sequencing technology on an ABI PRISM 310 Genetic Analyzer. The \textit{E. coli} host strain Rosetta-gami pRAREIq (Novagen, VWR International, Luzern, Switzerland) was transformed with pDS-6HisNdeI MT-SP1/matriptase. The expression was induced adding 0.2 mM IPTG to mid-log cells growing in LB medium at 37°C. Inclusion bodies were isolated and solubilized in 100 mM Tris-Cl pH 8.0, 6 M guanidine HCL, 100 mM β-mercaptoethanol. The solubilized proteins were renatured by rapid dilution into 20 mM Tris-Cl pH 8.6, 0.8 M arginine, 0.3 M NaCl, 20 mM glycine, 1 mM cysteine, 1 mM EDTA at 4°C. The refolding mixture was concentrated and dialyzed. MT-SP1/matriptase was purified chromatographically on a Benzamidine Sepharose, on a MonoQ (GE Healthcare) and on a Biosec column (Merck, VWR International, Luzern, Switzerland). The purification of MT-SP1/matriptase C122S/S195A was slightly modified. After purification on a Source Q (GE Healthcare) column, the pro-domain was cleaved by adding mature MT-SP1/matriptase at a ratio of 1:100. MT-SP1/matriptase C122S/S195A was further purified on a Benzamidine Sepharose and a Biosec column. Purified MT-SP1/matriptase was stored in 50% glycerol at -80°C. Functional activity of the C122S mutant was identical to wild-type.

\textit{Enzymatic activity}

Enzymatic activity of purified recombinant MT-SP1/matriptase proteins (1 nM) was detected using 25 µM of the synthetic fluorescent substrate Boc-Gln-Ala-Arg-AMC in 10 mM Tris-Cl pH 8.5, 150 mM NaCl, 1 mM EDTA and 5% glycerol. Fluorometric measurements were performed at $\lambda_{ex} = 360$ nm (Spectrafluor Plus, Tecan, Maennedorf, Switzerland).

\textit{Cell Culture}

Human umbilical vein endothelial cells (HUVEC, Promocell, Heidelberg, Germany) were cultured in EBM-2 medium (Clonetics, San Diego, USA). Peripheral blood mononuclear cells
(MNC) were isolated by Ficoll gradient centrifugation. Human coronary artery smooth muscle cells (SMC) were purchased from Clonetics and cultured in SmBM-2 medium (Clonetics). Mono-Mac-6 cells (MM6) were cultured in very-low endotoxin RPMI 1640 medium (Biochrom, Berlin, Germany), 10% FCS. Cells were starved in serum-free medium and then stimulated with MT-SP1/matriptase (20 nM or unless otherwise indicated) in serum-free medium for two hours to analyze RNA or over-night to measure cytokine release.

**Coincubation of HUVEC and MM6**

Coincubation was performed under static conditions at a 1:1 ratio. Cells were coincubated for 16 h, the supernatant was collected and subsequently subjected to immunoassay.

**Preparation of Human Atherectomy Samples**

Human atherectomies were obtained from carotid arteries (n = 26) and normal vessels from the external pudendal artery (n = 8). The institutional ethics committee approved the study protocol and all subjects gave informed consent. Samples were bisected and one part was immediately stored in RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) and homogenized in 600 µl RLT-Buffer (Qiagen) containing 10 µl/ml β-mercaptoethanol. Total RNA was prepared using RNeasy Mini Kit (Qiagen). For immunohistochemistry frozen tissue samples were embedded in Tissue Tek (Sakura, Zoeterwoude, NL) and 8 µm sections were stained.

**RNA interference (RNAi) targeting PAR-1, PAR-2 and MT-SP1/matriptase**

For down-regulation of PARs by RNAi 2.5*10^5 HUVEC were transfected with 2.5 µg annealed siRNA (PAR-1 sense: 5’-GGUCAAGAGCCGCGGUGGtt-3’, PAR-2 sense: 5’-GGAAGAAGCCUUAUUGGUAtt-3’, fluorescein-labeled non-silencing siRNA (siCtr) sense: 5’- UUCUCCGAACGUGACGACGUdTdT-3’, Ambion, Austin, Texas) and 7.5 µl RNAiFect
(Qiagen). For gene silencing of MT-SP1/matriptase \(5 \times 10^5\) MM6 were transfected with 1.3 \(\mu g\) annealed siRNA-Mix (containing MT-SP1 sense GCAACAAGAUCACAGUUCGtt and CCAGUUCACGUGCAAGAACtt) and 7.5 \(\mu l\) RNAiFect. Experiments were performed 48 hours after transfection.

**Transient transfection**

HUVEC were transfected with pcDNA3.1(+)-hPAR-1, pcDNA3.1(+)-hPAR-2 (UMR cDNA Resource Center, Rolla, USA) or pcDNA3.1(+) (Invitrogen, Karlsruhe, Germany) using Effectene transfection reagent (Qiagen). Transfection efficiency was monitored by fluorescence microscopy after co-transfection of a GFP-plasmid and was 30 ± 6%.

**Luciferase Assay**

HUVEC were transfected with pTAL-Luc as negative control and pNF-\(\kappa\)B-Luc (Clontech, Heidelberg) utilizing Effectene transfection reagent (Qiagen). Eight hours post transfection medium was changed and inhibitors were added to the medium containing 0.05% FCS. After one hour cells were treated with MT-SP1/matriptase (20 nM). 20 hours later cells were lysed with 250 \(\mu l\) 1x lysis buffer (Dual-Luciferase Reporter Assay System, Promega, Mannheim). 20 \(\mu l\) of the lysate were analyzed for reporter gene activity after addition of 100 \(\mu l\) luciferase assay reagent. Transfection efficiency was monitored by fluorescence microscopy following co-transfection with a GFP plasmid.

**Cytokine Release**

For cytokine release assays cells were seeded in 96-well plates and supernatants were analyzed using immunoassay (human IL-8, R&D Systems, Wiesbaden, Germany) or Cytometric Bead Array (CBA human Chemokine-I, Becton Dickinson, San Diego, USA).
Activation of Mitogen-activated protein kinases (MAPK)

After serum deprivation for three hours HUVEC were stimulated with 20 nM MT-SP1/matriptase. Cells were washed with ice-cold phosphate-buffered saline and scraped in 150 µl cell lysis buffer (1% Triton, 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin and 1 mM PMSF). Equal amounts of protein were resolved by SDS-PAGE, transferred to a PVDF membrane, blocked with 5% bovine serum albumin and then incubated with primary antibodies against p38 MAPK (1:1000), phospho-p38 MAPK (1:1000), p42/44 MAPK (1:1000) or phospho-p42/44 MAPK (1:2000) at 4°C overnight (Cell Signaling, Frankfurt, Germany). After washing the membrane was incubated with the secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences, Freiburg, Germany), washed and proteins were visualized with ECL (Amersham Biosciences).

Immunofluorescence

Human MT-SP1/matriptase was detected using M32 monoclonal antibody, kindly provided by C. Y. Lin (Georgetown University, Washington, USA). Ficoll-isolated PBMC were cytospined on slides and fixed in 1% paraformaldehyde. MT-SP1/matriptase was detected with M32 (5 µg/ml) followed by a secondary FITC-conjugated donkey-anti-mouse IgG (1:40, Jackson, Cambridgeshire, UK). Monocytes were identified by RPE-conjugated CD14 antibody (1:10, Dako, Hamburg, Germany). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI).

Immunohistochemistry

Immunohistochemical staining of atherectomy sections was performed using Vectastain ABC-AP Kit (Vector Labs, Gruenberg, Germany). M32 monoclonal antibody (10 µg/ml) and
Red Chromogen (Dako) were used for detection of MT-SP1/matriptase. Nuclei were stained with Mayer’s hemalaun.

**Semi-quantitative Reverse Transcription-PCR**

Total RNA was prepared using RNeasy Mini Kit (Qiagen). Reverse Transcription was performed using Superscript II (Invitrogen). Primers for amplification of β-actin and MT-SP1/matriptase were as follows: β-actin sense CTACGTCGCCCTGGACTTCGAG, β-actin antisense GATGGAGCCGCCGATCCACACGG, MT-SP1 sense GTGAACGTCGTCATTGTAC, MT-SP1 antisense CAGGTGGTCTGGTTGATGAC.

**Reverse Transcription and quantitative Real-Time PCR (qPCR)**

Total RNA was isolated using RNeasy Mini Kit (Qiagen) and reverse transcribed using Omniscript RT Kit (Qiagen). Transcript levels were detected by quantitative RT-PCR (TaqMan®, ABI PRISM 7700, Applied Biosystems, Weiterstadt, Germany). The PCR mixture contained 1x TaqMan Universal PCR Master Mix, 900 nM of each primer and 250 nM of a 6-carboxy-fluorescein (FAM)-labeled probe (Applied Biosystems). Assays-on-Demand containing specific primers and probe for GAPDH (Hs99999905_m1), MT-SP1/matriptase (Hs00222707_m1), IL-8 (Hs00174103_m1), IL-6 (Hs00174131_m1), PAR-1 (Hs00169258_m1) and PAR-2 (Hs00608346_m1) were purchased from Applied Biosystems. Fold increase was calculated using the ∆∆C_{t}-method by normalization on GAPDH. For absolute quantification qPCR was performed and C_{t}-values were analyzed based on standard curves established from pre-quantified PCR products specific for each target mRNA. PCR products were quantified by agarose gel electrophoresis in comparison to a DNA-standard of known concentration. mRNA copies were normalized on GAPDH copies present in the sample.
**Statistics**

Student’s t-test or ANOVA as appropriate were used to determine statistical significance between control and treated cells. P < 0.05 was considered significant. Shown are means ± SEM. Significant values are marked with an asterisk.
Suppl. fig. I. IL-6 induction in HUVEC by MT-SP1/matriptase

MT-SP1/matriptase C122S induced IL-6 release and transcription of the IL-6 gene, which was dependent on the catalytic activity of the enzyme as shown by use of the proteolytically inactive mutant MT-SP1/matriptase C122S/S195A.

A. HUVEC were starved for three hours and then treated with MT-SP1/matriptase C122S (20 nM). IL-6 release was determined by immunoassay. Each column represents the mean ± SEM of three independent experiments. Asterisk indicates p < 0.05.

B. HUVEC were serum-deprived for three hours and treated with 20 nM MT-SP1/matriptase C122S or proteolytically inactive MT-SP1/matriptase C122S/S195A (20 nM) for two hours. Total RNA was analyzed by TaqMan quantitative RT-PCR. Values were normalized on GAPDH and are shown as x-fold untreated cells. Shown is the mean ± SEM of at least three independent experiments. Asterisk indicates p < 0.05 versus unstimulated cells.
Suppl. fig. II. Independency of MT-SP1/matriptase-induced IL-8 expression of PAR-1

MT-SP1/matriptase C122S-induced IL-8 gene expression did not require PAR-1: In addition to gene silencing of PAR-1 by RNA interference, inhibition of PAR-1 by antibodies WEDE15 and ATAP2 did not abrogate the proinflammatory effect of MT-SP1/matriptase C122S either. In contrast, the inhibitory antibody mix rather amplified the effect of MT-SP1/matriptase C122S on IL-8 expression.

HUVEC were serum starved for two hours and then treated with inhibitory antibodies against PAR-1 WEDE15 and ATAP2 for one hour in a concentration of 20 µg/ml and 10 µg/ml, respectively. Then cells were stimulated with MT-SP1/matriptase C122S (20 nM) as indicated. Total RNA was prepared, subjected to reverse transcription and analyzed by quantitative PCR. Each column represents the mean ± SEM of three independent experiments.
**Suppl. fig. III. Assessment of PAR-1 cleavage by MT-SP1/matriptase**

A. and B. MT-SP1/matriptase C122S did not activate PAR-1 as compared to thrombin. Flow cytometry using activation-specific PAR-1 antibodies revealed that MT-SP1/matriptase C122S neither reduced the amount of intact PAR-1 nor of total PAR-1 on the surface of endothelial cells. This was shown in contrast to thrombin, which clearly reduced uncleaved PAR-1 levels as detected by SPAN12 and also diminished total PAR-1 levels, probably due to internalization of the receptor following its activation. Hence, we conclude that MT-SP1/matriptase C122S does not activate PAR-1, but specifically activates PAR-2.

HUVEC were kept serumfree for three hours and treated with 20 nM MT-SP1/matriptase C122S or 10 U/ml thrombin for two hours. Cells were collected non-enzymatically. Cleavage of PAR-1 was verified by flow cytometry using antibodies SPAN12-PE (A, 1:10) detecting intact PAR-1 and WEDE15-PE (B, 1:10) detecting both intact and cleaved PAR-1. Solid black line: untreated cells, red line: MT-SP1/matriptase C122S-treated cells, green line: thrombin-treated cells, dotted line: autofluorescence. Each panel shows one representative experiment out of three.
Suppl. fig. IV. Expression of MT-SP1/matriptase in MNC

A. Total RNA was prepared from SMC, mononuclear leukocytes (MNC) and HUVEC. Shown are the quantitative PCR curves obtained by TaqMan method. Target mRNAs GAPDH as internal control and MT-SP1/matriptase are indicated by arrows. HUVEC (green) and SMC (blue) do not show detectable MT-SP1/matriptase expression as compared to MNC (red).

B. Immunofluorescent staining for MT-SP1/matriptase in MNC. Nuclei were stained with DAPI (I), monocytes with anti-CD14-RPE (III) and MT-SP1/matriptase with M32 monoclonal anti-human MT-SP1/matriptase antibody (II).