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Matrix Metalloproteinase-10 Is Upregulated by Thrombin in Endothelial Cells and Increased in Patients With Enhanced Thrombin Generation

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Objective—Thrombin is a multifunctional serine protease that promotes vascular proinflammatory responses whose effect on endothelial MMP-10 expression has not previously been evaluated.

Methods and Results—Thrombin induced endothelial MMP-10 mRNA and protein levels, through a protease-activated receptor-1 (PAR-1)–dependent mechanism, in a dose- and time-dependent manner. This effect was mimicked by a PAR-1 agonist peptide (TRAP-1) and antagonized by an anti–PAR-1 blocking antibody. MMP-10 induction was dependent on extracellular regulated kinase1/2 (ERK1/2) and c-jun N-terminal kinase (JNK) pathways. By serial deletion analysis, site-directed mutagenesis and electrophoretic mobility shift assay an AP-1 site in the proximal region of MMP-10 promoter was found to be critical for thrombin-induced MMP-10 transcriptional activity. Thrombin and TRAP-1 upregulated MMP-10 in murine endothelial cells in culture and in vivo in mouse aorta. This effect of thrombin was not observed in PAR-1–deficient mice. Interestingly, circulating MMP-10 levels ($P<0.01$) were augmented in patients with endothelial activation associated with high (disseminated intravascular coagulation) and moderate (previous acute myocardial infarction) systemic thrombin generation.

Conclusion—Thrombin induces MMP-10 through a PAR-1–dependent mechanism mediated by ERK1/2, JNK, and AP-1 activation. Endothelial MMP-10 upregulation could be regarded as a new proinflammatory effect of thrombin whose pathological consequences in thrombin-related disorders and plaque stability deserve further investigation. **(*Arterioscler Thromb Vasc Biol*. 2009;29:2109-2116.)**

Key Words: thrombin ■ endothelium ■ MMP-10 ■ atherosclerosis ■ thrombosis

There is growing evidence for an intimate link between inflammatory and coagulation pathways in vascular disease. Inflammation appears to shift the hemostatic mechanisms in favor of thrombosis, and conversely, coagulation pathways influence inflammatory response.[1–3] Thrombin participates in this interplay and, besides its well established role in thrombosis and hemostasis, activates protease-activated receptors (PARs) and elicits multiple effects in a variety of cell types.[1] PARs have been implicated in the control of vascular tone, vascular permeability, and secretory activity of endothelial cells.[4–6] In addition, the activation of PARs induces the production of proinflammatory cytokines, upregulates cell adhesion molecules, and promotes leukocyte rolling in different models.[7–9] PAR-1 seems to be the major mediator of thrombin signaling in vascular endothelial cells (ECs) in mice and humans, and most of the actions of thrombin on ECs have been reproduced using the PAR-1 agonist peptides.[10]

Thrombin exerts powerful proinflammatory effects on vascular cells and upregulates some matrix degrading metalloproteinases (MMPs).[11–13] MMPs are zinc-dependent endopeptidases capable of degrading extracellular matrix (ECM) proteins, but also process a number of bioactive molecules. They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand), and chemokine in/activation. MMPs are also thought to play a major role on cell proliferation, migration, differentiation, angiogenesis, apoptosis, and host defense.[14,15] MMPs also participate in the maintenance of vascular patency after a thrombotic event, which requires fibrin removal

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J.M.-G. and J.A.P. contributed equally to this study.

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by a cooperative proteolytic processing involving plasmin and MMPs.[16]

Recently, we demonstrated that MMP-10 is associated with inflammation and subclinical atherosclerosis and is present in atherosclerotic lesions at rupture-prone sites.[17,18] MMP-10 (stromelysin-2) is a protease that processes various components of basement membrane matrix such as collagen type IV, laminin, and proteoglycans and activates other MMPs (MMP-1, -8, and -13) capable of degrading interstitial components of ECM.[19,20] However, little is known on its pathophysiological role in thrombotic disorders. In this study we investigated whether human thrombin is able to induce MMP-10 in ECs and in pathological conditions characterized by increased thrombin generation. Our results provide novel insights into the molecular mechanisms underlying vascular effects of thrombin and highlight the potential relevance of MMP-10 in processes associated to local and systemic thrombin generation.

Methods

Detailed explanation of the different experimental procedures is provided as supplementary material (available online at http://atvb.ahajournals.org).

Cell Cultures

Human umbilical vein endothelial cells (HUVECs), human aortic endothelial cells (HAECs), bovine aortic endothelial cells (BAECs), and mouse lung endothelial cells (MLECs) were arrested overnight and stimulated with human thrombin. When needed cells were pretreated for 30 minutes with different inhibitors of signaling pathways.

Real-Time PCR

RNA from cells was extracted and reverse-transcribed. Real-time PCR was performed using TaqMan gene expression assays-on-demand (Applied Biosystems).

MMP-10 Protein Secretion

MMP-10 levels were assayed in conditioned medium by ELISA (R&D Systems). Cell culture and serum samples were diluted 1:5 and 1:2 respectively. The assay recognizes the zymogens and active forms.

Western Blot Analysis

Protein extracts from HUVECs stimulated with thrombin in the presence or in the absence of hirudin and ATAP2 were analyzed by Western blot. Antibodies against ERK1/2 and JNK-1 (either phosphorylated forms of total protein) were used.

MMP-10 Promoter Constructs and Transient Transfection Assays

A 2.0-kb fragment of the human MMP-10 promoter was generated by PCR and cloned into pGL-3 vector (Promega). A series of promoter deletions were generated, and the putative AP-1 site present in MMP-10 promoter was mutated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). HUVECs or BAECs were transfected with the luciferase reporter plasmids and stimulated with thrombin in the presence or absence of inhibitors. Luciferase activity was measured in cell lysates using the Luciferase assay kit (Promega) and a luminometer (Orion I, Berthold Detection Systems). Results were normalized by β-galactosidase activity using the Enzyme Assay System (Promega).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays (EMSAs) were carried out using nuclear extracts from HUVECs stimulated with thrombin in the presence or in the absence of different pathway inhibitors. Doubled stranded probes containing the putative AP-1 element present in MMP-10 were labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase and protein-DNA complexes were resolved by electrophoresis.

Induction of MMP-10 by Thrombin in Mouse Aorta: In Vivo Assay

Thrombin (0.1 to 10 U) or TRAP-1 (6 mg/kg) were infused intravenously to wild-type mice (C57Bl/6). PAR-1-deficient (PAR-1$^{-/-}$) mice, kindly provided by Dr R. Chambers (University College, London, UK),[21] were also treated with thrombin or saline. MMP-10 in aorta was assessed by real-time PCR and immunohistochemistry. The research was performed in accordance with the European Community guidelines for ethical animal care and use of laboratory animals (Directive 86/609) and was approved by the University of Navarra Animal Research Review Committee.

Circulating MMP-10 Levels in Patients

MMP-10 was measured by ELISA (R&D Systems) in serum from 60 patients with previous acute myocardial infarction (previous AMI) and 12 patients with sepsis-induced disseminated intravascular coagulation (DIC). Another 2 groups of healthy subjects were included, matched for age and sex. Informed consent was obtained from all subjects according to the Declaration of Helsinki.

Statistical Analysis

Continuous variables were expressed as mean$\pm$SD (unless otherwise stated). Differences between groups were assessed by using appropriate statistical tests (SPSS version 11.0). Statistical significance was established as $P<0.05$.

Results

Thrombin Induces MMP-10 mRNA Levels in Human Endothelial Cells

Thrombin increased MMP-10 mRNA levels in HUVECs in a time- and dose-dependent manner (Figure 1A). The effect was statistically significant at 2 hours ($P<0.01$) reaching a maximum at 6 hours ($P<0.01$) compared to control, and was prevented by hirudin (a specific thrombin inhibitor). In contrast, no significant changes in TIMP-1 expression could be observed (data not shown). Thrombin also increased MMP-10 protein levels, an effect that was significant ($P<0.01$) 12 hours after stimulation (Figure 1B). Similarly, thrombin induced both MMP-10 mRNA and protein levels in adult HAECs, an effect that was prevented by hirudin (Figure 1C). In these cells, 0.1 U/mL thrombin promoted a 5-fold increase in MMP-10 mRNA at 12 hours and a 3-fold increase in MMP-10 protein. Actinomycin D blocked thrombin-induced MMP-10 upregulation, and no changes in MMP-10 mRNA half-life were observed (data not shown).

PAR-1 Mediates the Upregulation of MMP-10 by Thrombin

PAR-1 blocking antibody ATAP2 completely neutralized thrombin-induced MMP-10 mRNA and protein (Figure 2A), whereas a control antibody (N-19) did not produce any effect. Moreover, a short peptide agonist for human PAR-1 (TRAP-1) mimicked the effect of thrombin on endothelial

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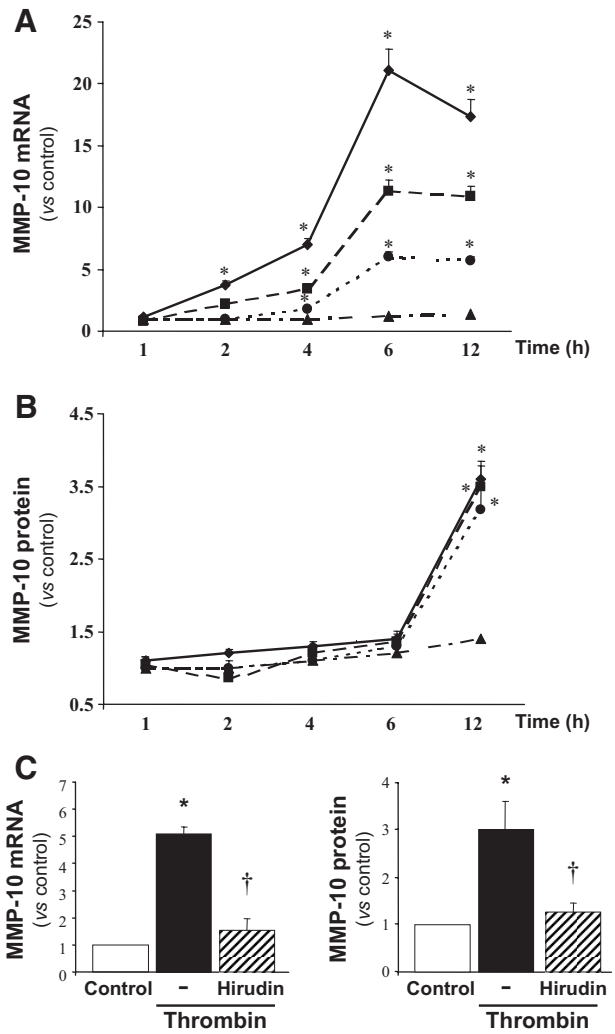

Figure 1. Thrombin induces MMP-10 mRNA and protein levels in vascular endothelial cells. A, Time-course and dose-response showing the increase of MMP-10 mRNA levels after incubation of HUVECs with thrombin (Thr, 0.1 to 5 U/mL) for 1 to 12 hours in the presence or absence of hirudin (Hir): circles (Thr 0.1 U/mL); squares (Thr 2.5 U/mL); diamonds (Thr 5 U/mL); triangles (Thr 0.1 U/mL + Hir). B, MMP-10 protein in the conditioned medium from cells treated as indicated in A. C, Thrombin (0.1 U/mL, 12 hours) increased MMP-10 mRNA (left) and protein (right) by human aortic endothelial cells (HAECs), effects that were prevented by hirudin. Data (mean±SEM, n=4) are presented as percentage from controls. $P<0.01$: * vs control; † vs thrombin alone.

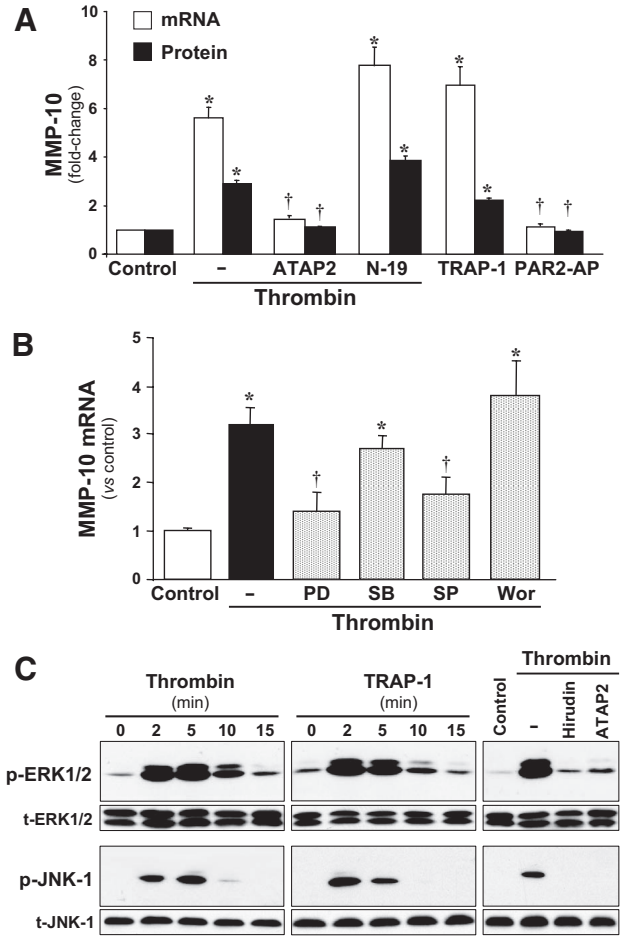

Figure 2. Thrombin upregulates MMP-10 through a PAR-1–dependent mechanism. A, PAR-1 mediates thrombin-induced MMP-10 expression in endothelial cells. The effect of thrombin (0.1 U/mL,12 hour) on MMP-10 mRNA (white bars) and protein (black bars) was mimicked by a PAR-1 agonist peptide (TRAP-1) and inhibited by a PAR-1 blocking antibody (ATAP2). The absence of effect of a control antibody against PAR-1 (N-19) or a PAR-2 agonist (PAR2-AP) is also shown. $P<0.01$: * vs control; † vs thrombin alone. B, ERK1/2 and JNK signaling pathways are involved in thrombin-induced MMP-10 expression. Real-time PCR showing MMP-10 mRNA levels in HUVECs induced with thrombin in the presence of different inhibitors: PD98059 (PD), SP600125 (SP), SB203580 (SB), and wortmannin (Wor). Data are mean±SD (n=5). $P<0.05$: * vs controls; † vs thrombin alone. C, Thrombin activates ERK1/2 and JNK signaling pathways. Western blot analysis showing the time-dependent activation of ERK1/2 and JNK-1 in HUVECs stimulated with thrombin or TRAP-1. Activation of ERK1/2 and JNK-1 by thrombin (5 minutes) is prevented by incubation with either hirudin or ATAP2. Unchanged levels of total ERK1/2 (t-ERK1/2) and total JNK-1 (t-JNK-1) are shown.

MMP-10 expression, whereas a peptide agonist for human PAR-2 (PAR2-AP) had no effect (Figure 2A). Taken together, these results indicate that thrombin upregulates endothelial MMP-10 via PAR-1.

MAPK Pathways Are Involved in Thrombin-Induced MMP-10 Gene Expression

Because thrombin activates different signaling pathways in ECs, specific inhibitors were used to address which of these pathways are involved in MMP-10 induction. Results shown in Figure 2B indicate that MMP-10 induction by thrombin was dependent on ERK1/2 (inhibited by PD98059) and JNK (inhibited by SP600125). By contrast, SB203580 (inhibitor of p38 MAPK) and wortmannin (inhibitor of PI3K) did not significantly affect MMP-10 response to thrombin. In the absence of thrombin, inhibitors did not produce any effect on MMP-10 levels (data not shown). Thrombin and TRAP-1 activated ERK1/2 and JNK-1 phosphorylation in a time-dependent manner, whereas hirudin and ATAP2 completely prevented thrombin-induced activation of these signaling kinases (Figure 2C). Therefore, these results indicate that thrombin upregulates MMP-10 through a PAR-1–dependent mechanism mediated by ERK1/2 and JNK.

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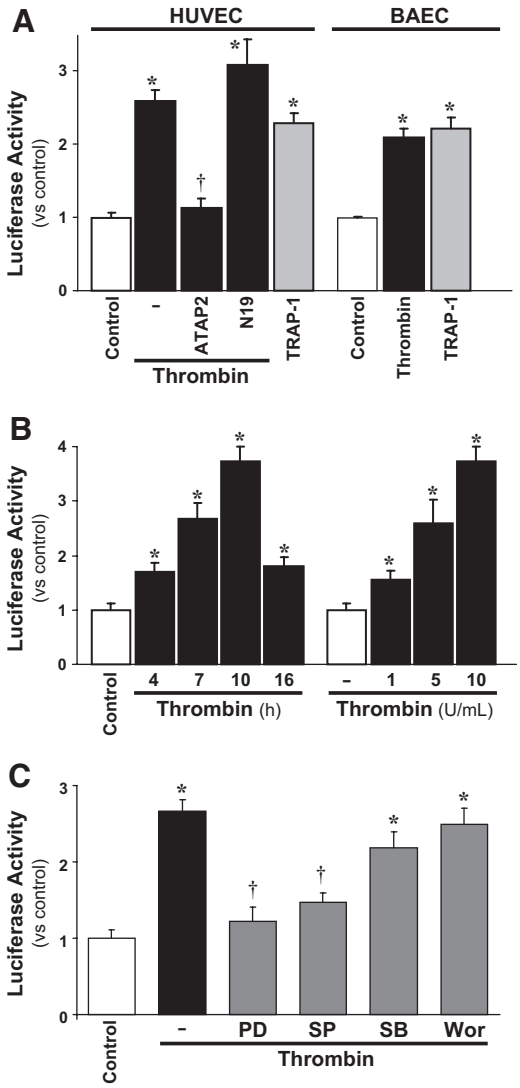

Figure 3. Thrombin induces MMP-10 promoter activity in endothelial cells. The effect of thrombin (5 U/mL) on the activity of MMP-10 promoter (construct pMMP10/-1934) was assayed in transient transfection experiments. A, Thrombin and TRAP-1 increase MMP-10 promoter activity in both HUVECs and BAECs. This effect was completely prevented by a PAR-1 blocking antibody (ATAP2). The absence of effect of a control antibody against PAR-1 (N-19) is also shown. B, Time- and dose-dependent effect of thrombin on MMP-10 promoter activity in HUVECs. C, Effect of inhibitors of different signaling pathways on MMP-10 promoter activity in HUVECs transfected with pMMP10/-1934 and treated with thrombin for 10 hours. PD98059 (PD), SB203580 (SB), SP600125 (SP), and wortmannin (Wor) were used as described in Figure 2 legend. Data are mean±SD (n=5). $P<0.05$: * vs controls; † vs thrombin alone.

Involvement of AP-1 in the Induction of MMP-10 by Thrombin

To further characterize the mechanisms underlying MMP-10 upregulation by thrombin we performed transient transfection assays using a luciferase reporter plasmid containing MMP-10 promoter (pMMP10/-1934). In these experiments thrombin (5 U/mL) increased MMP-10 promoter activity more than 2-fold over controls in both HUVECs and BAECs, an effect that was mimicked by TRAP-1 (Figure 3A). In addition, ATAP2 but not N19 abrogated the upregulation of MMP-10 promotor activity induced by thrombin in HUVECs. The effect of thrombin was dose- and time-dependent, reaching a maximum 10 hours after thrombin stimulus (Figure 3B). Thrombin-induced activity of MMP-10 promoter was prevented by PD98059 and SP600125, but not by SB203580 or wortmannin (Figure 3C). To determine the element responsive to thrombin in the MMP-10 promoter, we constructed a series of promoter deletions and examined their activity in transient transfection experiments (Figure 4A). The constructs pMMP-10/-1934, -456, -350, and -251 exhibited similar basal promoter activity that was increased by 2-fold after thrombin induction. However, the construct pMMP-10/-47 showed a markedly reduced basal activity with no significant changes after thrombin treatment. We identified several potential transcription factor binding sites within the proximal promoter region of MMP-10, spanning nucleotides -251 to -47 including a cAMP response element (CRE) and a response element for AP-1 (Jun/Fos heterodimer). Mutation of the CRE site did not affect thrombin-induced MMP-10 transcriptional activity (data not shown). By contrast, mutation of the AP-1 site ([-67]TGAATCA[-61]) by site-directed mutagenesis significantly reduced both basal and thrombin-induced MMP-10 promoter activities (Figure 4A). Consistent with this, NDGA (AP-1 inhibitor) significantly reduced both thrombin-induced mRNA and the transcriptional activity of MMP-10 promoter in transient transfection assays (Figure 4B and 4C). By EMSA we show that thrombin increases the binding to this AP-1 response element in a time-dependent manner. AP-1 binding was verified by antibody *supershift* assays and was abrogated by site-directed mutagenesis (Figure 4D). Finally, AP-1 binding to this response element was reduced by PD98059, SP600125, and NDGA, compounds that inhibit ERK1/2, JNK-1, and AP-1, respectively, and reduce thrombin-induced MMP-10 mRNA levels.

Thrombin Induces Vascular MMP-10 Expression In Vivo

Thrombin increased MMP-10 mRNA levels in mouse endothelial cells in a dose-dependent manner (Figure 5A). This effect was mimicked by TRAP-1 and was completely prevented by hirudin. MMP-10 mRNA upregulation by thrombin and TRAP-1 was also reduced by PD98059 and SP600125. To determine whether thrombin is able to induce MMP-10 in vivo, we analyzed the expression of MMP-10 in the aorta of mice 6 hours after i.v. administration of thrombin (0.1 to 10 U) or TRAP-1 (6 mg/kg). The expression of MMP-10 in the aorta of saline-treated control mice was undetectable by real-time PCR, but thrombin strongly induced MMP-10 mRNA in a dose-dependent manner (Figure 5B). The effect, mimicked by TRAP-1, was observed even at the lowest dose of thrombin administered (0.1 U) and was maximum at the higher dose used (10 U), although the later was associated with a high mortality rate (2 of 4 mice) likely as a result of DIC, as assessed by the presence of diffuse petechial intra-abdominal hemorrhage. By contrast, high doses of thrombin were unable to induce MMP-10 mRNA in PAR-1$^{-/-}$ mice. MMP-10 mRNA induction by thrombin was followed by an increase in MMP-10 protein levels. Indeed, immunohistochemical analysis clearly showed the increase of MMP-10 in

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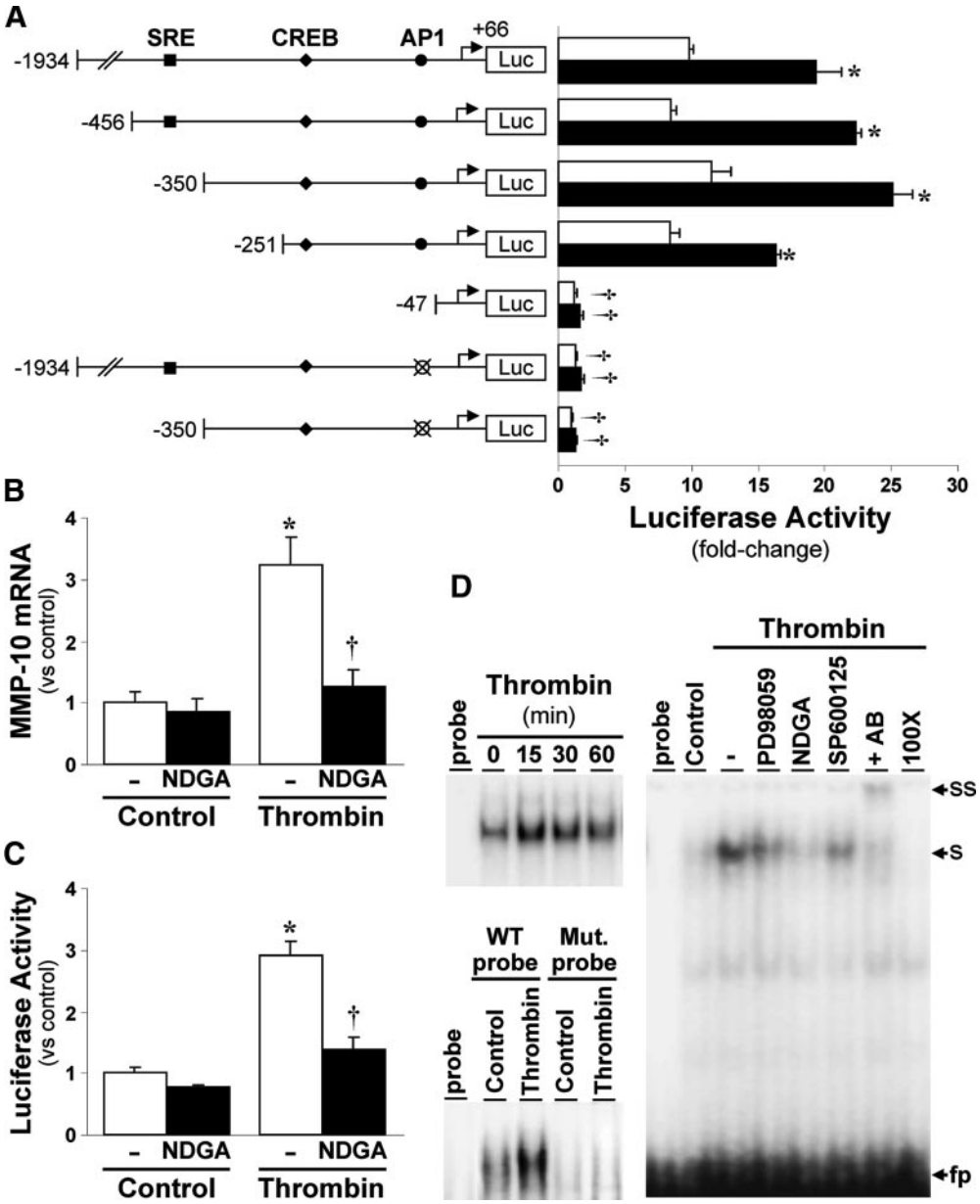

Figure 4. AP-1 is involved in thrombin-induced MMP-10 upregulation. HUVECs were transiently transfected with MMP-10 constructs and stimulated with thrombin (5 U/mL, 10 hours). A, MMP-10 promoter activity of various deletion mutants in the presence (black bars) or in the absence (white bars, controls) of thrombin. Constructs are numbered and named according to their length upstream of the transcription-initiation site. The activity of pMMP10/-1934 and pMMP10/-350 constructs and the same constructs mutated in the AP-1 site (-67/-61) is shown. $P<0.05$: * vs control cells transfected with the same construct; † vs cells transfected with the corresponding wild-type construct (nonmutated) and induced with thrombin. Data are mean±SD (n=5). The location of the putative response elements is indicated: serum response element (SRE), cAMP response element binding protein (CREB), and activator protein-1 (AP-1) site. Luciferase activity is expressed as fold-change using pMMP10/-350 (mutated in AP-1 in the absence of stimulus) as a reference value. B and C, Effect of NDGA on MMP-10 mRNA levels (B) and MMP-10 promoter activity (C) induced by thrombin (5 U mL for 10 hours). $P<0.05$: * vs controls; † vs thrombin. Data are mean±SD (n=5). D, Thrombin induces the binding activity to a putative AP-1 site (-67/-61) present in MMP-10 promoter. The binding to this site was evaluated by EMSA using nuclear extracts from HUVECs stimulated with thrombin (5 U/mL) for increasing times (upper left panel). The binding observed using the wild-type sequence of MMP-10 promoter (WT probe) was abolished when the AP-1 site was mutated (Mut probe; lower left panel). The effect of PD98059, NDGA, and SP600125 on AP-1 binding was also analyzed. A supershifted band was observed on addition of a specific antibody against Jun (+AB). Competition using a 100-fold (100×) excess of unlabeled probe is also shown. Arrowheads indicate the position of the specific shifted band (s), the supershifted band (ss), and the free probe (fp).

the intima (endothelial cells and subendothelium) of aortic sections from thrombin-treated animals (Figure 5C), indicating that thrombin is able to induce vascular MMP-10 expression in vivo.

Circulating Levels of MMP-10 in Thrombin-Related Clinical Disorders

To further demonstrate whether increased thrombin generation is associated with elevated MMP-10 levels in patholog-

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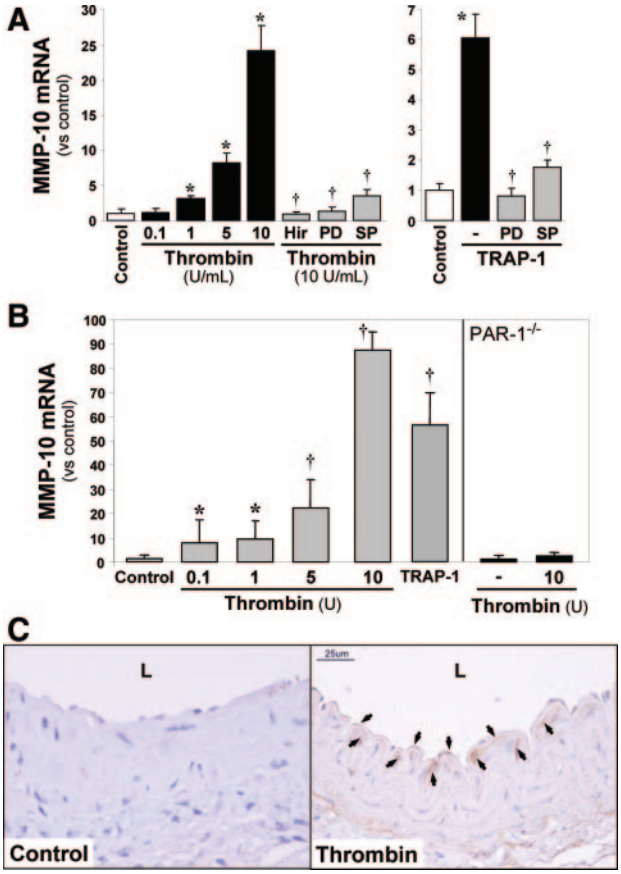

Figure 5. Thrombin induces MMP-10 mRNA levels in both mouse lung endothelial cells (MLECs) and mouse aorta. A, Thrombin increases MMP-10 mRNA levels in MLECs in a concentration-dependent manner. This effect was prevented by either hirudin, PD98059 (PD), and SP600125 (SP). The induction of MMP-10 expression by the PAR-1 agonist peptide (TRAP-1) and its prevention by PD and SP is also shown. Data are mean±SD (n=5). B, i.v. thrombin injection induces MMP-10 mRNA levels in the aorta of wild-type mice 6 hours after administration (shaded bars) but had no effect in PAR-1$^{-/-}$ mice (black bars). TRAP-1 (6 mg/kg) elicits a similar response to thrombin. Because after 40 cycles of amplification no expression was detected in vessels from control animals (Control), a reference value of 1 was assigned to these samples. $P<0.05$: * vs controls; † vs 0.1 and 1 U/mL thrombin. C, Immunohistochemistry for MMP-10 in murine aortic sections. MMP-10 immunoreactivity was clearly detected in endothelial cells and subendothelium (arrows) of aortic sections from mice receiving an i.v. injection of 5 U thrombin (right) but not in the arterial wall of control animals (left). L indicates vessel lumen.

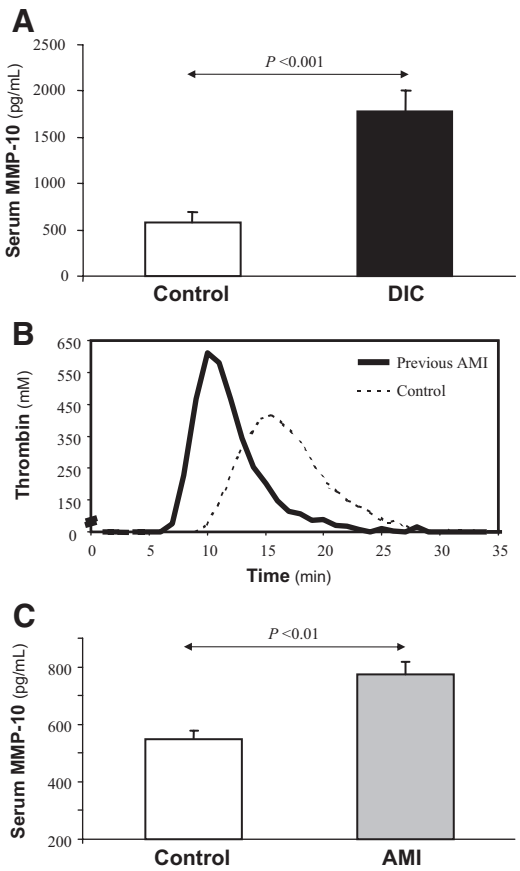

Figure 6. Circulating levels of MMP-10 are increased in patients with high systemic thrombin generation. A, Circulating MMP-10 levels (pg/mL, mean±SEM) in patients with disseminated intravascular coagulation (DIC) and controls after adjusting for age and sex. B, Representative thrombogram profiles as assessed by the thrombin generation test in control subjects and patients with previous acute myocardial infarction (AMI). C, Circulating MMP-10 levels (pg/mL, mean±SEM) in AMI patients and controls.

ical situations, a small series of patients with well characterized DIC-related sepsis as well as patients with previous AMI were analyzed and compared with age- and sex-matched healthy subjects. Patients with DIC (n=12) showed reduced platelet count (139.7±92.6 $\times 10^9$/L), decreased prothrombin (57.8±19.7%) and antithrombin activities (35.7±8.3%), and elevated levels of C-reactive protein (hsCRP) (16.6±13.0 mg/L) and plasma D-dimer (2044.3±647.8 ng/mL), indicating an acute inflammatory status associated with systemic thrombin generation and fibrin degradation, typical of overt DIC. MMP-10 levels were significantly higher than in controls (n=50) when crude analysis was performed (1682.6±1713.5 [median=1412.2, IQR=1574.3] versus 573.1±170.0 pg/mL [median=557.9, IQR=237.7], $P<0.05$). Further ANCOVA analysis, controlling for age and sex, revealed highly statistically significant differences in MMP-10 levels (1799.1±237.4 versus 542.7±116.0 pg/mL, $P<0.001$) between groups (Figure 6A). To assess indirectly whether plasmin, which is increased in DIC, could affect MMP-10 activity, in vitro experiments were carried out coincubating rMMP-10 and plasmin. Western blot analysis showed a single band of 45 kDa corresponding to fully activated MMP-10 after plasmin addition, which was corroborated in an MMP-10 activity assay (supplemental Figure I).

We also measured MMP-10 levels in a group of patients with previous AMI (n=60) and enhanced thrombin generation (thrombin peak, 615.5±26.1 nmol/L versus 497.4±37.2 nmol/L, $P<0.01$)[22] (Figure 6B). MMP-10 levels were increased in these patients as compared to controls (765.3±386.7 pg/mL versus 546.12±119.0 pg/mL, $P<0.01$), which remained statistically significant after adjustment for age and sex ($P<0.01$, Figure 6C). In patients with previous AMI, MMP-10 positively correlated with inflammatory marker hsCRP ($r=0.30$, $P<0.05$) as well as with the thrombin generation index ($r=0.31$, $P<0.05$). Finally, as a control of

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endothelial activation we show that circulating levels of von Willebrand factor (vWF), a marker of endothelial activation/injury that has been shown to be induced by thrombin, were increased in patients with either DIC or previous AMI (supplemental Table I).

Discussion

MMP-10 (stromelysin-2) degrades a broad range of ECM proteins and plays an important role in cancer progression, invasion, and metastasis, liver fibrogenesis, capillary tube regression, wound repair, and maintenance of vascular integrity.[23–26] Recently, our group reported that CRP induces MMP-10 in human ECs, linking inflammation and proteolysis at cellular level.[17] We also found that circulating MMP-10 levels correlate with systemic inflammatory markers and carotid intima-media thickness (IMT) in apparently healthy subjects.[17,18] Here we report that thrombin, via PAR-1, markedly enhances expression and secretion of MMP-10 in ECs, both in vitro and in vivo, and that circulating MMP-10 levels are increased in patients with high (DIC) and moderate (previous AMI) systemic thrombin generation.

Thrombin is a multifunctional serine protease involved not only in the cleavage of fibrinogen to fibrin and clot formation but also in activating a variety of cell types, including ECs.[27] Thrombin signaling in the endothelium results in a variety of phenotypic changes, including alterations in cell shape, permeability, migration, angiogenesis, and hemostasis.[28] Thrombin selectively enhanced MMP-10 mRNA and protein levels in human ECs, with no changes in TIMP-1 expression. The specificity of the thrombin effect was assessed in vitro by the following experiments: (1) addition of direct thrombin inhibitor hirudin completely blocked the increase of MMP-10 induced by thrombin, (2) the effect was mimicked by a synthetic PAR-1 agonist, and (3) MMP-10 induction was inhibited by a PAR-1 blocking antibody (ATAP2). Therefore, our results show that MMP-10 induction by thrombin requires PAR-1. Because activation of PAR-1 promotes proinflammatory, proangiogenic, and transforming pathways,[29,30] it might be suggested that MMP-10 could be a mediator of thrombin-induced proinflammatory responses. Finally, under our experimental conditions thrombin was also able to induce MMP-9 mRNA and protein levels (supplemental Figure II), in agreement with previous data reported in other cell types,[31] emphasizing the potential role of thrombin in vascular remodeling by modulating MMP expression.

Regarding the molecular mechanisms by which thrombin, via PAR-1, leads to endothelial MMP-10 upregulation, our results involve JNK and ERK1/2. The inhibition of thrombin-induced MMP-10 mRNA upregulation by actinomycin D indicates that thrombin increases MMP-10 transcription rate. Indeed, in transient transfection experiments thrombin induced the transcriptional activity of a MMP-10 promoter construct, an effect that was prevented by inhibitors of ERK1/2 pathway and JNK. It has been shown that thrombin is able to modulate gene expression in vascular cells by activating a number of transcription factors including AP-1 and CRE binding protein (CREB).[6] In silico analysis of the proximal region of the MMP-10 promoter reveals the presence of putative response elements for several transcription factors, including CREB and AP-1.[15] By mutation deletion analysis and site-directed mutagenesis we identified an AP-1 site that is critical in thrombin-induced MMP-10 expression. Furthermore, EMSA studies demonstrate that thrombin increases the binding of AP-1 to this site through the activation of ERK1/2 and JNK pathways. Therefore, our results agree with those demonstrating that PAR-1 stimulates ERK1/2 and JNK pathways and activates AP-1, a transcription factor involved in thrombin-induced gene expression associated to different cellular effects triggered by this serine protease, including proinflammatory, proangiogenic, and transforming pathways.[29,30]

We then used a mouse model to study MMP-10 expression in the vascular wall and find out whether thrombin was able to induce MMP-10 after intravenous injection. Thrombin upregulated MMP-10 mRNA levels in the aorta of wild-type mice and increased MMP-10 protein in the subendothelium. In vivo MMP-10 induction was reproduced by TRAP-1 administration but was not observed in PAR-1$^{-/-}$ mice treated with thrombin, further supporting the involvement of PAR-1 and suggesting that MMP-10 could be implicated in processes associated with thrombin generation and ECM degradation in vivo.[3] In this regard, to assess MMP-10 in a clinical setting, circulating MMP-10 levels were measured in patients with thrombin-related disorders, namely sepsis-related DIC and AMI, both characterized by increased thrombin generation and endothelial activation.[22] Interestingly, we found a 3-fold increase in MMP-10 levels in DIC patients, as compared to age- and sex-matched control subjects. The ability of MMPs to destroy isolated clotting factors and inhibitors suggests that in certain types of DIC direct proteolysis rather than consumption of clotting factors may be operational.[32,33] Other MMPs, such as MMP-3, the highest one homologous with MMP-10, have been shown to play a role in the regulation of cellular fibrinolysis.[34] In addition, MMP-10 was enhanced in patients with previous AMI and correlated with hsCRP as well as with the thrombin generation index, suggesting that MMP-10 may be a link between systemic inflammation and thrombin generation in atherothrombotic syndromes.[35]

In summary, the present findings confirm and extend our previous observations pointing out MMP-10 as a novel biomarker for cardiovascular disorders and show for the first time the molecular mechanisms that connect thrombin with MMP-10, which has recently emerged as a new player in inflammation and vascular integrity.[25,26,36] Further studies are needed to establish whether MMP-10 is critical for mediating the effects of thrombin on vascular remodeling, inflammation, or angiogenesis and to define the role of this MMP in the pathophysiology of thrombin-related disorders and plaque stability.

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Disclosures

None.

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Correction

In the article, "Matrix Metalloproteinase-10 Is Upregulated by Thrombin in Endothelial Cells and Increased in Patients With Enhanced Thrombin Generation" by Orbe et al, which appeared in the December 2009 issue of the journal (*Arterioscler Thromb Vasc Biol*. 2010;29:2109–2116; DOI: 10.1161/ATVBAHA.109.194589), P.S. and M.V. should have been included in the affiliation ". . .Center for Applied Medical Research, University of Navarra, Pamplona, Spain"

The online version has been corrected.

The publisher sincerely regrets the error.

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SUPPLEMENT MATERIAL. METHODS

Cell cultures

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords by digestion with collagenase A (Invitrogen), cultured as described and used between passages 3 and 5[1]. Human artery endothelial cells (HAEC, Cambrex BioScience) and bovine aortic endothelial cells (BAEC, Cambrex BioScience) were grown in endothelial growth medium (EGM-2, Cambrex) according to the manufacturer's instructions. Mouse lung endothelial cells (MLEC) were isolated from the lungs of C57BL/6 mice by collagenase digestion followed by selection with ICAM-2-coated magnetic beads. Briefly, lungs were excised and finely minced prior to collagenase A (0.1%) digestion at 37ºC for 1 hour under shaking. Digested tissue was homogeneized by a 14G syringe and homogenates were centrifuged at 200 ×*g* for 5 min. Then, the cell pellet was extensively washed and seeded in a gelatin-coated flask. After 6 days in culture, endothelial cells were recovered and selected using Dynabeads™ M-450 (Dynal Biotech) coupled to anti-ICAM-2 (Pharmingen). MLEC were cultured in DMEM:F12 supplemented with 20% FCS, 30 µg/mL endothelial cell growth supplement (ECGS, Sigma), 100 µg/mL heparin (Sigma) and antibiotics. Cell purity was assessed by FACS analysis using antibodies against ICAM-2 (Dako) and CD31 (Dako).

Cells were seeded in multi-well plates and at confluency were arrested overnight, afterwards were stimulated with human recombinant thrombin (0.1-10 U/ml; Enzyme Research Laboratory) or agonists in the presence or absence of inhibitors. Thrombin effects were inhibited using recombinant hirudin (2 µM, Refludan, Schering AG) or antibodies against PAR-1 (2.5 µg/mL, ATAP2, Santa Cruz)[2]. Control antibody (2.5 µg/mL, N-19, Santa Cruz) against an N-terminal epitope of PAR-1 was used.

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PAR-1 and PAR-2 agonist peptides, TRAP-1 (TFLLRNPNDK; 50 μM) and PAR2-AP (SLIGRL; 100 μM) respectively, generated as described[3], were also used. When needed, cells were pre-treated with inhibitors of signaling pathways for 30 minutes (unless otherwise stated). The inhibitors used were: 10 μM PD98059 (a mitogen-extracellular kinase-1/2 [MEK1/2] inhibitor, Calbiochem); 10 μM SB203580 (a p38 mitogen-activated protein kinase [MAPK] inhibitor, Calbiochem), 10 μM SP600125 (a c-Jun N-terminal kinase [JNK] inhibitor, BioSource), 1 μM wortmannin (a phosphatidylinositol-3 kinase [PIK3] inhibitor, Sigma), and 0.5 μg/mL nordihydroguaiaretic acid (NDGA, an inhibitor of activator protein-1 [AP-1], Sigma). Finally, actinomycin D (4 μM, Sigma) was used to determine whether thrombin effects were due to changes in transcription rate.

Real-Time PCR

RNA from cells was extracted using a semi-automated system for the isolation and purification of nucleic acids (Abi Prism 6100, Applied Biosystems) and reverse transcription was performed with 1 μg of total RNA, random primers and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen). Real-time PCR was performed on an ABI PRISM 7900 sequence detector (Applied Biosystems) using TaqMan™ gene expression assays-on-demand (Applied Biosystems) for MMP-10 (human: Hs00233987_m1; mouse: Mm00444630_m1), MMP-9 (Hs00957562_m1) and TIMP-1 (Hs00171558_m1). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 4326317E) or murine β-actin (4352341E) were used to normalize results.

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MMP-10 protein secretion

MMP-10 levels were assayed in conditioned medium and serum by ELISA (Quantikine, R&D systems). Cell culture and serum samples were diluted 1:5 and 1:2 respectively, following the manufacturer's instructions. MMP-9 levels were assayed in conditioned medium by ELISA (Quantikine, R&D systems). The assays recognize the zymogens and active forms. Inter and intra-assay coefficients of variation for the ELISA were <6%.

Western blot analysis

HUVEC were stimulated with thrombin or TRAP-1 in the presence or in the absence of hirudin and ATAP2 and protein extracts were analyzed by western blot as described[4]. Briefly, cell cultures were washed twice with wash buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 100 mM NaF, 10 mM NaPPi, 10 mM EDTA, 2 mM Na_3VO_4) and lysed with lysis buffer (wash buffer containing 1 mM PMSF, 5 µM leupeptin, 1% triton X-100). Protein concentration was measured by the bicinchoninic acid (BCA) protein assay™ (Pierce). Proteins were separated by SDS-PAGE (12.5% acrylamide:bisacrylamide 37.5:1) and electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories). Blots were incubated with antibodies against human phosphorylated ERK1/2 (p-ERK1/2; Cell Signaling Technology), total human ERK1/2 (t-ERK1/2; Cell Signaling Technology), human phosphorylated JNK-1 (p-JNK-1; Cell Signaling Technology) or total human JNK-1 (Santa Cruz Biotechnology, Inc). Bound antibody was detected using the appropriate horseradish peroxidase-conjugated antibody (Dako) and a chemiluminescent detection system (Supersignal West Dura™, Pierce).

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Construct of MMP-10 promoter

A 2.0 kb fragment corresponding to nucleotides –1934 to +66 of the human MMP-10 promoter was generated by PCR and cloned into pGL-3 vector (Promega). The primers used were: 5´-GGATCCGAATTCGAGCTCTCGTATGGCAGCACAGTAGG-3' (forward) and 5'-GGATCCGAATTCCTCGAGACAGCACAAGGAATGCAAGA-3' (reverse) (*Sac*I and *Xho*I restriction enzyme cloning sites are underlined). A series of promoter deletions were generated using the reverse primer indicated above and the following forward primers: p-456, 5'-CTCAAGAGCTCAAGAAGTGAAATATTTG-3' (*SacI* site is underlined); p-350, 5'-GCGTTGGTACCTGCTTATATTTAAGTTT-3' (*KpnI* site is underlined); p-251, 5-TGTCAGCTAGCTTTAAGTAAATACAGAGACGTGAA-3' (*NheI* site is underlined); p-47, 5'-TTGGTGAGCTCAGCATTGTCTCTGTATA-3' (*SacI* site is underlined). The putative AP-1 site present in MMP-10 promoter was mutated using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) and the following oligonucleotide: (-88)-CAGACTTAAAAAACACATGCAgGAAcCATACTGTTGGTGATCTCAGC-(-42) (changes introduced are indicated in lower case letters). The new sequence was analyzed by different promoter analysis softwares to confirm that no new response elements were generated.

Transient transfection and luciferase reporter assay

HUVEC or BAEC were seeded on six-well plates (180,000 cells/well) and were transfected with the luciferase reporter plasmids as described[5]. Briefly, the transient transfection assays were performed with 1 µg/well of plasmid and 4 µL of Lipofectamine™ Reagent (Invitrogen, CA, USA). The complexes DNA/liposome were

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added to the cells for 4 h, which were washed and incubated with complete medium for 8 h. Afterwards, cells were incubated overnight in 5% FCS medium and stimulated with thrombin in the presence or absence of inhibitors. Luciferase activity was measured in cell lysates using the Luciferase assay kit (Promega) and a luminometer (Orion I, Berthold detection systems) according to the manufacturer. Results were normalized by β-galactosidase activity using the Enzyme Assay System™ (Promega).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from HUVEC stimulated with thrombin in the presence or in the absence of different pathway inhibitors were obtained using the NucBuster Protein Extraction Kit (Novagen) according to the manufacturer's recommendations. Proteins were quantified by the BCA protein assay™ (Pierce) and nuclear extracts were aliquoted and stored at -80ºC until used. The double-stranded DNA probe corresponding to the putative AP-1 binding site in MMP-10 promoter (in bold) and a mutated AP-1 probe (underlined bases) were generated from the annealing of the following single-stranded complementary oligonucleotides: AP-1-wild-type (WT) upper strand: 5'-CACATGCA**TGAATCA**TACTGTTG-3' and AP-1-WT lower strand: 5'-CAACAGTA**TGATTCA**TGCATGTG-3'; AP-1-mutated (mut) upper strand: 5'-CACATGCA**<u>G</u>GAA<u>C</u>CA**TACTGTTG-3' and AP-1-mut lower strand: 5'-CAACAGTA**TG<u>G</u>TTC<u>C</u>**TGCATGTG-3'. Probes were end-labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase as described [6]. EMSA assays were performed using the Novagen's EMSA Accessory kit. Briefly, nuclear proteins (5 µg) were incubated for 15 min on ice with 0.01 U poly-d [I-C] and 1 µl sonicated Salmon Sperm DNA in 20 mM HEPES (pH 8), 0.2 mM EDTA, 100 mM KCl, 20% glycerol, and 0,5 mM DTT. Approximately 50000 cpm of the appropriate ^{32}P end-labeled probe was then added to

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the reaction mixture and incubated for 30 min on ice in a final volume of 20 µl. For supershift assays, an anti-c-jun antibody (4 µg; sc-45x; Abcam) was added to the reactions. Protein-DNA complexes were resolved by electrophoresis at 4ºC on 5% polyacrylamide gels in 0.5X TBE. Gels were dried and were subjected to autoradiography using a Storage Phosphor Screen (GE Healthcare). Shifted bands were detected using a Typhoon 9400 scanner (GE Healthcare).

Induction of MMP-10 by thrombin in mouse aorta: *in vivo* assay

C57Bl/6 mice (6 months old male, 30–35 g, n = 18) were procured from the colony at CIMA animal facilities, originating from breeders purchased from Charles River, and fed a normal chow diet. The research was performed in accordance with the European Community guidelines for ethical animal care and use of laboratory animals (Directive 86/609), and was approved by the University of Navarra Animal Research Review Committee. In order to assess the role of thrombin on MMP-10 *in vivo*, mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), and thrombin (0.1-10 U, Enzyme Research Laboratory) was infused intravenously (tail injection). PAR-1-deficient mice in C57Bl/6 background were treated in a similar fashion either with saline (n=4) or 10 U thrombin (n=4). Another set of C57Bl/6 mice (n=3) received TRAP-1 (6 mg/kg body weight) intravenously. Animals were sacrificed by CO_2 inhalation 6 h after treatment and aortic arteries were obtained. MMP-10 mRNA levels in aorta were quantified by real-time PCR. Immunohistochemical analysis for MMP-10 was also performed (polyclonal antibody P-16, Santa Cruz Biotechnology) in aorta from mice treated with 5 U thrombin (n = 3) or saline (n = 3) and sacrificed 24 h after i.v. injection[7]. Tissue sections were deparaffinized, treated with 10% H_2O_2, immersed in 10 mM boiling citrate buffer for 10

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min, and incubated in 10% normal horse serum for 30 min at room temperature. Samples were incubated with a polyclonal antibody against MMP-10 (P-16; Santa Cruz Biotechnology), further incubated with biotinylated anti-goat antibodies (GE Healthcare), and then followed by the ABC kit (Dako) with diaminobenzidine (Dako).

Circulating levels of vWF and MMP-10 in patients

Blood samples from patients with sepsis-induced disseminated intravascular coagulation (DIC, n = 12) were collected into 0.13 mol/L sodium citrate (9/1, v/v) kept on ice for no longer than 2 h and the platelet-poor plasma, obtained by centrifugation at 2000×g for 20 min at 4°C, was stored at −70°C until used. Serum fraction of blood was also obtained after blood clotting (1 h), and further centrifuged (1,800 x g for 10 min) at room temperature. The diagnosis of overt DIC was made according to the criteria established by Scientific Subcommittee of DIC of the International Society on Thrombosis and Hemostasis (ISTH) if the score was ≥7[8]. Specific clinical conditions (bleeding symptoms, organ dysfunction), and results of coagulation profile (platelet count, prothrombin time, fibrinogen, fibrin degradation products/D-dimer) and were assessed in plasma samples. Levels of von Willebrand factor (vWF) were determined in citrated plasma samples by ELISA following the manufacturers' instructions (vWF; Asserachrom, Diagnostica Stago, Gennevilliers, France), whereas MMP-10 levels were measured in serum samples by ELISA (R&D Systems). A group of 50 healthy subjects (mean age 58.2 ± 8.6 years) with no clinical history of thrombosis and normal coagulation screening profile, matched for age and sex (4 or 5 matches by case), were used as controls. In addition, we studied 60 consecutive patients (90 % men, mean age 56 years) with previous acute myocardial infarction (previous AMI) and no history of malignant or autoimmune disease, coagulation disorders or presence of chronic renal

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insufficiency (creatinine level ≥ 124 μmol/L), who were under observation at the Cardiology Department of the University Hospital (Pamplona, Spain). Samples were taken between 3 and 11 months after the initial diagnosis (mean 6 months). All patients were treated with aspirin indefinitely and none of them was receiving oral anticoagulant treatment. A high proportion were also taking statins and antihypertensive therapy (>80%) and had increased thrombin generation[9]. As controls, a similarly aged group (n=100, 86% men, mean age 59 years) with no history of coronary artery disease (CAD), normal physical examination and resting electrocardiogram was enrolled. Informed consent was obtained from all patients according to the Declaration of Helsinki.

MMP-10 activity assay

Human recombinant MMP-10 (R&D systems) was incubated for 1 h at 37 ºC with or without 20 nM plasmin (Enzyme Research) and MMP-10 activity was measured in 96-well microplates by using the fluorogenic peptide (MCA-Arg-Pro-Lys-Pro-Val-Glu-Nval-Trp-Arg-Lys-[DNP]-NH2, R&D systems). Fluorescence (320 nm excitation and 405 nm emission) was kinetically recorded on a spectrophotofluorometer (SpectraMAX GeminiXS, Molecular Devices). Plasmin alone was included as control.

Statistical analysis

Continuous variables were expressed as mean ± SD (unless otherwise stated). Differences between controls and stimulated endothelial cultures were assessed by Mann-Whitney-U test. Data from mice were analyzed by one-factor ANOVA to compare differences among groups. Differences in the baseline characteristics between DIC and control groups were evaluated by the Student's t-test. Analysis of co-variance

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(ANCOVA) was used to examine the association of MMP-10 and thrombin related disorders, with age and sex as covariables. Relationships between inflammatory marker levels, thrombin generation and MMP-10 in AMI patients were examined by Pearson correlation analysis, applying Bonferroni's correction for multiple comparisons. Differences in functional studies between samples with recombinant MMP-10 (rMMP-10) and controls were analyzed by the Mann-Whitney-U test. Statistical significance was established as $p < 0.05$ (SPSS version 11.0).

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