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Matrix Metalloproteinase-10 Effectively Reduces Infarct Size in Experimental Stroke by Enhancing Fibrinolysis via a Thrombin-Activatable Fibrinolysis Inhibitor–Mediated Mechanism

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Background—The fibrinolytic and matrix metalloproteinase (MMP) systems cooperate in thrombus dissolution and extracellular matrix proteolysis. The plasminogen/plasmin system activates MMPs, and some MMPs have been involved in the dissolution of fibrin by targeting fibrin(ogen) directly or by collaborating with plasmin. MMP-10 has been implicated in inflammatory/thrombotic processes and vascular integrity, but whether MMP-10 could have a profibrinolytic effect and represent a promising thrombolytic agent is unknown.

Methods and Results—The effect of MMP-10 on fibrinolysis was studied in vitro and in vivo, in MMP-10–null mice ($Mmp10^{-/-}$), with the use of 2 different murine models of arterial thrombosis: laser-induced carotid injury and ischemic stroke. In vitro, we showed that MMP-10 was capable of enhancing tissue plasminogen activator–induced fibrinolysis via a thrombin-activatable fibrinolysis inhibitor inactivation–mediated mechanism. In vivo, delayed fibrinolysis observed after photochemical carotid injury in $Mmp10^{-/-}$ mice was reversed by active recombinant human MMP-10. In a thrombin-induced stroke model, the reperfusion and the infarct size in sham or tissue plasminogen activator–treated animals were severely impaired in $Mmp10^{-/-}$ mice. In this model, administration of active MMP-10 to wild-type animals significantly reduced blood reperfusion time and infarct size to the same extent as tissue plasminogen activator and was associated with shorter bleeding time and no intracranial hemorrhage. This effect was not observed in thrombin-activatable fibrinolysis inhibitor–deficient mice, suggesting thrombin-activatable fibrinolysis inhibitor inactivation as one of the mechanisms involved in the MMP-10 profibrinolytic effect.

Conclusions—A novel profibrinolytic role for MMP-10 in experimental ischemic stroke is described, opening new pathways for innovative fibrinolytic strategies in arterial thrombosis. (***Circulation***. 2011;124:2909-2919.)

Key Words: fibrinolysis ■ metalloproteinases ■ stroke ■ TAFI ■ thrombolysis

S troke is a leading cause of death and disability in developed countries.[1] Permanent brain damage after a stroke induces death of brain cells and causes irreversible neurological damage. The majority of strokes are ischemic, caused by a thrombotic or embolic blood clot that leads to suddenly decrease blood flow in a major cerebral artery, commonly the middle cerebral artery (MCA). Prompt treatment with thrombolytic drugs to remove the clot can restore blood flow before major brain damage occurs and improves recovery after stroke[2]; however, these drugs can also cause serious bleeding in the brain, which can be fatal. Recombinant tissue plasminogen activator (rtPA), a main activator of

fibrinolytic system, is the only drug licensed for use in highly selected patients within 3 to 4.5 hours of stroke.[3]

Clinical Perspective on p 2919

The fibrinolytic and matrix metalloproteinase (MMP) systems cooperate in thrombus dissolution.[4] Besides MMP activation by the plasminogen/plasmin system, several studies have suggested that multiple MMPs may participate in the dissolution of fibrin deposits by targeting fibrin(ogen).[5,6]

In this study, we focused on the role of MMP-10 (stromelysin-2) in thrombosis and fibrinolysis. MMP-10 is capable of degrading various components of the extracellular

matrix and activating other MMPs and has emerged as a new player in inflammation and vascular integrity.[7–9] Our group has shown that vascular MMP-10 expression can be induced by inflammatory stimuli,[10] and that serum MMP-10 is associated with carotid intimal-medial thickness, a surrogate marker of subclinical atherosclerosis.[8] Moreover, we have described that thrombin, via specific receptors (eg, protease-activated receptor-1), markedly enhances endothelial MMP-10 expression in vitro and in vivo. In addition, increased circulating levels of MMP-10 are observed in patients with high or moderate thrombin generation.[11,12] These data suggest that MMP-10 is involved in the homeostasis of vascular system; however, a role for MMP-10 in clot formation and lysis has not been assessed.

In vitro studies presented here demonstrate that MMP-10 enhances tPA fibrinolytic activity through inactivation of thrombin-activatable fibrinolysis inhibitor (TAFI) without affecting thrombus formation. We further tested the hypothesis that MMP-10 functions as a profibrinolytic agent in vivo using 2 mouse models of arterial thrombosis: (1) laser-induced carotid thrombosis and (2) thrombin-induced stroke. Experiments were performed in MMP-10 knockout ($Mmp10^{-/-}$), TAFI knockout ($TAFI^{-/-}$), and wild-type (WT) mice to gain insights into the possible role of MMP-10 on thrombus dissolution. We found that the absence of active MMP-10 limits fibrinolysis, shifting the hemostatic equilibrium toward hypofibrinolysis. We also show that MMP-10 treatment improves the time of blood reperfusion and reduces infarct size in a stroke model, involving MMP-10–mediated TAFI inactivation. We propose that MMP-10 can be a new profibrinolytic agent, thus representing an innovative therapeutic approach to arterial thrombosis.

Methods
A detailed description is presented in the online-only Data Supplement.

Expression and Purification of Recombinant Human MMP-10
The full-length human proMMP-10 was amplified with the following primers: 5′-ATGATGCATCTTGCATTCCTT-3′ (forward) and 5′-GCAATGTAACCAGCTGTTACT-3′ (reverse) with the use of the KOD Hot Start DNA polymerase enzyme (Novagen). The insert was cloned into the pcDNA 3.1-V5-His (Invitrogen) expression vector, between the *Bst*XI and the *Eco*RV sites in frame with a c-myc epitope and 6 histidines (His) tag to express the human proMMP-10 fused with this tag at the C-terminal end. The vector was grown and used to transfect HEK293 before purification of protein from cell supernatant.

In Vitro Studies
Effect of MMP-10 on Rate of Clot Formation and Lysis
The effect of MMP-10 on clot formation and lysis was studied by monitoring changes in turbidity in normal and TAFI-deficient plasma (Affinity Biologicals Inc, Kordia) with the use of a microplate reader (Fluostar Optima, BMG Labtech).

MMP-10 Activity Assay
MMP-10 activity was measured with the use of a fluorogenic peptide for stromelysins (ES002, Fluorogenic Peptide Substrate II, R&D Systems).

Fibrin Plate Assay
In a first set of experiments, macroscopic fibrin plate assay was used to examine the effects of MMP-10 on in vitro fibrinolysis. The area of the lysis zones was determined to assess fibrinolytic activity. In a second set of experiments, gold-labeled fibrin clots[13] were used to measure fibrin lysis-front velocity of tPA alone or combined with recombinant human MMP-10 (rhMMP-10).

Fibrinolytic Substrates of MMP-10
To identify potential substrates for MMP-10, several proteins of the fibrinolytic system (plasminogen, fibrinogen, TAFI, and urokinase plasminogen activator) were incubated with rhMMP-10 (1:10 molar ratio) at 37°C for 24 hours in assay buffer (100 mmol/L NaCl, 5 mmol/L CaCl$_2$, 20 mmol/L Tris-HCl, pH 8). Digestion products were analyzed by Tris-tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with GelCode Blue stain reagent (Pierce).

Active TAFI (TAFIa) and thrombin activity were determined by chromogenic assays, and functional fibrinogen was assayed by turbidimetric analysis.

Determination of TAFI Cleavage Site
MMP-10–cleaved TAFI was determined by proteomic analysis after sodium dodecyl sulfate polyacrylamide gel electrophoresis and trypsin digestion. Resulting peptides were separated by reverse-phase capillary chromatography for tandem mass spectrometry analysis.

MMP-10 Cytotoxicity
To assess cytotoxicity, we measured lactate dehydrogenase release (Roche Applied Science) from human umbilical vein endothelial cells after treating them with different concentrations of MMP-10 (5 pmol/L to 200 nmol/L).

In Vivo Studies
Animals
$Mmp10^{-/-}$ mice generated by removing MMP-10 catalytic domain (exons 3–5) and crossbred for 10 generations with C57BL/6 mice were generated at the Center for Lung Biology,[9] University of Washington, Seattle, and bred in the Center for Applied Medical Research (CIMA) animal facilities. $TAFI^{-/-}$ mice were generated[14] and kindly provided by Dr J.C.M. Meijers (Experimental Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands) and bred in CIMA animal facilities. Experiments were performed in accordance with European Communities Council Directives (86/609/European Economic Community) guidelines for the care and use of laboratory animals and were approved by the University of Navarra Animal Research Review Committee.

Tail Bleeding Assay
Time to cessation of bleeding was measured up to 30 minutes in 2-month-old WT C57Bl6 (n=15) and $Mmp10^{-/-}$ (n=15) mice by removing the tail tip.

Murine Carotid Artery Laser Thrombosis Model
Laser-induced arterial injury was performed in 8- to 10-week-old WT and $Mmp10^{-/-}$ male mice. Anesthetized animals (50/10 mg/kg of ketamine/xylazine) were injected with rose bengal (100 mg/kg), and the left carotid artery was exposed to 1.5-mW green light laser (540 nm; Melles Griot Inc). Blood flow was recorded for 2 hours with a pulse Doppler flow probe (Transonic, Sidney, Australia).

Mouse Model of In Situ Thromboembolic Stroke and Reperfusion
Animals (aged 4 months) were anesthetized with 2.5% isoflurane. A catheter was inserted into the tail vein to allow the intravenous administration of saline (200 μL), tPA (10 mg/kg), or active rhMMP-10 (2 nmol/L, ≈6.5 μg/kg). Thrombin clot formation and assessment of infarct size were performed by thrombin injection in the MCA, as described previously.[15]
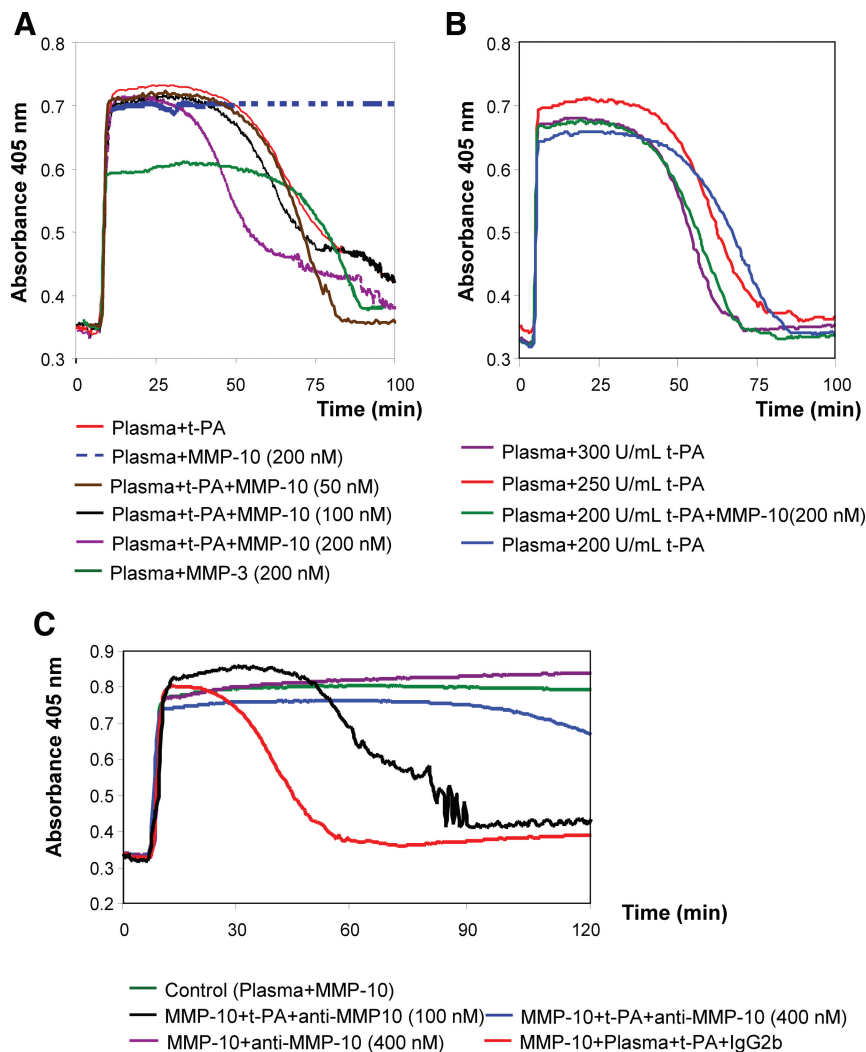
Figure 1. Role of matrix metalloproteinase (MMP)-10 in in vitro fibrin clot formation and lysis. **A**, Kinetics of plasma clot formation in presence or absence of tissue plasminogen activator (tPA) (300 U/mL) and different MMP-10 concentrations (50–200 nmol/L) in a turbidimetric assay. MMP-10 alone did not promote clot lysis but increased the tPA-induced clot lysis in a dose-dependent manner. However, MMP-3 (200 nmol/L) reduced fibrin clot formation. **B**, Turbidimetric study for different concentrations of tPA (200–300 U/mL) shows a dose-dependent reduction in the clot lysis time and the adjuvant effect of MMP-10 (200 nmol/L) in combination with tPA (200 U/mL). **C**, The addition of monoclonal antibody anti-MMP-10 (monoclonal antibody 100 and 400 nmol/L) to recalcified plasma containing MMP-10 (200 nmol/L) blocked the tPA-induced (300 U/mL) clot lysis compared with similar concentrations of isotype control antibody (IgG2b) (3 independent experiments, performed in triplicate).

Statistical Analysis

Data from mice were analyzed by the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U* test with the Bonferroni correction. Continuous variables were expressed as mean±SD and skewed variables as median and interquartile range. Differences in the in vitro experiments between presence and absence of MMP-10 were evaluated by Mann-Whitney *U* test. Statistical significance was established as $P<0.05$ (SPSS version 15.0).

Results

MMP-10 Is Required for tPA-Induced Clot Lysis In Vitro

To elucidate whether MMP-10 plays a role in thrombin-dependent clot formation and lysis, we performed a turbidimetric analysis of recalcified plasma supplemented with tPA in the presence or absence of MMP-10. As shown in Figure 1A, MMP-10 alone had no effect on clot formation or lysis, whereas in combination with tPA, it increased the rate of clot lysis in a dose-dependent manner compared with tPA alone. Accordingly, clot lysis time was significantly reduced from 72.3±17.7 minutes (tPA alone) to 61.2±13.2 minutes (tPA+100 nmol/L MMP-10; $P<0.05$) and to 52.3±12.7 minutes (tPA+200 nmol/L MMP-10; $P<0.01$). In contrast, an equimolar amount of MMP-3 (stromelysin-1) did not

shorten clot lysis time but significantly decreased turbidity, indicating impaired clot formation likely by fibrinogen degradation. Interestingly, clot lysis time achieved with two thirds of the tPA dose (200 U/mL) plus MMP-10 (200 nmol/L) was comparable to that achieved with the full tPA dose (300 U/mL) (Figure 1B). These data suggest that MMP-10 in combination with a lower dose of tPA has the same fibrinolytic effect as a full dose of tPA.

The net effect of MMP-10 on the rate of clot lysis was further assessed by using MMP-10 and 2-fold molar excess of an antibody (MAB9101) that completely abolished MMP-10 activity (Figure I in the online-only Data Supplement). In the presence of this antibody, tPA-induced clot lysis was markedly slowed compared with an isotype control antibody (Figure 1C). Together, these data suggest that MMP-10 significantly enhances the efficiency of tPA-mediated fibrin lysis rate.

The in vitro profibrinolytic effect of MMP-10 was also assessed on fibrin plates. As shown in Figure 2A, no lysis was induced by MMP-10 alone, whereas the combination of tPA and MMP-10 increased lytic areas compared with tPA alone (210.5±27.3%), and this effect was again prevented with anti-MMP-10 antibody. By scanning confocal microscopy,
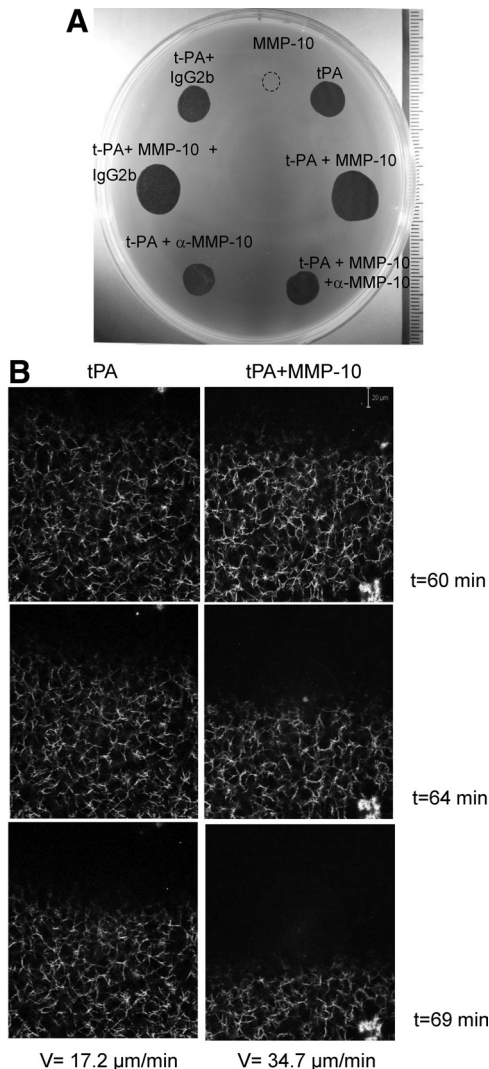
Figure 2. Fibrinolytic activity of matrix metalloproteinase (MMP)-10 on fibrin plates. **A**, Representative fibrin plate (n=5) in presence of MMP-10 alone (200 nmol/L) or combined with tissue plasminogen activator (tPA) (1 U/mL). Samples containing both MMP-10 and tPA showed increased lytic areas, whereas inhibition of MMP-10 activity with a monoclonal antibody (α-MMP10, 400 nmol/L) reduced tPA-induced lysis compared with control antibody (IgG2b, 400 nmol/L). **B**, Series of micrographs showing the dynamic lysis of fibrin with tPA (1 U/mL, left) and tPA plus MMP-10 (200 nmol/L, right). Lysis-front motion, visualized as a straight and sharp line in clot, was scanned at different times and shows higher velocity in tPA combined with MMP-10 compared with tPA alone.

lysis-front velocity induced by the combination of tPA and MMP-10 was 2-fold faster than that induced by tPA alone, indicating a higher fibrinolysis rate (Figure 2B).

Fibrinolytic Substrates for MMP-10

Active MMP-10 was incubated with purified recombinant proteins involved in fibrinolysis (ratio enzyme/substrate 1/10) to explore the mechanisms responsible for its profibrinolytic effect. As shown in Figure 3A, MMP-10 cleaved TAFI, resulting in a lower-molecular-mass fragment of ≈55 kDa. This fragment was trypsin-digested and analyzed by mass spectrometry, showing C-terminal cleavage of TAFI and the

absence of Glu[363], 1 of the 3 catalytic amino acids (data not shown). The specificity of the proteolytic cleavage was confirmed with the use of the MMP inhibitor GM6001.

We further analyzed the functionality of the cleaved substrate by measuring its capacity to generate TAFIa. As shown in Figure 3B, MMP-10 inhibited TAFI activation by thrombin/thrombomodulin to the level of carboxipeptidase potato inhibitor (CPI), a specific TAFI inhibitor. In contrast, active MMP-3 did not affect TAFI activation. Interestingly, once TAFI is activated by the thrombin/thrombomodulin complex, MMP-10 cannot reduce its activity. Both proteins CPI and MMP-10 inhibited TAFIa generation with similar IC_{50} of 207.5 and 163.5 nmol/L, respectively (Figure 3C). The inhibitory effect was also confirmed when plasmin was used as an activator of TAFI (Figure IIA in the online-only Data Supplement).

The proteolysis of TAFI (10–2000 nmol/L) by MMP-10, in the presence of thrombin/thrombomodulin as activator, caused a marked reduction in V_{max} (0.09 versus 0.22 A_{405nm}/min; $P<0.001$) with similar K_m (815 versus 865 nmol/L) (Figure 3D). The catalytic efficiency (K_{cat}/K_m) of TAFI cleaved by MMP-10 was ≈2.5-fold lower, consistent with results obtained on tricine gels. To exclude that MMP-10 cleaves and inactivates thrombin or thrombomodulin, we incubated active MMP-10 with thrombin or thrombomodulin for 24 hours at 37°C. MMP-10 neither cleaved thrombin or thrombomodulin on tricine gels nor modified thrombin activity assessed by chromogenic substrate (Figure IIB and IIC in the online-only Data Supplement).

Clot lysis experiments were performed in the presence of CPI to analyze whether the profibrinolytic effect of MMP-10 in plasma samples was due to regulation of the TAFI pathway. Figure 3E shows that the inhibition of TAFIa by CPI significantly shortened the clot lysis time, similar to the effect obtained with MMP-10. This profibrinolytic effect was not detected when MMP-10 was added to TAFI-depleted plasma, indicating that MMP-10 enhanced fibrinolysis through a TAFI-mediated mechanism (Figure 3F).

In regard to other fibrinolytic substrates, MMP-10 did not cleave plasminogen, urokinase plasminogen activator, and plasmin (data not shown); however, it partially digested fibrinogen after 24 hours by cleaving part of the native fibrinogen α chain but without affecting its ability to form a fibrin clot (Figure IIIA and IIIC in the online-only Data Supplement). In contrast, MMP-3 clearly cleaved plasminogen and fibrinogen, preventing clot formation (Figure IIIB and IIIC in the online-only Data Supplement). These data suggest that MMP-3 cleaves these substrates more efficiently than MMP-10.

MMP-10–Related Cytotoxicity

The effect of MMP-10 on endothelial cell viability was analyzed with an in vitro cytotoxicity assay to assess a range of MMP-10 concentrations for in vivo experiments. As shown in Figure IV in the online-only Data Supplement, MMP-10 concentrations from 5 pmol/L to 10 nmol/L did not modify lactate dehydrogenase levels, whereas concentrations >10 nmol/L induced significant cell mortality. Therefore, we chose a 2-nmol/L dose (≈6.5 μg/kg) for in vivo experiments as a therapeutic dose of MMP-10 without triggering cytotoxicity.
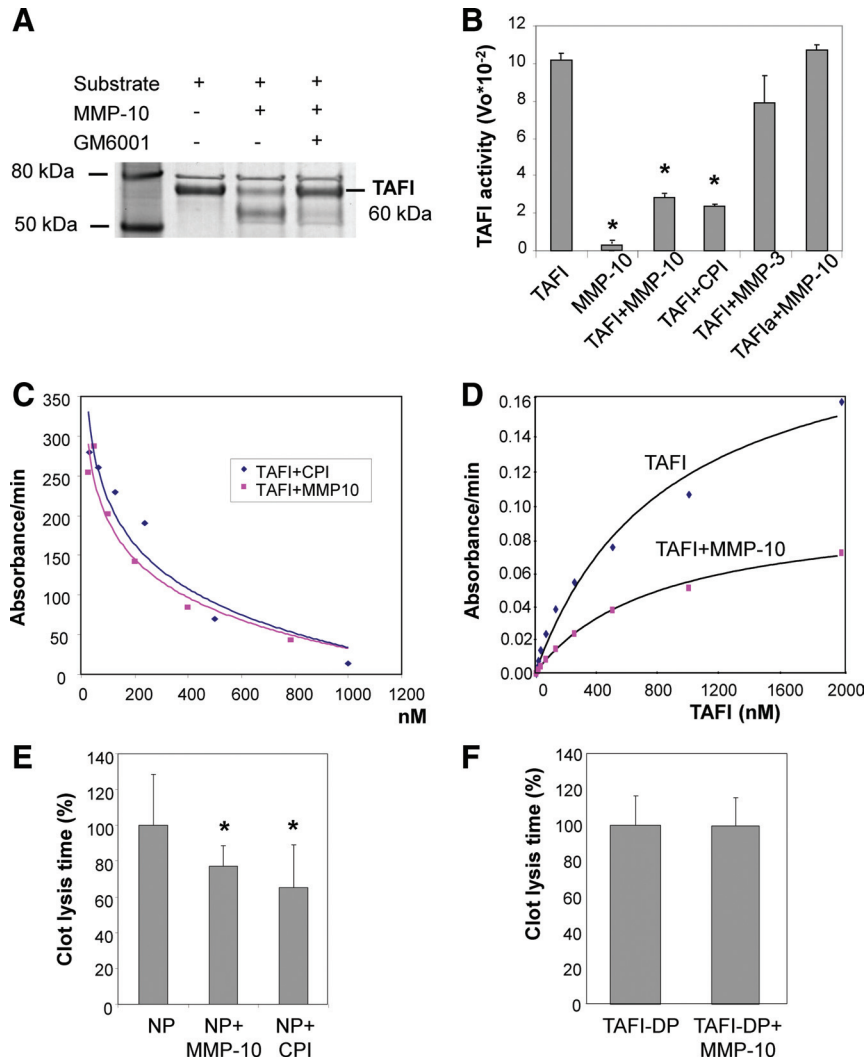
Figure 3. Effect of matrix metalloproteinase (MMP)-10 on thrombin-activatable fibrinolysis inhibitor (TAFI). **A,** Tricine gel showing TAFI incubated for 24 hours at 37°C alone, with active MMP-10, or with active MMP-10 plus the MMP inhibitor GM6001. MMP-10 proteolytically cleaved TAFI, resulting in a smaller fragment ($\approx$55 kDa) that was prevented by GM6001. **B,** TAFI activity assayed with a chromogenic substrate (3 independent experiments performed in duplicate). Bars represent the initial velocity (Vo) of TAFI (75 nmol/L) activation with thrombin/thrombomodulin (Pefablock activator) incubated for 2 hours at 37°C in presence or absence of active MMP-10 (200 nmol/L) and controls: active MMP-10 (200 nmol/L) and TAFI with carboxipeptidase potato inhibitor (CPI) (1 μmol/L). Active TAFI (TAFIa) was unaffected by MMP-10. *$P<0.01$. **C,** Inhibition of TAFI activity (30 nmol/L TAFI) by different concentrations of CPI (25–1000 nmol/L) and MMP-10 (30–800 nmol/L). Curves fitting the concentration of CPI and MMP-10 and rate of TAFI activation allow calculation of the concentrations required for 50% reduction of TAFI activation (IC$_{50}$; 3 independent experiments performed in duplicate). **D,** TAFI (0–2 μmol/L), in the presence or absence of active MMP-10 (200 nmol/L), was activated by thrombin-thrombomodulin. The initial lysis rates (Vo) of a TAFI-sensitive chromogenic substrate in both conditions were calculated. Each point represents the mean value of 3 independent experiments. **E,** Turbidimetric analysis of normal plasma (NP) with TAFI inhibitor CPI (1 μmol/L) shows reduction in clot lysis time similar to that with tPA (300 U/mL) combined with MMP-10 (200 nmol/L). *$P<0.05$ vs NP. **F,** The profibrinolytic effect of MMP-10 (200 nmol/L) was no longer detected when the analysis was performed with TAFI-depleted plasma (TAFI-DP) (3 independent experiments performed in triplicate).

Effect of MMP-10 on Bleeding Time

Bleeding time was determined in *Mmp10*$^{-/-}$ mice to assess whether this MMP affects hemostasis in vivo. Tail tip transection bleeding time of *Mmp10*$^{-/-}$ mice was significantly shorter (38.5 [29.5] versus 70.5 [76.5] seconds), and the blood lost was significantly reduced (11.3 [1.4] versus 31.9 [21.7] μL) than in WT mice. Intravenous injection of active rhMMP-10 (6.5 μg/kg) in *Mmp10*$^{-/-}$ mice increased the bleeding time and the blood lost (99.6 [29.5] seconds and 15.4 [4.9] μL) without affecting platelet count (1032$\times$10^3 versus 1157$\times$10^3 cells per microliter) (Figure 4A and 4B). These results suggest that MMP-10 plays a role in hemostasis in vivo.

Effect of MMP-10 on Fibrinolysis

Furthermore, we looked for differences in plasma fibrinolytic activity between WT and *Mmp10*$^{-/-}$ mice, analyzing euglobulin fractionated plasma in fibrin plates. Although fibrin lytic areas were evident in both groups, a significant reduction in fibrinolytic activity was observed in *Mmp10*$^{-/-}$ mice. Addition of rhMMP-10 (200 nmol/L) increased euglobulin fibrinolytic activity, especially in *Mmp10*$^{-/-}$ animals, confirming an abnormal hypofibrinolytic state in the absence of MMP-10 that can be restored by addition of rhMMP-10 (Figure 4C). Moreover, turbidimetric analysis of mouse euglobulin fractionated plasma showed that
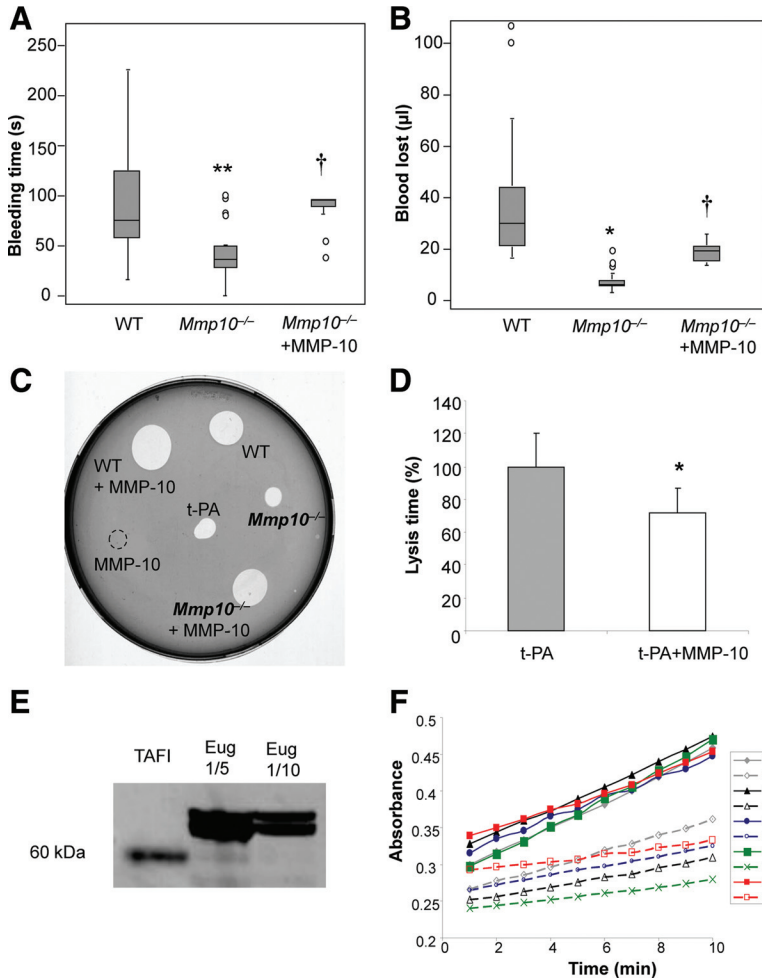
Figure 4. Matrix metalloproteinase-10–null (*Mmp10*$^{-/-}$) mice exhibit shortened bleeding time. **A**, Boxplot of the bleeding time in wild-type (WT) (n=15) and *Mmp10*$^{-/-}$ (n=15) mice injected with saline or active recombinant human MMP-10 (6.5 μg/kg; n=6) (*Mmp10*$^{-/-}$+MMP-10). **B**, Boxplot of blood lost (μL) in the same groups of animals showing a significant reduction in the *Mmp10*$^{-/-}$ group. Both parameters were increased after administration of active recombinant human MMP-10 (6.5 μg/kg). *$P<0.05$, **$P<0.01$ vs WT; †$P<0.05$ vs *Mmp10*$^{-/-}$. **C**, Fibrinolytic activity of plasma euglobulins from WT and *Mmp10*$^{-/-}$ mice on fibrin plates (n=3). Euglobulins from WT animals showed increased lytic areas compared with *Mmp10*$^{-/-}$. Addition of active recombinant human MMP-10 (200 nmol/L) to euglobulins increased the fibrin lysis, although the effect was higher in euglobulins from *Mmp10*$^{-/-}$ mice. tPA (1 U/mL) was used as positive control. **D**, MMP-10 shortens the lysis time of euglobulin in turbidimetric analysis with mouse plasma in the presence tPA (n=3 independent experiments in triplicate). **E**, Western blot showing thrombin-activatable fibrinolysis inhibitor (TAFI) expression in human plasma euglobulins (Eug) (diluted 1/5 and 1/10) and recombinant human TAFI as control. **F**, Representative graph of TAFI activation in human plasma (n=5; P1 to P5) and their corresponding euglobulin fractions (E1 to E5). Thrombin/thrombomodulin can induce active TAFI generation in plasma-fractionated euglobulins, although this activity is much higher in whole plasma.

MMP-10 in combination with tPA reduced clot lysis time (Figure 4D).

We performed Western blot and TAFI activity experiments to confirm that TAFI is present in euglobulin fraction (Figure 4E). As shown in Figure 4F, TAFI present in euglobulin fraction can be activated by thrombin/thrombomodulin. Mean values of TAFIa in 10 different plasmas and their corresponding euglobulins showed a significant reduction of TAFIa in euglobulins (46.2±5.0% versus plasma).

Effect of MMP-10 on Carotid Thrombosis
We conducted several in vivo experiments to assess more directly the effect of MMP-10 on arterial thrombosis by using a classic laser-induced carotid model. As shown in Figure 5A, the formation of an occlusive thrombus occurred faster in *Mmp10*$^{-/-}$ mice than in WT mice (27.5 [22.2] versus 60.5 [24.8] min), and thrombus lysis was significantly delayed (4.6 [3.1] versus 2.7 [1.4]) (Figure 5B). Interestingly, intravenous administration of active rhMMP-10 (6.5 μg/mL) to *Mmp10*$^{-/-}$ mice reversed both end points (ie, occlusion, 46.6 [24.7] min, $P<0.05$; reperfusion, 2.9 [1.9] min, $P<0.05$).

Effect of MMP-10 on In Situ Thromboembolic Stroke Model
An experimental stroke model was induced in WT and *Mmp10*$^{-/-}$ mice by in situ thrombin injection (1 U/μL) in the

MCA. All animals showed infarct areas restricted to the cortex without differences in the mean lesion volume between WT and *Mmp10*$^{-/-}$ mice (12.8 [12.1] versus 10.2 [12.1] mm³). Average reduction in cerebral blood flow was similar in both genotypes (WT: 81.8±11.5%; *Mmp10*$^{-/-}$: 74.8±14.6%). However, spontaneous reperfusion was less frequent in *Mmp10*$^{-/-}$ animals (28.6% versus 68.7%; $P<0.05$). Additional experiments with rtPA-based thrombolytic therapy (10 mg/kg) showed a decreased infarct volume in WT mice compared with control animals reperfused with saline (45% reduction; $P<0.05$). In contrast, *Mmp10*$^{-/-}$ mice treated with tPA showed no significant changes in infarct area compared with null mice receiving saline (Figure 6A), together with a significant delay in reperfusion compared with WT (38.6±4.7 versus 21.9±4.7 minutes; $P<0.05$) (Figure 6B).

To analyze the thrombolytic effect of MMP-10 infusion, we performed the stroke model and increased the thrombin concentration to generate a more stable clot. After injection of 2 U/μL thrombin, only 3 of 10 WT animals showed spontaneous reperfusion after saline infusion. Under these conditions, 30 WT animals were divided into 3 groups (n=10) that received saline, tPA (10 mg/kg), or active rhMMP-10 (6.5 μg/kg) through the tail vein. As expected, treatment with tPA significantly shortened reperfusion time (25±3 versus 52±5 minutes; $P<0.01$). Interestingly, MMP-10 administration also significantly reduced reperfusion time (29.6±5.3 min-
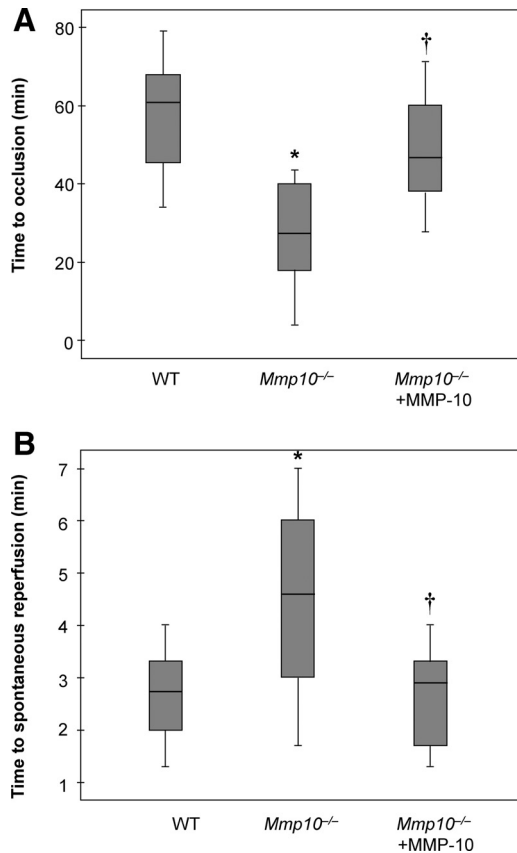
Figure 5. Boxplot of matrix metalloproteinase (MMP)-10 effect on laser-induced carotid thrombosis model. **A**, Time to occlusion was significantly decreased in $Mmp10^{-/-}$ compared with wild-type (WT) mice (n=10; *$P<0.05$). **B**, Time to lysis was clearly increased in $Mmp10^{-/-}$ compared with WT mice (n=10; *$P<0.05$). Both parameters were reversed after administration of active recombinant human MMP-10 (6.5 μg/kg) (n=6; †$P<0.05$ vs $Mmp10^{-/-}$).

utes; $P<0.01$ versus saline) without differences with tPA (Figure 6C). Moreover, infarct volume in both tPA and MMP-10 groups was significantly reduced (55% and 60%, respectively; $P<0.05$) compared with control. These data indicate that MMP-10 shows efficacy similar to that of tPA in terms of shortening reperfusion time and reducing infarct size (Figure 6D).

To analyze whether MMP-10 in vivo effects are mediated by TAFI, an experimental stroke model was performed in $TAFI^{-/-}$ mice. Thirty $TAFI^{-/-}$ mice (aged 2–3 months) were divided into 2 groups that received saline or active rhMMP-10 (6.5 μg/kg) through the tail vein. Interestingly, treatment with MMP-10 did not modify the reperfusion time (45.2 ± 3.2 versus 50.5 ± 6.1 minutes; Figure 6C) compared with saline. Moreover, infarct volume in both groups was also similar (7.1 [16.3] versus 11.5 [9.1] mm³; Figure 6D).

Effects of MMP-10 on Hemorrhage and Bleeding Time

To determine whether MMP-10 confers advantages over tPA, we analyzed hemorrhage by Perls' staining in cryostat brain sections and also determined bleeding time in WT animals injected with tPA (1 and 10 mg/kg), active rhMMP-10 (6.5

μg/kg), or saline. No evidence of intracranial hemorrhage was observed under these conditions in any of the animals tested compared with positive controls performed with collagenase type VII (data not shown). However, WT animals (n=5) receiving an experimental dose of tPA (10 mg/kg) exceeded the maximum bleeding time allowed (30 minutes), whereas those injected with the experimental dose of MMP-10 (6.5 μg/kg; n=6) showed a much shorter bleeding time (10 minutes; $P<0.05$), although this was still longer than in controls. When the dose of tPA was reduced to therapeutic levels in humans (1 mg/kg), 3 of 5 animals still exceeded 30 minutes, and 2 animals stopped bleeding at 25 and 28 minutes (Figure 6E).

Effect of MMP-10 on Circulating TAFI Activity In Vivo

To assess the in vivo effect of MMP-10 on TAFI, plasma samples were taken from $Mmp10^{-/-}$ and WT animals before and 24 hours after experimental stroke. As shown in Figure 7A, $Mmp10^{-/-}$ mice exhibited higher basal TAFI activity than WT animals ($30.9\pm6.8\%$ versus $21.7\pm8.8\%$; $P<0.01$). TAFI activity was also higher after experimental stroke in $Mmp10^{-/-}$ mice, suggesting that MMP-10 impairs activation of TAFI in vivo.

Moreover, WT animals (n=10) were injected with active rhMMP-10 or saline, and plasma samples were collected at different times (0–30 minutes) to measure TAFI activity. Figure 7B shows a significant reduction in TAFIa generation as early as 15 minutes, reaching 60% reduction 30 minutes after MMP-10 injection ($P<0.05$), suggesting in vivo lower activatable TAFI in the presence of MMP-10.

Discussion

We demonstrate herein an unexpected role for MMP-10 in promoting the dissolution of fibrin thrombi in a model of experimental stroke. Our data demonstrate that MMP-10 enhanced tPA-induced fibrinolysis in vitro and in vivo by impairing TAFI activation. Our results also support a possible physiological role for MMP-10 in hemostasis and the clinical use of MMP-10 as an innovative approach to enhance fibrinolysis.

Pharmacological thrombolysis consists of the dissolution of a blood clot by intravenous infusion of plasminogen activators to activate the fibrinolytic system. The clinical benefits of thrombolytic therapy in patients with acute myocardial infarction and ischemic stroke are well documented. However, available thrombolytic agents have significant shortcomings, including the need for large therapeutic doses, limited fibrin specificity, and, most importantly, a significant associated bleeding tendency. These issues are particularly relevant in ischemic stroke, in which tPA is the only available agent for clinical use with limitations such as the narrow time window, neurotoxicity, and bleeding complications, which all restrict its clinical potential.[16]

The plasminogen activator/plasmin axis is often assumed to serve as the sole determinant of clot lysis, given the efficiency of plasmin for fibrin degradation. However, several studies have suggested that multiple MMPs may participate in the dissolution of fibrin deposits by targeting fibrin(ogen).[5,6,17]
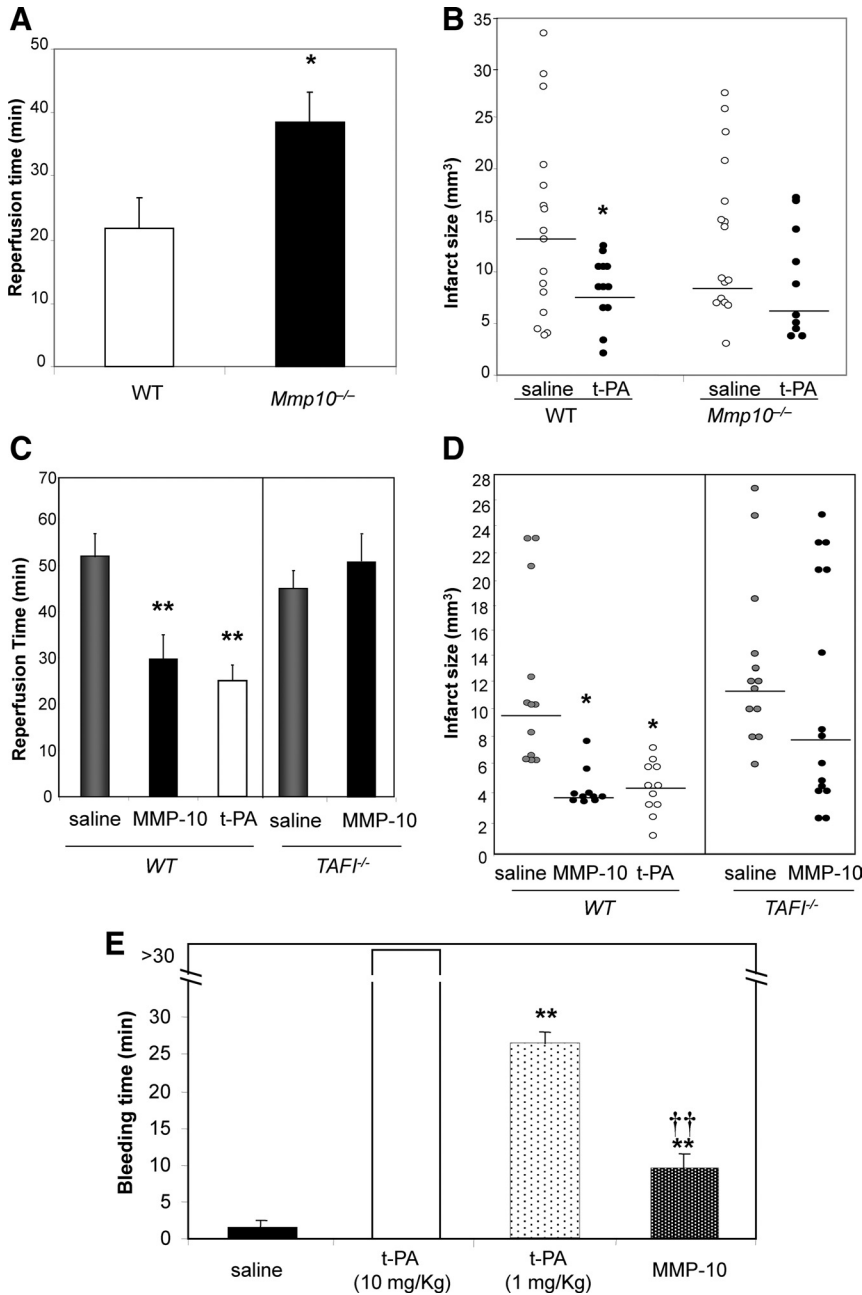
Figure 6. Effect of matrix metalloproteinase (MMP)-10 on ischemic stroke model. **A**, Reperfusion time in wild-type (WT) and $Mmp10^{-/-}$ mice after tissue plasminogen activator (tPA) treatment, measured by laser Doppler sonography. The reperfusion time was significantly delayed in $Mmp10^{-/-}$ mice compared with WT (*$P<0.05$). **B**, Dot plot of the infarct size in WT and $Mmp10^{-/-}$ mice after treatment with saline (n=16) and tPA (n=12). **C**, MMP-10 and tPA treatments improved reperfusion time in WT mice compared with controls (**$P<0.01$) but not in thrombin-activatable fibrinolysis inhibitor (TAFI)$^{-/-}$ mice (n=15). **D**, Similarly, MMP-10 and tPA treatments reduced the infarct size in WT mice (n=10; *$P<0.05$) but not in TAFI$^{-/-}$ mice (n=15). **E**, Bleeding time of WT animals after saline (n=5), tPA (10 and 1 mg/kg; n=5), or active MMP-10 (6.5 μg/kg; n=6) infusion. **$P<0.01$ vs saline; ††$P<0.01$ vs tPA.

We hypothesized that MMP-10 could behave as a profibrinolytic agent by acting on fibrinolytic proteins. Experiments performed to validate this hypothesis demonstrated that MMP-10 was capable of (1) enhancing tPA-induced fibrinolysis by preventing TAFI activation in vitro, (2) protecting against laser-induced carotid artery thrombus formation, and (3) reducing infarct size in a thrombin-induced murine model of stroke.

Profibrinolytic Effect of MMP-10 In Vitro

Results of in vitro experiments showed that MMP-10 favored tPA-induced clot lysis, allowing a one-third reduction of tPA dose while maintaining the full-dose fibrinolytic efficiency. Experiments with fibrin plates and confocal microscopy also revealed that addition of MMP-10 to tPA after clot formation was still efficient for increasing the velocity and lysis of fibrin clot. This suggests that the combination of both proteins in thrombolytic therapy may improve fibrinolysis, allowing reduction of the tPA dose and thereby reducing its side effects. The specificity of MMP-10 profibrinolytic effect was assessed and compared with that of MMP-3 because they share a high degree of homology (82%) but are differently regulated and distributed.[18,19] Unlike MMP10, MMP-3 did not affect clot lysis but rather inhibited thrombus formation. Our results confirm previous data showing a very rapid degradation of fibrinogen with MMP-2 and MMP-3[5] and suggest that MMP-3 can digest fibrinogen, whereas MMP-10 displays a limited capacity to cleave this substrate, allowing clot formation and subsequent lysis.
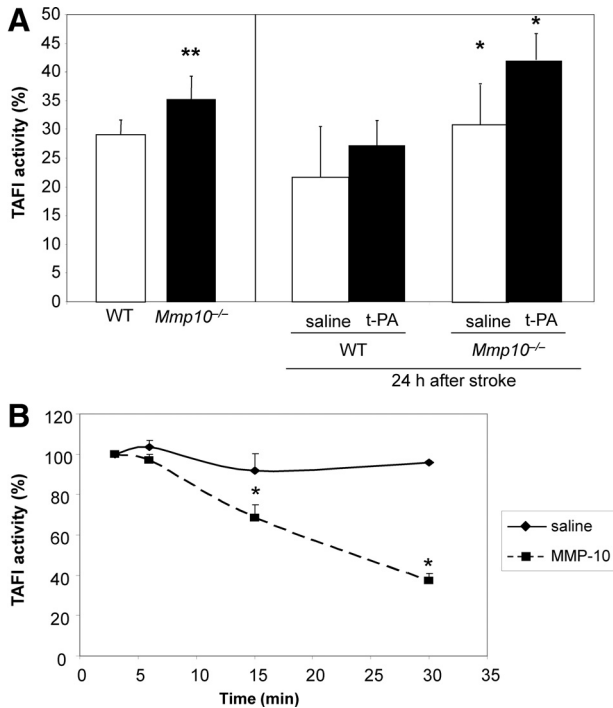
Figure 7. In vivo circulating thrombin-activatable fibrinolysis inhibitor (TAFI) activity. **A**, Plasma TAFI activation measured with chromogenic substrate (Pefakit) in matrix metalloproteinase-10–null ($Mmp10^{-/-}$) mice was significantly higher than in wild-type (WT) mice at baseline and 24 hours after stroke in saline or tissue plasminogen activator (tPA)–treated groups. *$P<0.05$ vs WT; n=10. **B**, Fast inhibition of active TAFI generation after treatment with active recombinant human matrix metalloproteinase (MMP)-10 (6.5 μg/kg) in WT mice (n=10). *$P<0.05$ vs saline.

MMP-10 Substrates in the Fibrinolytic System

Previous data on fibrinolytic substrates for MMPs indicate that MMP-3, membrane type 1 MMP, MMP-7, and MMP-11 hydrolyze fibrin(ogen). Furthermore, MMP-9 has been reported to degrade fibrin, and MMP-3 specifically hydrolyzes urokinase plasminogen activator, α_2-antiplasmin, plasminogen, and plasminogen activator inhibitor-1.[4] We show herein that the profibrinolytic activity of MMP-10 involves TAFI degradation that prevents its activation by plasmin and thrombin/thrombomodulin complexes and thereby the inhibition of fibrinolysis.[20] The role of TAFI in the regulation of fibrinolysis has been shown previously in several in vitro and in vivo studies.[21,22] Activation of TAFI during clot lysis results in removal of fibrin C-terminal lysines and consequently reduces plasmin production, leading to a several-fold prolongation of clot lysis time. Moreover, in vivo studies have demonstrated that inhibition of TAFI activity with CPI enhanced tPA-induced thrombolysis as well as endogenous fibrinolysis.[23,24] TAFI is proteolytically activated to TAFIa by cleavage at Arg-92, which results in release of the activation peptide from the catalytic domain. MMP-10 proteolyses TAFI but not its active form TAFIa, suggesting that proteolytic activity of MMP-10 might only be possible for a specific conformational structure of TAFI. Several studies have demonstrated that TAFIa undergoes conformational rearrangements[25] that could hide the cleavage site for MMP-

10. In a similar manner, thrombin cleaves and inactivates prourokinase but not active urokinase. Furthermore, cleavage of prourokinase by thrombin prevents its activation by plasmin.[26]

Profibrinolytic Effect of MMP-10 In Vivo

The role of MMP-10 in vivo was first demonstrated by a significant 2-fold shortening of the mouse tail bleeding time in $Mmp10^{-/-}$ mice and its restoration after intravenous injection of rhMMP-10, indicating a role in normal hemostasis. A profibrinolytic effect of MMP-10 on arterial thrombus formation was further confirmed in vivo with the use of 2 different experimental models: carotid thrombosis and ischemic stroke. Faster carotid thrombus formation and delayed clot lysis in $Mmp10^{-/-}$ mice were restored by intravenous MMP-10 administration, suggesting that there is limited fibrinolysis in the absence of active MMP-10, shifting the hemostatic equilibrium toward hypofibrinolysis.

The profibrinolytic effect of MMP-10 was also evident in a thrombin-induced murine stroke model, in which intravenous administration of MMP-10 showed a thrombolytic efficacy similar to that of tPA in terms of infarct size and reperfusion time without significant brain hemorrhage. It has been demonstrated that after ischemic brain injury, there is an increase in endogenous tPA activity within the ischemic tissue.[27] Therefore, it is also possible that MMP-10 is acting in conjunction with endogenous tPA to exert its profibrinolytic effect in vivo. The relevance of MMP-10 as endogenous profibrinolytic agent has been further demonstrated by the lower reperfusion rate observed after clot formation in the MCA of $Mmp10^{-/-}$ mice.

We demonstrate here that MMP-10 inhibits TAFI activation in vitro, and, consequently, higher TAFI activity observed in knockout mice suggests that TAFI inhibition by MMP-10 might represent a dominant mechanism controlling thrombus resolution in vivo. Thrombolysis with tPA failed to reduce lesion volume and reperfusion time in $Mmp10^{-/-}$ mice. Our results agree with previous studies describing that higher levels of TAFI require higher tPA concentration to obtain the same clot lysis time.[28] In addition, TAFI levels are inversely associated with recanalization rates and worse outcome in ischemic stroke patients treated with tPA.[22] Finally, in our stroke model, no differences in reperfusion time or in lesion volume were observed in TAFI$^{-/-}$ mice after MMP-10 administration. Although our experimental design did not allow for direct comparison between WT and TAFI$^{-/-}$ mice, available data from Kraft et al[29] show that TAFI deficiency does not protect from acute ischemic stroke in an experimental model of transient MCA occlusion. It is evident from previous studies in which TAFI inhibitors were used, that TAFI plays a regulatory role in tPA-induced thrombolysis,[30] however, its effect on endogenous fibrinolysis may be more subtle. Moreover, studies on TAFI$^{-/-}$ mice backcrossing to a heterozygous plasminogen background indicate that TAFI can modulate the in vivo functions of plasmin(ogen) in fibrinolysis but that redundancy in the regulation of the fibrinolytic system may mask the phenotype of TAFI$^{-/-}$. All of these data suggest that the profibrinolytic effect of MMP-10 is mediated by TAFI-dependent mecha-

nisms in vivo, although other pathways activated by MMP-10 (eg, the activation of other MMPs) cannot be excluded.

Currently, there are no data regarding the use of MMPs in thrombolytic therapy. At most, several MMPs have been reported to be expressed in brain,[31] but only MMP-2, MMP-9, and MMP-3 have been implicated in tPA side effects.[32] Decreased cerebral hemorrhage and brain injury after treatment with tPA were described in experimental embolic stroke models involving MMP knockout animals and MMP inhibitors.[33,34] However, controversy still exists because stroke outcomes are made worse with broad-acting MMP inhibitors.[35] Indeed, MMP activity is required for microvascular recanalization through embolus extravasation.[36]

Effect of MMP-10 on Bleeding

Interestingly, significant improvement in arterial reperfusion and reduction in infarct size in MMP-10–treated mice was achieved with no bleeding complications, particularly intracranial hemorrhage. In contrast, supratherapeutic and therapeutic doses of tPA showed off-scale bleeding times that were not observed in mice treated with MMP-10, suggesting a significant advantage over tPA. Whether MMP-10 could also act as an adjuvant of the fibrinolytic effect of tPA, allowing for a reduced dose administration, requires additional investigation.

Our findings are limited to in vitro and in vivo experimental models. Stroke models in animals allow testing of a mechanistic hypothesis but do not mimic the human condition entirely. Another limitation of this study includes the management of healthy animals, in which comorbidities, sex, and aging are clinically relevant factors affecting the stroke outcome.

In conclusion, our study demonstrates that MMP-10 is a new profibrinolytic agent in vivo and in vitro and reveals that TAFI inactivation is at least one of the mechanisms involved. We have demonstrated that MMP-10 markedly reduces infarct size in a murine model of stroke, indicating that, either alone or as fibrinolytic adjuvant, it may be a powerful agent for the treatment of ischemic cerebrovascular events in humans.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The majority of strokes, the third leading cause of death worldwide, are ischemic in nature. It is estimated that 1 of every 16 deaths is due to stroke, ranking as the No. 1 cause of adult disability with an estimated cost of $74 billion in 2010. With an aging population, these numbers are likely to rise. Intravenous fibrinolysis with recombinant tissue plasminogen activator (tPA) remains the only Food and Drug Administration–approved treatment for stroke patients presenting within 3 hours after onset, which can be extended to 4.5 hours in selected patients. Recombinant tPA, although effective in reducing disability, does not improve mortality. Indeed, most stroke centers use recombinant tPA in only ≈5% of stroke patients. The major adverse effect after recombinant tPA administration is intracerebral hemorrhage, seen in ≈6% to 7% of cases and thus remaining an important clinical issue. Because of the potential side effects of recombinant tPA, efforts are being made to improve recanalization after stroke by using new fibrinolytics or mechanical revascularization therapies. Fibrinolysis and matrix metalloproteinase–mediated proteolysis act in concert to degrade the occlusive fibrin clot. We have demonstrated that matrix metalloproteinase-10 reduces infarct size and favors fibrinolysis through a thrombin-activatable fibrinolysis inhibitor–mediated mechanism in an experimental stroke model in mice, with much lower effect on bleeding than tPA. This novel thrombolytic strategy can open new perspectives for the treatment of stroke, likely reducing the impact of this enormous economic and social burden provided that it can be translated to humans.

SUPPLEMENTAL MATERIAL

Supplemental methods

Expression of recombinant human pro-MMP-10

The full-length human proMMP-10 was amplified with the following primers: 5´-ATGATGCATCTTGCATTCCTT-3´ (forward) and 5´-GCAATGTAACCAGCTGTTACT-3´ (reverse), by using the *KOD Hot Start DNA polymerase enzyme* (Novagen). The insert was cloned into the pcDNA 3.1-V5-His (Invitrogen) expression vector, between the BstXI and the EcoRV sites in frame with a c-myc epitope and six histidines (His) tag in order to express the human proMMP-10 fused with this tag at the C-terminal end. TOP 10 cells were transformed with the plasmid and its isolation from cell cultures was carried out with the *Qiagen Plasmid Mini Kit* (Qiagen) and then subjected to sequencing. The purified plasmid was used to transfect the HEK293 *(Human Embryonic Kidney fibroblasts)* cells with 4 μg of pcDNA 3.1-V5-His using Lipofectamine (Invitrogen). The transfected cells were grown in the presence of DMEM containing 2 mg/ml of the selective antibiotic geneticin (Calbiochem) to select the more efficient clones.

Once the selection was performed, supernatants were screened for the production of proenzyme hMMP-10 by ELISA (R&D Systems) and Western blot with an anti-MMP-10 antibody directed to the catalytic domain of the protein (MAB9101, R&D Systems).

Purification of recombinant human MMP-10 (rhMMP-10)

The cell culture was expanded to grow in T-175 cm^2 flasks (Costar; "Cell Bind") and cells were cultured in low foetal bovine serum medium (Advanced DMEM; GIBCO). Every 48 hours the supernatants were collected and maintained on ice during filtration, and concentration (Vivaflow 200; cut off 30 kDa). Finally they were stored at -20ºC until the purification process.

The ice-cold concentrated supernatant was applied to 1 ml Co-MAC column (Novagen). After washing the non-specifically retained proteins with binding buffer (20 mM Tris-HCl, 5 mM Imidazole and 500 mM NaCl; pH=7.9), bound proteins were eluted with elution buffer (20 mM Tris-HCl, 500 mM Imidazole and 500 mM NaCl; pH=7.9). Eluted fractions were pooled and subjected to an immunoaffinity chromatography in a HiTrap NHS-activated HP column (Amersham Biosciences, USA) coupled with an anti-His antibody (R&D systems). Non-specific proteins were removed from the column by washing with binding buffer (20 mM Tris-HCl and 500 mM NaCl; pH= 7.5) and the pro-rhMMP-10 was eluted with Glycine 0,1 M pH=2.9. The eluted fractions were immediately neutralized with Tris-HCl, pH=8. Peak collected fractions were concentrated, dialysed against TNB buffer (50 mM Tris-HCl pH =7.5, 150 mM NaCl and 0,05% Brij 35) and stored at -80 ºC. The purity of the sample was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Gelcode Blue Stain Reagent (Thermo Scientific, USA). All the purification steps were carried out using a FPLC System (GE Healthcare) at room temperature but maintaining the sample on ice and measuring the absorbance at 280 nm. Each batch of purified proMMP-10 was tested for: i) protein concentration with both an ELISA assay (R&D Systems) and Nanodrop ND-1000 measurement (Thermo Scientific, USA) applying an extinction coefficient of $A_{280}=1,497$ $M^{-1}cm^{-1}$ based on the primary sequence of rhMMP-10 and with; ii) protein activation with a kinetic assay using fluorogenic peptide for stromelysins (ES002, Fluorogenic Peptide Substrate II, R&D systems) and active rhMMP-10 for the standard curve; iii) protein purity and activatability by western blot with anti-MMP10 antibody (R&D Systems) as previously mentioned; iv) fibrinolytic activity by fibrin plate assay.

Effect of MMP-10 on the rate of clot formation and lysis

The effect of MMP-10 during clot formation and lysis was studied by monitoring the change in turbidity in normal and TAFI deficient plasma (Affinity Biologicals Inc., Kordia) using a microplate reader (Fluostar Optima, BMG Labtech). $CaCl_2$ (10 mM final concentration) was added to citrated plasma diluted 1:2 in HEPES buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 6 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 0.1% BSA, pH=7.5). After mixing, samples were incubated at 37ºC, and turbidity at 405 nm measured for 2 h every 30 seconds. To study the effect of MMP-10 on fibrin formation, rMMP-10 (10-200 nM, R&D System), previously activated by 24 h incubation with 10 mM $CaCl_2$ at 37ºC (Supplemental Figure 1A), was added to the plasma mixture. The rate of clot lysis was further examined by simultaneous addition of 300 U/mL of tissue plasminogen activator (t-PA, Actilyse, Boehringer Ingelheim), and rhMMP-10 (10-200 nM), in presence or absence of either MAB9101 (at the concentrations that block MMP-10 activity) or control antibody.

Additional experiments were also performed with recombinant MMP-3 (200 nM, R&D Systems) previously activated with 1 mM p-aminophenylmercuric acetate (APMA, Sigma). Clot lysis time was calculated as the time from initiation of clot formation to the time at which maximal absorbance falls to 50%.

MMP-10 Activity Assay

MMP-10 activity was measured in 96-well microplates, precoated with a human anti-MMP-10 specific monoclonal antibody (Clone 110343), by using a fluorogenic peptide for stromelysins (ES002, Fluorogenic Peptide Substrate II, R&D Systems). Fluorescence (320 nm excitation and 405 nm emission) was kinetically recorded on a spectrophotofluorometer (SpectraMAX GeminiXS, Molecular Devices). Plasma

samples were incubated with or without rhMMP-10 (10 nM) and different concentrations of MAB9101 to determine the concentration that blocks MMP-10 activity, established at 2-fold molar excess of the antibody.

Fibrin plate Assay

A solution containing 9.5 µM of fibrinogen from human plasma (Sigma) was laid over dishes and clotted by the addition of 20 U human thrombin (Enzyme Research Laboratory). Plates were incubated at 37ºC for 1 h and kept at 4ºC until used. 30 µl of recombinant t-PA (1 U/mL) and rhMMP-10 (200 nM) alone or plus t-PA and plasma euglobulins were pipetted onto the surface of the fibrin plate (n=5), and further incubated at 37ºC for 18 h. Plasma euglobulins were obtained as follows: pooled plasma (200 µL) was diluted 1:10 with distilled water and acidified to pH 5.9 with acetic acid. After 30 min on ice, the precipitate was centrifuged and redissolved in 200 µL HEPES buffer (pH 7.5). The lytic areas were determined as a measurement of fibrinolytic activity.

In a second set of experiments, gold-labelled fibrin clots were loaded with 1 U/mL of t-PA alone or with active rhMMP-10 (200 nM). After 1h of incubation at 37 ºC in a moist chamber, the edge of the thrombus was visualized with a confocal microscope (Zeiss LSM 510 Meta) set up in the reflection mode. At the fibrin network level, scanning was performed at 40x magnification (Zeiss 40x Plan-Neofluar) every minute, and the fibrin lysis-front velocity of t-PA alone or combined with MMP-10 was recorded (Zeiss Axiovert 200M).

TAFI and thrombin activity and fibrinogen functionality

TAFI activity was determined by chromogenic assay (Pefakit, Pentapharma). Briefly, TAFI alone (0 nM-2 µM) or combined with rhMMP-10 (200 nM) were incubated with

activator (Pefakit, thrombin/thrombomodulin) or plasmin (15 nM, Enzyme Research Laboratories) for 3 min at 37ºC. Start reagent (Substrate, Tris, Mannit) was added and substrate conversion was measured for 10 min at 22ºC by monitoring absorbance at 405 nm. Calibrator and control plasmas were used for validation of the assay (Pefakit TAFI calibrator and controls, Pentapharma).

Thrombin activity was measured with a chromogenic substrate (S2238, Chromogenix) on thrombin (0,1 U/mL) and thrombin with MMP-10 (ratio 1/10) incubated at 37 ºC for 24 h.

Fibrinogen functionality was assayed on turbidimetric analysis using fibrinogen (2 mg/mL, Sigma) incubated alone or with rhMMP-10 or rhMMP-3 (ratio enzyme/substrate 1/10), in the presence of thrombin (5U/mL, Enzyme Research Laboratories) and $CaCl_2$ (25 mM). Clot formation was studied by monitoring the change in turbidity at 405 nm.

Determination of TAFI cleavage site

For proteomic analysis, TAFI alone or combined with rhMMP-10 and incubated 24 h at 37 ºC were run on a 12 % SDS-PAGE, cut from the gel, digested with trypsin, and the resulting peptides were separated by reverse phase capilar chromatography using a capLC (Waters) connected through a nanospray picotip probe to a Q TOF micr (Waters) for MS/MS analysis. MS and MS/MS data were processed with MassLynx and protein matching and peptide assignment was done with ProteinLynx Global Server and Phenyx using UniprotKB database.

MMP-10 cytotoxicity

R6

To assess cytotoxicity, we measured LDH released (Roche Applied Science) using a scanning multiwell spectrophotometer enzyme-linked immunosorbent assay (ELISA) reader at 492 nm. Human umbilical vein endothelial cells (HUVECs) were seeded in a 96-well multiplate (10^4 cells/well) and allowed to attach for 24 hours. After this period, cells were placed in endothelial serum free media containing different concentrations of MMP-10 (5 pM-200 nM). After 24 hours, an ELISA reader was used to measure LDH in the supernatant.

Tail bleeding assay

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<u>**Supplemental Figures**</u>

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SUPPLEMENTAL MATERIAL

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Supplemental methods

Expression of recombinant human pro-MMP-10

The full-length human proMMP-10 was amplified with the following primers: 5´-ATGATGCATCTTGCATTCCTT-3´ (forward) and 5´-GCAATGTAACCAGCTGTTACT-3´ (reverse), by using the *KOD Hot Start DNA polymerase enzyme* (Novagen). The insert was cloned into the pcDNA 3.1-V5-His (Invitrogen) expression vector, between the BstXI and the EcoRV sites in frame with a c-myc epitope and six histidines (His) tag in order to express the human proMMP-10 fused with this tag at the C-terminal end. TOP 10 cells were transformed with the plasmid and its isolation from cell cultures was carried out with the *Qiagen Plasmid Mini Kit* (Qiagen) and then subjected to sequencing. The purified plasmid was used to transfect the HEK293 *(Human Embryonic Kidney fibroblasts)* cells with 4 µg of pcDNA 3.1-V5-His using Lipofectamine (Invitrogen). The transfected cells were grown in the presence of DMEM containing 2 mg/ml of the selective antibiotic geneticin (Calbiochem) to select the more efficient clones.

Once the selection was performed, supernatants were screened for the production of proenzyme hMMP-10 by ELISA (R&D Systems) and Western blot with an anti-MMP-10 antibody directed to the catalytic domain of the protein (MAB9101, R&D Systems).

Purification of recombinant human MMP-10 (rhMMP-10)

The cell culture was expanded to grow in T-175 cm^2 flasks (Costar; "Cell Bind") and cells were cultured in low foetal bovine serum medium (Advanced DMEM; GIBCO). Every 48 hours the supernatants were collected and maintained on ice during filtration, and concentration (Vivaflow 200; cut off 30 kDa). Finally they were stored at -20ºC until the purification process.

The ice-cold concentrated supernatant was applied to 1 ml Co-MAC column (Novagen). After washing the non-specifically retained proteins with binding buffer (20 mM Tris-HCl, 5 mM Imidazole and 500 mM NaCl; pH=7.9), bound proteins were eluted with elution buffer (20 mM Tris-HCl, 500 mM Imidazole and 500 mM NaCl; pH=7.9). Eluted fractions were pooled and subjected to an immunoaffinity chromatography in a HiTrap NHS-activated HP column (Amersham Biosciences, USA) coupled with an anti-His antibody (R&D systems). Non-specific proteins were removed from the column by washing with binding buffer (20 mM Tris-HCl and 500 mM NaCl; pH= 7.5) and the pro-rhMMP-10 was eluted with Glycine 0,1 M pH=2.9. The eluted fractions were immediately neutralized with Tris-HCl, pH=8. Peak collected fractions were concentrated, dialysed against TNB buffer (50 mM Tris-HCl pH =7.5, 150 mM NaCl and 0,05% Brij 35) and stored at -80 ºC. The purity of the sample was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Gelcode Blue Stain Reagent (Thermo Scientific, USA). All the purification steps were carried out using a FPLC System (GE Healthcare) at room temperature but maintaining the sample on ice and measuring the absorbance at 280 nm. Each batch of purified proMMP-10 was tested for: i) protein concentration with both an ELISA assay (R&D Systems) and Nanodrop ND-1000 measurement (Thermo Scientific, USA) applying an extinction coefficient of A_{280}=1,497 $M^{-1}cm^{-1}$ based on the primary sequence of rhMMP-10 and with; ii) protein activation with a kinetic assay using fluorogenic peptide for stromelysins (ES002, Fluorogenic Peptide Substrate II, R&D systems) and active rhMMP-10 for the standard curve; iii) protein purity and activatability by western blot with anti-MMP10 antibody (R&D Systems) as previously mentioned; iv) fibrinolytic activity by fibrin plate assay.

Effect of MMP-10 on the rate of clot formation and lysis

The effect of MMP-10 during clot formation and lysis was studied by monitoring the change in turbidity in normal and TAFI deficient plasma (Affinity Biologicals Inc., Kordia) using a microplate reader (Fluostar Optima, BMG Labtech). $CaCl_2$ (10 mM final concentration) was added to citrated plasma diluted 1:2 in HEPES buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 6 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 0.1% BSA, pH=7.5). After mixing, samples were incubated at 37ºC, and turbidity at 405 nm measured for 2 h every 30 seconds. To study the effect of MMP-10 on fibrin formation, rMMP-10 (10-200 nM, R&D System), previously activated by 24 h incubation with 10 mM $CaCl_2$ at 37ºC (Supplemental Figure 1A), was added to the plasma mixture. The rate of clot lysis was further examined by simultaneous addition of 300 U/mL of tissue plasminogen activator (t-PA, Actilyse, Boehringer Ingelheim), and rhMMP-10 (10-200 nM), in presence or absence of either MAB9101 (at the concentrations that block MMP-10 activity) or control antibody.

Additional experiments were also performed with recombinant MMP-3 (200 nM, R&D Systems) previously activated with 1 mM p-aminophenylmercuric acetate (APMA, Sigma). Clot lysis time was calculated as the time from initiation of clot formation to the time at which maximal absorbance falls to 50%.

MMP-10 Activity Assay

MMP-10 activity was measured in 96-well microplates, precoated with a human anti-MMP-10 specific monoclonal antibody (Clone 110343), by using a fluorogenic peptide for stromelysins (ES002, Fluorogenic Peptide Substrate II, R&D Systems). Fluorescence (320 nm excitation and 405 nm emission) was kinetically recorded on a spectrophotofluorometer (SpectraMAX GeminiXS, Molecular Devices). Plasma

samples were incubated with or without rhMMP-10 (10 nM) and different concentrations of MAB9101 to determine the concentration that blocks MMP-10 activity, established at 2-fold molar excess of the antibody.

Fibrin plate Assay

A solution containing 9.5 µM of fibrinogen from human plasma (Sigma) was laid over dishes and clotted by the addition of 20 U human thrombin (Enzyme Research Laboratory). Plates were incubated at 37ºC for 1 h and kept at 4ºC until used. 30 µl of recombinant t-PA (1 U/mL) and rhMMP-10 (200 nM) alone or plus t-PA and plasma euglobulins were pipetted onto the surface of the fibrin plate (n=5), and further incubated at 37ºC for 18 h. Plasma euglobulins were obtained as follows: pooled plasma (200 µL) was diluted 1:10 with distilled water and acidified to pH 5.9 with acetic acid. After 30 min on ice, the precipitate was centrifuged and redissolved in 200 µL HEPES buffer (pH 7.5). The lytic areas were determined as a measurement of fibrinolytic activity.

In a second set of experiments, gold-labelled fibrin clots were loaded with 1 U/mL of t-PA alone or with active rhMMP-10 (200 nM). After 1h of incubation at 37 ºC in a moist chamber, the edge of the thrombus was visualized with a confocal microscope (Zeiss LSM 510 Meta) set up in the reflection mode. At the fibrin network level, scanning was performed at 40x magnification (Zeiss 40x Plan-Neofluar) every minute, and the fibrin lysis-front velocity of t-PA alone or combined with MMP-10 was recorded (Zeiss Axiovert 200M).

TAFI and thrombin activity and fibrinogen functionality

TAFI activity was determined by chromogenic assay (Pefakit, Pentapharma). Briefly, TAFI alone (0 nM-2 µM) or combined with rhMMP-10 (200 nM) were incubated with

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activator (Pefakit, thrombin/thrombomodulin) or plasmin (15 nM, Enzyme Research Laboratories) for 3 min at 37ºC. Start reagent (Substrate, Tris, Mannit) was added and substrate conversion was measured for 10 min at 22ºC by monitoring absorbance at 405 nm. Calibrator and control plasmas were used for validation of the assay (Pefakit TAFI calibrator and controls, Pentapharma).

Thrombin activity was measured with a chromogenic substrate (S2238, Chromogenix) on thrombin (0,1 U/mL) and thrombin with MMP-10 (ratio 1/10) incubated at 37 ºC for 24 h.

Fibrinogen functionality was assayed on turbidimetric analysis using fibrinogen (2 mg/mL, Sigma) incubated alone or with rhMMP-10 or rhMMP-3 (ratio enzyme/substrate 1/10), in the presence of thrombin (5U/mL, Enzyme Research Laboratories) and $CaCl_2$ (25 mM). Clot formation was studied by monitoring the change in turbidity at 405 nm.

Determination of TAFI cleavage site

For proteomic analysis, TAFI alone or combined with rhMMP-10 and incubated 24 h at 37 ºC were run on a 12 % SDS-PAGE, cut from the gel, digested with trypsin, and the resulting peptides were separated by reverse phase capilar chromatography using a capLC (Waters) connected through a nanospray picotip probe to a Q TOF micr (Waters) for MS/MS analysis. MS and MS/MS data were processed with MassLynx and protein matching and peptide assignment was done with ProteinLynx Global Server and Phenyx using UniprotKB database.

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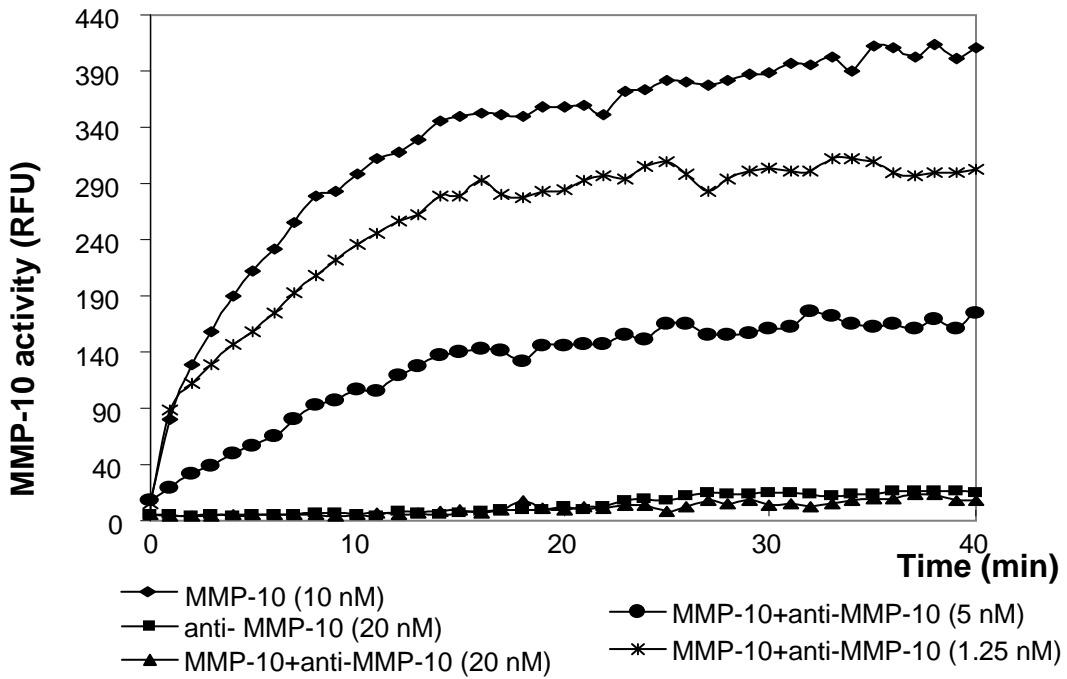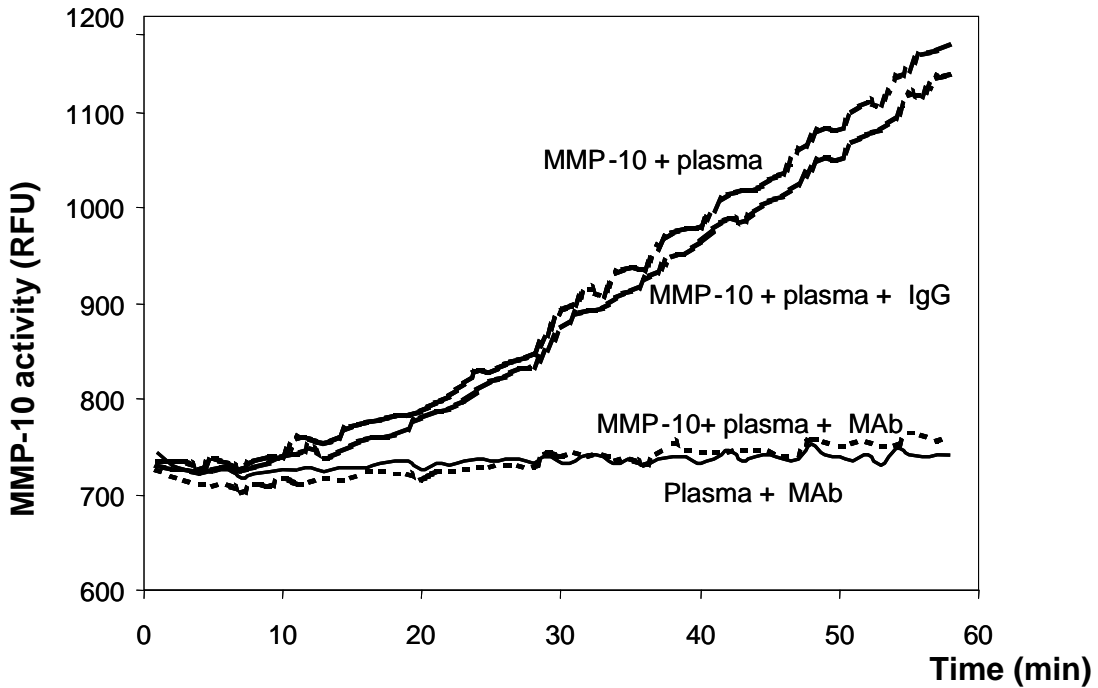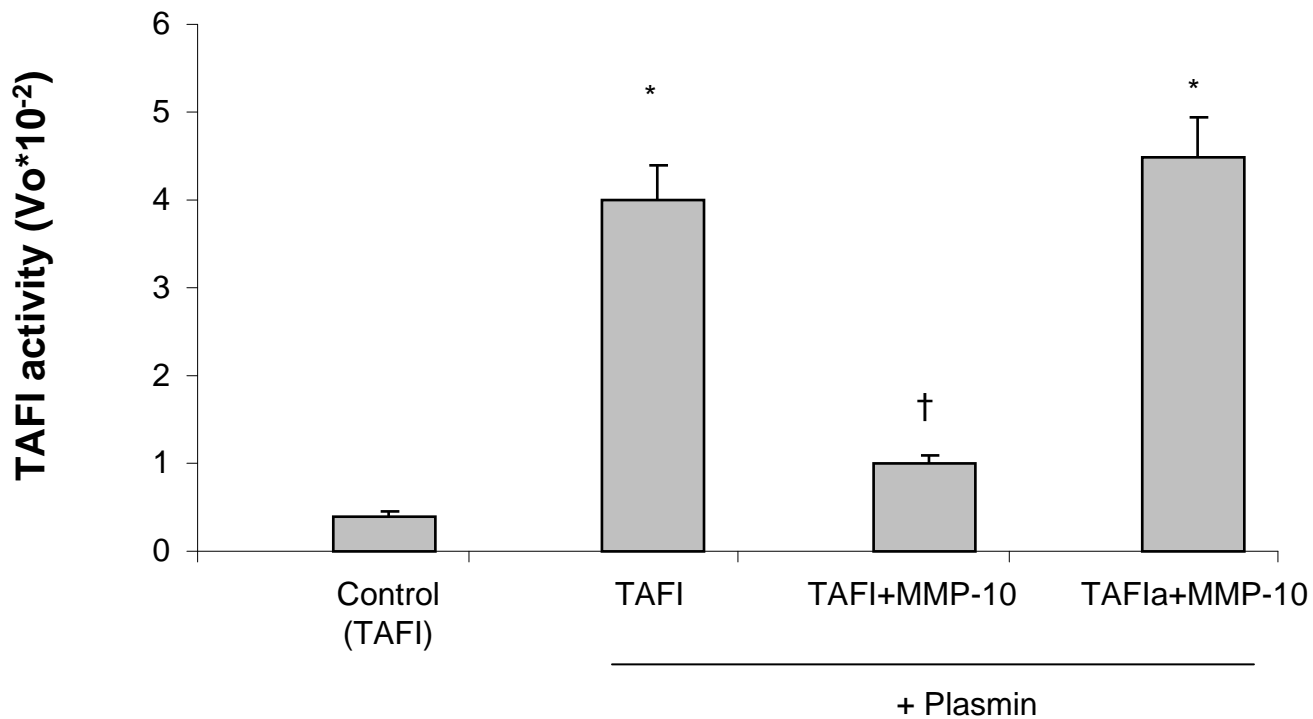
Supplemental Figures

Figure 1s

A

rMMP-10　rMMP-10a　rMMP-10　rMMP-10a

55 kDa

45 kDa

B

- ◆ MMP-10 (10 nM)
- ■ anti- MMP-10 (20 nM)
- ▲ MMP-10+anti-MMP-10 (20 nM)
- ● MMP-10+anti-MMP-10 (5 nM)
- ✳ MMP-10+anti-MMP-10 (1.25 nM)

C

MMP-10 + plasma

MMP-10 + plasma + IgG

MMP-10+ plasma + MAb

Plasma + MAb

Figure 2s

A

B

C

Figure 3s

A

MMP-10	-	-	-	+	+
GM6001	-	-	+	-	+
MMP-3	-	+	+	-	-

Fibrinogen

— 66.5 kDa
— 52.0 kDa
— 46.5 kDa

B

MMP-10	-	-	-	+	+
GM6001	-	-	+	-	+
MMP-3	-	+	+	-	-

Plasminogen

— 85 kDa

C

Control
MMP-10

MMP-3

Absorbance 405 nm

Time (min)

Figure 4s

MMP-10