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<sup>−/−</sup>), 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<sup>−/−</sup> 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<sup>−/−</sup> 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 ■ TAFII ■ thrombolysis

Stroke is a leading cause of death and disability in developed countries. 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; 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.

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The fibrinolytic and matrix metalloproteinase (MMP) systems cooperate in thrombus dissolution. 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). 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...
expressed in a stroke model, involving MMP-10–mediated TAFI improves the time of blood reperfusion and reduces infarct hypofibrinolysis. We also show that MMP-10 treatment limits fibrinolysis, shifting the hemostatic equilibrium toward decreased circulating levels of MMP-10 are observed in patients activated receptor-1), markedly enhances endothelial MMP-10 expression can be induced by inflammatory stimuli, and that serum MMP-10 is associated with carotid intimal-medial thickness, a surrogate marker of subclinical atherosclerosis. 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. 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 (Mnp10−/−), 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'-GAATGTAACCAGCTGTTACT-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 BstXI and the EcoRV 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 Biologics Inc, Kordia) with the use of a microplate reader (Fluorost 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 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 CaCl2, 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 (TAFIα) 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
Mnp10−/− mice generated by removing MMP-10 catalytic domain (exons 3–5) and crossed for 10 generations with C57BL/6 mice were generated at the Center for Lung Biology, University of Washington, Seattle, and bred in the Center for Applied Medical Research (CIMA) animal facilities. TAFI−/− mice were generated 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 Mnp10−/− (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 Mnp10−/− 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.
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 (IgG2b) (3 independent experiments, performed in triplicate).
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\[^{163}\]. 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 \(V_{\text{max}}\) (0.09 versus 0.22 A\(_{405}\)nm/min; \(P<0.001\)) with similar \(K_{\text{m}}\) (815 versus 865 nmol/L) (Figure 3D). The catalytic efficiency (\(K_{\text{cat}}/K_{\text{m}}\)) 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, 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 \(\alpha\) chain but without affecting its ability to form a fibrin clot (Figure IIA and IIC in the online-only Data Supplement). In contrast, MMP-3 clearly cleaved plasminogen and fibrinogen, preventing clot formation (Figure IIB and IIC 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 \(\mu\)g/kg) for in vivo experiments as a therapeutic dose of MMP-10 without triggering cytotoxicity.
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] L) than in WT mice. Intravenous injection of active rhMMP-10 (6.5 g/kg) in Mmp10−/− mice increased the bleeding time and the blood lost (99.6 [56.3] seconds and 15.4 [9.7] L) without affecting platelet count (1032 × 10^3 versus 1157 × 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 fibrinolytic 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
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 TAFIα in 10 different plasmas and their corresponding euglobulins showed a significant reduction of TAFIα 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] minutes) (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%; P<0.05). 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 minutes; P<0.05).
MMP-10 groups was significantly reduced (55% and 60%, respectively; \( P < 0.05 \)) 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 \( \mu g/kg; n=6 \)) 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±6.8% versus 21.7±8.8%; \( P < 0.01 \)). 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.

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
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-35 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.
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 (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.

Figure 7. In vivo circulating thrombin-activatable fibrinolysis inhibitor (TAFI) activity. A, Plasma TAFI activity 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.

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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 al29 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, but only MMP-2, MMP-9, and MMP-3 have been implicated in tPA side effects. Decreased cerebral hemorrhage and brain injury after treatment with tPA were described in experimental embolic stroke models involving MMP knockout animals and MMP inhibitors. However, controversy still exists because stroke outcomes are made worse with broad-acting MMP inhibitors. Indeed, MMP activity is required for microvascular recanalization through embolus extravasation.

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.

References

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 $\approx$5% of stroke patients. The major adverse effect after recombinant tPA administration is intracerebral hemorrhage, seen in $\approx$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.
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Supplemental methods

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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)
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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.
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For proteomic analysis, TAFI alone or combined with rhMMP-10 and incubated 24 h at 37 °C 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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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.

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as the difference between 50 % blood flow recovery (reperfusion time) minus the time at which initial blood flow falls to 50% (coagulation time).

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Thrombin clot was formed using a micropipette filled with 1 µL of purified murine alpha-thrombin (1 NIH U/µl or 2 U/µl, to induce more stable clot) by applying negative pressure. Mice were placed in a stereotaxic device, the skin between the right eye and the right ear was incised, and the temporal muscle was retracted. A small craniotomy was performed, the dura excised, and the middle cerebral artery (MCA) was exposed. The pipette was introduced into the lumen of the MCA bifurcation and thrombin was pneumatically injected by applying positive pressure to induce in situ clot formation. The pipette was removed 10 min after the injection to allow clot stabilisation.

To induce thrombolysis, t-PA (10 mg/kg; Actilyse) was intravenously injected (tail vein, 10% bolus, 90% perfusion during 40 minutes) 20 minutes after the injection of thrombin. The control group received the same volume of saline under identical conditions. Cerebral blood velocity was determined by laser Doppler Flowmetry using a fiberoptic probe (Oxford Optronix) glued to the skull in the MCA territory. Cerebral blood velocity was measured before the injection of thrombin (100% baseline) and throughout the experiment (75 min).

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After 24 hours, mice were euthanized and brains removed and frozen in isopentane. Cryostat-cut coronal brain sections (20 µm) were stained with thionine and analysed with an image analyser (Image J, National Institutes of Health, USA). For volume
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Data from mice were analyzed by the non-parametric Kruskall-Wallis test followed by the Mann-Whitney U test with the Bonferroni correction. The Friedman test was used when the same parameter was measured at different times on the same animals. Continuous variables were expressed as mean±SD and skewed variables as median and interquartile range (IQR). 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).

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SUPPLEMENTAL MATERIAL
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incial velocity (Vo=A405/min) of TAFI (75 nM) activation by plasmin (15 nM) incubated for 2 h at 37 °C in the presence or absence of active rhMMP-10 (200 nM). Plasmin-activated TAFI (TAFIa) incubated with rhMMP-10 proved to be uninhibited by MMP-10. * p<0.01 vs control, †p<0.01 vs TAFIa. B: Tricine gel showing that MMP-10 does not cleave thrombin (T, upper part) or thrombomodulin (TM, lower part) when they are incubated for 24h at 37 °C with active rhMMP-10. C: MMP-10 does not modify thrombin activity assayed with a chromogenic substrate (S2238). 0.1 U/mL Thrombin; 4.4 ng/mL MMP-10.

Figure 3S. Fibrinolytic substrates for MMP-10. A.: Tricine gel showing a different pattern of bands of fibrinogen incubated alone or with rhMMP-10 or rhMMP-3. Differences were restored after addition of MMPs inhibitor GM6001. B. Tricine gel showing a different pattern of bands when plasminogen was incubated with rhMMP-10 or rhMMP-3. Differences were restored after addition of MMPs inhibitor GM6001. C. Turbidimetric assay showing clot formation of fibrinogen alone (2 mg/mL, control) or preincubated with rhMMP-10 and rhMMP-3 (1:10). Normal clot formation was observed with fibrinogen preincubated with rhMMP-10, whereas no clot was formed after preincubation with rhMMP-3.

Figure 4S. Effect of MMP-10 on cultured HUVEC determined by LDH cytotoxicity assay. Cells were treated with different concentrations of MMP-10 (5 pM-200 nM) for 24h hours in serum free media. Graph represents the mean values obtained from 6 replicates of 3 different experiments. *p<0.05 and **p<0.01 vs control.
Supplemental Figures

Figure 1s

A

![Supplemental Figure A](image135x609 to 403x715)

B

![Supplemental Figure B](image35x309)

C

![Supplemental Figure C](image416x365)
Figure 2s

**A**

![Graph showing TAFI activity](image)

Control (TAFI) | TAFI | TAFI+MMP-10 | TAFIa+MMP-10
--- | --- | --- | ---
\[0 \times 10^{-2}\] | **\[4 \times 10^{-2}\]** | **\[5 \times 10^{-2}\]** | \[0 \times 10^{-2}\]

†

**B**

![Image of protein bands](image)

MMP-10 | T | T+MMP-10
--- | --- | ---
37 kDa | \[\text{protein bands}\] | \[\text{protein bands}\]

MMP-10 | TM | TM+MMP-10
--- | --- | ---
68 kDa | \[\text{protein bands}\] | \[\text{protein bands}\]

**C**

![Absorbance graph](image)

Absorbance 405 nm

0.00 | 0.05 | 0.10 | 0.15 | 0.20 | 0.25
--- | --- | --- | --- | --- | ---
0 | 50 | 100 | 150 | \(\text{Time (s)}\)

- Thrombin
- Thrombin+MMP-10

**Notes:**

- *: Statistically significant difference.
- †: Significantly different from control.
Figure 3s

A

<table>
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<tr>
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<th>MMP-10</th>
<th>GM6001</th>
<th>MMP-3</th>
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<tbody>
<tr>
<td>Fibrinogen</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td></td>
<td>-</td>
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Fibrinogen
- 66.5 kDa
- 52.0 kDa
- 46.5 kDa

B

<table>
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<th>MMP-3</th>
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Plasminogen
- 85 kDa

C

![Graph showing absorbance over time for MMP-10 and MMP-3](image)

Absorbance 405 nm

Time (min)

Control MMP-10

MMP-3
Figure 4s

Cells viability (%) vs MMP-10 concentration.

Control, 5 pM, 10 pM, 50 pM, 500 pM, 1 nM, 10 nM, 100 nM, 200 nM.