Inhibition of Thrombin-Activatable Fibrinolysis Inhibitor and Plasminogen Activator Inhibitor-1 Reduces Ischemic Brain Damage in Mice

Frederik Denorme, MSc*; Tine Wyseure, PhD*; Miet Peeters, BSc; Nele Vandeputte, BSc; Ann Gils, PhD; Hans Deckmyn, PhD; Karen Vanhoorelbeke, PhD; Paul J. Declerck, PhD*; Simon F. De Meyer, PhD*

Background and Purpose—Cerebral ischemia and reperfusion is associated with activation of the coagulation cascade and fibrin deposition in cerebral microvessels. Both thrombin-activatable fibrinolysis inhibitor (TAFI) and plasminogen activator inhibitor-1 (PAI-1) attenuate fibrinolysis and are therefore attractive targets for the treatment of ischemic stroke.

Methods—TAFI and PAI-1 were inhibited by monoclonal antibodies in a mouse model of transient middle cerebral artery occlusion. Twenty-four hours after stroke, mice were neurologically scored, cerebral thrombotic burden was assessed, and brain infarct sizes were calculated.

Results—Inhibition of TAFI or PAI-1 significantly decreased cerebral infarct sizes by 50% 24 hours after stroke. This reduction in cerebral damage was associated with a significant decrease in fibrin(ogen) deposition in the ischemic brain. Concurrently, functional recovery of the animals was improved. Interestingly, combined targeting of TAFI and PAI-1 using low, and by themselves inactive, doses of antibodies improved cerebral blood flow and reduced cerebral fibrin(ogen) deposition and infarct sizes by 50%. When dual treatment was delayed to 1 hour after the start of reperfusion, it still reduced brain injury; however, this was not statistically significant.

Conclusions—Targeting of PAI-1 and TAFI is protective in an ischemic stroke model by attenuating fibrin(ogen) deposition, thereby improving reperfusion. Combined inhibition has a co-operative effect that could become useful in ischemic stroke therapy. (Stroke. 2016;47:2419-2422. DOI: 10.1161/STROKEAHA.116.014091.)

Key Words: fibrinolysis ■ plasminogen activator inhibitor-1 ■ reperfusion injury ■ stroke ■ thrombin-activatable fibrinolysis inhibitor

In ischemic stroke, cerebral ischemia and subsequent reperfusion cause brain damage via a complex interplay of thrombotic and inflammatory processes. A key aspect of ischemia/reperfusion injury is intravascular fibrin(ogen) deposition.1,3 Plasmin-mediated fibrinolysis is counteracted by both thrombin-activatable fibrinolysis inhibitor (TAFI) and plasminogen activator inhibitor-1 (PAI-1).4 TAFI removes C-terminal lysine residues from partially degraded fibrin, reducing plasminogen binding to fibrin and subsequent activation by tissue-type plasminogen activator. PAI-1 is the main inhibitor of tissue-type plasminogen activator by forming an inactive complex with tissue-type plasminogen activator. Importantly, high levels of TAFI and PAI-1 increase the risk for stroke.5,6 Moreover, concentrations of both TAFI and PAI-1 increase in the acute phase of stroke, and this increase has been linked to worse clinical outcomes and lower survival rates.7,8

We recently showed a profibrinolytic effect of a novel bispecific inhibitor against TAFI and PAI-1.9 However, the precise contributions of inhibiting PAI-1 and TAFI during ischemic stroke remain largely unknown. In this study, we therefore used well-characterized monoclonal antibodies (MAs) that inhibit either TAFI or PAI-1 to dissect their role in cerebral ischemia/reperfusion injury.

Materials and Methods

Mice were subjected to 1 hour of cerebral ischemia followed by 23 hours of reperfusion, after which neurological and motor outcome, edema-corrected brain infarct sizes, and cerebral fibrin(ogen) deposition were assessed. Cerebral blood flow was monitored via laser Doppler flowmetry. Treatment was given 5 or 60 minutes after the start of reperfusion and consisted of anti-TAFI MA-TCK26D6,10 anti-PAI-1 MA-33H1F711 or a combination of both. A detailed description of Materials and Methods can be found in the online only Data Supplement.

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From the Laboratory for Thrombosis Research, KU Leuven Campus Kulak Kortrijk, Belgium (F.D., N.V., H.D., K.V., S.F.D.M.); and Laboratory for Therapeutic and Diagnostic Antibodies, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Belgium (T.W., M.P., A.G., P.J.D.).

*Drs Denorme and Wyseure contributed equally and are joint first authors, and Drs Declerck and De Meyer contributed equally and are joint last authors.

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Correspondence to Simon F. De Meyer, PhD, Laboratory for Thrombosis Research, KU Leuven Campus Kulak Kortrijk, E. Sabbelaan 53, 8500 Kortrijk, Belgium. E-mail simon.de.meyer@kuleuven.be

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Results

Using the transient middle cerebral artery occlusion stroke model, mice were treated 5 minutes after the start of reperfusion with different doses of anti-TAFI MA-TCK26D6, anti-PAI-1 MA-33H1F7, or a combination. Brains were isolated 23 hours later to determine infarct volumes (Figure 1A and 1B). Mice were treated with 2, 6 or 25 mg/kg control IgG MA developed infarct sizes of 76.9±33.4, 75.6±28.6, and 78.2±23.4 mm³, respectively. Treatment with anti-PAI-1 or anti-TAFI MAs decreased brain injury. Indeed, a dose of 6 mg/kg anti-PAI-1 MA-33H1F7 resulted in a 50% reduction of the cerebral infarct volume (33.7±21.2 mm³; \( P<0.01 \)), whereas a lower dose of 1 mg/kg MA-33H1F7 had no effect (68.5±30.4 mm³; \( P=0.97 \)). Similarly, 25 mg/kg anti-TAFI MA-TCK26D6 reduced mean infarct volumes (35.4±20.2 mm³; \( P<0.01 \)), whereas 6 and 1 mg/kg anti-TAFI MA-TCK26D6 had little or no effect (56.1±21.1 mm³; \( P=0.51 \) and 66.3±31.7 mm³; \( P=0.92 \), respectively). In agreement with the smaller ischemic brain lesions, the highest doses of MAs led to improved neurological and motor outcome (Figure 1C and 1D), although this only reached statistical significance for the anti-TAFI MA. To study the effect of dual inhibition of PAI-1 and TAFI,
a combination of low concentrations of both MAs (1 mg/kg) was given to mice subjected to stroke. Interestingly, although individual doses of 1 mg/kg were ineffective, combined targeting of PAI-1 and TAFI using these doses led to a 50% reduction of brain lesions (40.5±25.1 mm³; \(P < 0.01\)). Correspondingly, a trend toward improved neurological and motor outcome was observed. To further assess the effect of combined inhibition of PAI-1 and TAFI, we monitored middle cerebral artery regional cerebral blood flow during ischemia and reperfusion (Figure 2). Blocking of both PAI-1 and TAFI resulted in better reperfusion of the affected tissue of treated mice than that of control mice. Six hours after the start of reperfusion, cerebral blood flow was restored to 67.1±11.9% in treated mice versus 44.4±10.5% in control mice (\(P < 0.05\)). This difference in cerebral blood flow was even more pronounced the next day (68.4±18.0% versus 27.2±16.4%; \(P < 0.005\)).

Delayed administration of TAFI and PAI-1 MAs 1 hour after the initiation of reperfusion was less effective. Indeed, combined targeting of TAFI and PAI-1 at this later time point still reduced cerebral infarct sizes; however, this was not statistically significant (51.9±39.1 versus 68.5±33.6 mm³ in control mice; \(P = 0.15\); Figure 1 in the online-only Data Supplement).

To evaluate the effect of PAI-1 and TAFI inhibition on fibrin formation, cerebral fibrin(ogen) deposition was examined 1 day after stroke via Western blotting (Figure 3). In mice treated with control MA, fibrin(ogen) deposition in the infarcted hemisphere was 4 times higher than in the contralateral hemisphere (4.3±0.6-fold increase). High doses of anti-TAFI MA-TCK26D6 (25 mg/kg) or anti-PAI-1 MA-33H1F7 (6 mg/kg) significantly reduced fibrin(ogen) deposition in the infarcted hemisphere (1.7±0.9- and 1.9±0.8-fold increase, respectively; \(P < 0.01\)). Lower doses of MA-33H1F7 (1 mg/kg) or MA-TCK26D6 (6 and 1 mg/kg) did not reduce fibrin(ogen) deposition. Again, however, dual targeting of PAI-1 and TAFI via a combination of the individually ineffective dose of 1 mg/kg resulted in a 2-fold reduction of fibrin(ogen) deposition in the infarcted brain hemisphere (1.9±0.9-fold increase; \(P < 0.05\)). Of note, no cerebral bleeding was observed in any of the treated mice.

**Discussion**

Our present study demonstrates a protective effect of PAI-1 or TAFI inhibition on cerebral ischemia/reperfusion injury.
A stronger effect was observed when targeting both factors, indicative for a co-operative effect. Although fast restoration of blood vessel patency is key in ischemic stroke management, subsequent progressive stroke still develops in many patients. This has been attributed to reperfusion injury, which involves complex thromboinflammatory processes. We here show that inhibition of PAI-1 and TAFI can protect mice from ischemia/reperfusion injury by reducing cerebral fibrinogen deposition and improving cerebral reperfusion. Remarkably, using the same model, Kraft et al found that TAFI-deficient mice were unprotected. A plausible explanation could be the fact that MA–TCK26D6 specifically impairs the antifibrinolytic capacity of TAFI while preserving the anti-inflammatory potency of TAFI. The latter may also contribute to limiting brain injury. Our observation that PAI-1 inhibition can improve stroke outcome is in line with results of Nagai et al who used transgenic mice overexpressing PAI-1 and observed larger cerebral infarctions after thrombotic stroke. The observed difference in effective dose for the anti-PAI-1 and anti-TAFI MAs can possibly be explained by the much higher plasma concentrations of TAFI compared with PAI-1. As a key finding of this study, combining individually ineffective doses of MAs against PAI-1 and TAFI leads to a protective effect. These results are strongly suggestive for a co-operative effect of PAI-1 and TAFI in ischemic stroke and further support the use of a bispecific diabody targeted against both TAFI and PAI-1.

Massberg et al showed that as early as 5 minutes after initiation of reperfusion, fibrinogen is accumulating in the postischemic vasculature, actively recruiting platelets and inflammatory cells. Our findings further support the idea that early fibrinogen generation hampers efficient reperfusion of the affected brain tissue. Early inhibition of the antifibrinolytic factors, TAFI and PAI-1, prevents excessive accumulation of fibrinogen, limiting ischemic brain damage.

In conclusion, we demonstrated that both single and combined inhibition of PAI-1 and TAFI is protective in a mouse model of cerebral ischemia/reperfusion and thus constitute relevant targets (either individually or combined) for ischemic stroke treatment. Future preclinical testing in other relevant animal models is necessary to further evaluate the safety and efficacy of this strategy.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

Inhibition of TAFI and PAI-1 reduces ischemic brain damage in mice

Frederik Denorme MSc, Tine Wyseure PhD, Miet Peeters BSc, Nele Vandeputte BSc, Ann Gils PhD, Hans Deckmyn PhD, Karen Vanhoorelbeke PhD, Paul J. Declerck PhD, Simon F. De Meyer PhD
Supplemental Materials & Methods

Antibodies
The monoclonal antibodies (MA) MA-TCK26D6 (raised against human TAFI-ACIIYQ and cross-reacting with human and mouse TAFI) and MA-33H1F7 (raised against the human PAI-1/tPA complex and cross-reacting with human and mouse PAI-1) have been described previously.1,2 The control IgG antibody, MA-NB27B3, was raised against a nanobodies mix (Declerck PJ, unpublished).

Animals
Transient mechanical middle cerebral artery occlusion (tMCAO) was performed on 10-week-old male and female C57BL/6J mice (The Jackson laboratory, Bar Harbor, ME). All experiments were approved by the local ethical committee (P081/2014 KU Leuven, Leuven, Belgium) and were performed following the ARRIVE guidelines (www.nc3rs.org.uk), including randomization of treatment as well as surgery and analysis blind to the treatment.

Cerebral ischemia and reperfusion injury model
tMCAO was performed as described previously.3 Anesthesia was induced by inhalation of 5% isoflurane and maintained by inhalation of 2% isoflurane. After a midline incision in the neck, the proximal common carotid artery and the external carotid artery were ligated and a standardized silicon rubber–coated 6.0 nylon monofilament (6021; Doccol Corp, Redlands, CA) was inserted and advanced via the right internal carotid artery to occlude the origin of the right MCA. The intraluminal suture was left in situ during 60 minutes. Next, animals were re-anesthetized and the occluding monofilament was withdrawn to allow reperfusion. Surgery time did not exceed 10 minutes per animal. Five minutes or 60 minutes after reperfusion, MAs were administered as a bolus intravenously.

The following conditions excluded mice from endpoint analyses (exclusion criteria): (1) death within 24 hours after tMCAO, (2) operation time > 10 minutes or (3) when surgical complications occurred. Of the 192 mice subjected to MCAO, 23 mice (12%) met at least 1 of the exclusion criteria after randomization. Ten were excluded because they died during the time of the experiment (exclusion criterion (1)). No difference in mortality rate was observed between the groups assessed. The remaining 13 mice were excluded due to surgical complications determined after sacrifice (exclusion criterion (3)). During these experiments, no mice needed to be eliminated due to exclusion criterion (2): operation time > 10 minutes.

Neurological tests
Twenty-four hours after induction of tMCAO; mice were subjected to the modified Bederson test4 and the grip test5 to assess global neurological and motor function, respectively as described.6

Cerebral lesion quantification and assessment of bleeding
To measure cerebral infarct volumes mice were euthanized 24 hours after induction of tMCAO. Brains were quickly isolated and cut into 2-mm-thick coronal sections using a mouse brain slice matrix. The slices were stained with 2% 2,3,5-triphenyl-tetrazolium chloride (TTC, Sigma-Aldrich) to distinguish healthy tissue from unstained infarctions.
Stained slices were photographed with a digital Nikon D70 camera and infarct areas (white) were measured using Image J software (National Institutes of Health, Bethesda, MD) by an operator blinded for treatment condition. Edema-corrected infarct sizes were calculated by use of the following equation: $V_{corrected} = V_{uncorrected} \times \left(1 - \frac{V_i - V_c}{V_c}\right)$ with $V_i$ the volume of ipsilateral hemisphere and $V_c$ the volume of the contralateral hemisphere. The presence of cerebral hemorrhages was macroscopically assessed upon brain isolation as well as via careful visual analysis of the coronal brain sections after cutting.

**Quantification of intracerebral fibrin(ogen) deposition**

Intracerebral fibrin(ogen) deposition was quantified as described previously. Briefly, TTC-stained coronal brain sections were separated in contra- and ipsilateral hemispheres and homogenized. Immunoreactivity for fibrin(ogen) (rat anti-fibrin(ogen) pAb; 1:500; Acris Antibodies GmbH, Herford, Germany) and actin (murine anti-actin; 1:500; Merck Millipore, Darmstadt, Germany) was detected by Western blot analysis and quantified by densitometry using Image J software. The signal obtained for fibrin(ogen) was normalized with its corresponding actin signal, which served as a loading control. The ratio of both normalized signals from the ipsilateral and contralateral side served as a measurement of relative fibrin(ogen) deposition (fold increase).

**Monitoring of cerebral blood flow**

MCA blood flow was determined by laser Doppler flow (LDF) measurements (moorVMS-LDF1; Moor Instruments; Devon, UK) via a fiber optic probe placed in the vascular territory of the right MCA. Changes in blood flow were recorded using a PowerLab 8/35 data acquisition unit (ADInstruments; Oxford, UK) and calculated using LabChart software (v8.0.5; ADInstruments). Blood flow measured for 10 min before induction of ischemia was set as baseline (100%). Blood flow was monitored directly after insertion of the monofilament and before removal of the monofilament to confirm 1 hour of ischemia. Subsequently, blood flow was monitored 2, 4, 6 and 24 hours after reperfusion (averaged blood flow measured during 10 min was calculated and expressed as %CBF).

**Statistical analysis**

All data are presented as mean and standard deviation (SD) except for the Bederson and the grip-test score, which are depicted as scatter plots including median with the interquartile range (IQR). Statistical analysis was performed with Graph Pad Prism Version 6.0c. The number of experimental animals in each group was based on power calculations with infarct volume as primary parameter and with mean differences and standard deviations taken from available data from the same tMCAO model (power of 80% and $\alpha$ of 0.05). Prior to statistical analysis, a D’Agostino and Pearson normality test was used to check data distribution. One-way ANOVA with Dunnett’s post hoc test or a Mann-Whitney test was used for statistical comparison of lesion volumes and cerebral fibrin(ogen) deposition when applicable. In the case of non-parametric data (Bederson and grip-test score) a Kruskal–Wallis test with post hoc Dunn correction was performed. Changes in laser Doppler blood flow were analyzed and compared by repeated measures ANOVA.
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**Supplemental Figure**

**Figure I: Effect of delayed TAFI and PAI-1 treatment on cerebral ischemia/reperfusion injury.** Mice were treated with either a combination of MA-33H1F7 and MA-TCK26D6 (both 1mg/kg (n=9)) or control IgG (MA-NB27B3; 2 mg/kg (n=10)) one hour after the onset of reperfusion. (A) Edema-corrected brain infarct volumes were quantified by planimetric analysis. A Mann-Whitney test was used to compare experimental mice with control treated mice (IgG MA at a dose of 2 mg/kg). (B) Neurological outcome was assessed using the Bederson test. (C) Motor function was examined using the grip test. For these functional tests, a Kruskal–Wallis test with post hoc Dunn correction was used to compare experimental mice with control treated mice (IgG MA at a dose of 2 mg/kg).
Figure I

A

B

C

D