Enhanced activity of an ADAMTS-13 variant (R568K/F592Y/R660K/Y661F/Y665F) against platelet agglutination in vitro and in a murine model of acute ischemic stroke

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Essentials

- ADAMTS13 requires a substrate-induced conformational change to attain full activity in vitro.
- The efficacy of wild type ADAMTS13 in models of thrombosis/stroke may be enhanced by pre-activation.
- A pre-activated ADAMTS13 variant exhibits enhanced proteolysis of platelet agglutinates.
- This ADAMTS13 variant is protective in a murine model of stroke at a lower dose than WT ADAMTS13.

Summary. Background: ADAMTS-13 circulates in a closed conformation, only achieving full proteolytic activity against von Willebrand factor (VWF) following a substrate-induced conformational change. A gain-of-function (GoF) ADAMTS-13 variant (R568K/F592Y/R660K/Y661F/Y665F) is conformationally preactivated. Objectives: To establish how the hyperactivity of GoF ADAMTS-13 is manifested in experimental models mimicking the occlusive arterial thrombi present in acute ischemic stroke. Methods: The ability of GoF ADAMTS-13 to dissolve VWF–platelet agglutinates was examined with an assay of ristocetin-induced platelet agglutination and in parallel-flow models of arterial thrombosis. A murine model of focal ischemia was used to assess the thrombolytic potential of GoF ADAMTS-13. Results: Wild-type (WT) ADAMTS-13 required conformational activation to attain full activity against VWF-mediated platelet capture under flow. In this assay, GoF ADAMTS-13 had an EC50 value more than five-fold lower than that of WT ADAMTS-13 (0.73 ± 0.21 nM and 3.81 ± 0.97 nM, respectively). The proteolytic activity of GoF ADAMTS-13 against preformed platelet agglutinates under flow was enhanced more than four-fold as compared with WT ADAMTS-13 (EC50 values of 2.5 ± 1.1 nM and 10.2 ± 5.6 nM, respectively). In a murine stroke model, GoF ADAMTS-13 restored cerebral blood flow at a lower dose than WT ADAMTS-13, and partially retained the ability to recanalize vessels when administration was delayed by 1 h. Conclusions: The limited proteolytic activity of WT ADAMTS-13 in in vitro models of arterial thrombosis suggests an in vivo requirement for conformational activation. The enhanced activity of the GoF ADAMTS-13 variant translates to a more pronounced protective effect in experimental stroke.

Keywords: ADAMTS-13 protein; conformational activation; hemostasis; stroke; von Willebrand factor.

Introduction

Evidence has been accumulating that implicates von Willebrand factor (VWF) as an important factor in the pathophysiology of acute ischemic stroke (AIS) [1]. VWF is an adhesive plasma glycoprotein (GP) that is secreted and circulates as large globular multimers [2–5]. In this globular conformation, VWF is quiescent and unable to capture platelets. At sites of vascular damage, VWF is tethered to the vessel wall through its constitutively exposed collagen-binding site in the A3 domain [6,7]. Under the shear force of flowing blood, the unfolding of its A2 domain [8–10] induces conformational changes at a macromolecular scale [11], and local conformational changes in the A1 domain expose the previously cryptic
Protein expression and purification

VWF
tele plug is dependent on its multimeric size, and is regulated by the plasma metalloprotease ADAMTS-13 [12,13].

Experimental studies in VWF−/− and ADAMTS13−/− mice have confirmed the importance of the VWF–ADAMTS-13 axis for the extent of ischemic injury [14–17]. Recombinant ADAMTS-13 (rADAMTS-13) has systemic antithrombotic activity in ADAMTS13−/− mice [16]. Furthermore, rADAMTS-13 has been shown to have protective effects in murine stroke models without the risk of intracranial hemorrhage associated with recombinant tissue-type plasminogen activator (rt-PA) [18]. The VWF content of patient clots has been suggested to be a determinating factor in rt-PA resistance [19]. Moreover, a recent study identified VWF-rich patient clots and demonstrated the efficacy of rADAMTS-13 against rt-PA-resistant, VWF-rich thrombi in mice [20].

Recently, our understanding of ADAMTS-13 function has developed with the identification of a substrate-induced conformational activation mechanism [21–24]. In this model, ADAMTS-13 circulates in a quiescent conformation, maintained by an autoinhibitory interaction between its N-terminal spacer domain and its C-terminal CUB domains [21]. This autoinhibition is relieved upon binding of ADAMTS-13 to the D4-CK domains of globular VWF, exposing the crucial spacer domain exosite in readiness for VWF A2 domain unfolding. Exposure of ADAMTS-13 to the VWF D4-CK domains results in an approximately three-fold enhancement of its activity. A gain-of-function (GoF) ADAMTS-13 spacer domain variant (R568K/F592Y/4660K/Y661F/Y665F), which was first described by Jian et al. [25], is preactivated because of the mutagenic disruption of the autoinhibitory spacer–CUB domain interaction. This results in enhanced activity, even in the absence of VWF D4-CK. This variant also has the potential for broadened substrate specificity, and there are reports of in vitro proteolytic activity against both fibrinogen and plasminogen [26,27].

We hypothesized that the previously identified, dose-dependent activity of wild-type (WT) ADAMTS-13 in murine models of stroke may be further improved by conformational preactivation. We tested this hypothesis by using an in vitro parallel-flow model of platelet clumping under arterial shear conditions, before comparing the efficacy of WT ADAMTS-13 with that of GoF ADAMTS-13 in in vitro models of thrombosis and in a murine model of AIS.

**Materials and methods**

**Protein expression and purification**

Recombinant human ADAMTS-13 with a C-terminal Myc/His6 tag in pCDNA3.1 [28] was used to generate GoF ADAMTS-13 (R568K/F592Y/R660K/Y661F/ Y665F) by sequential site-directed mutagenesis. For in vitro experiments ADAMTS-13 was expressed in HEK293S stable cell lines and quantified, in conditioned medium, with an in-house ELISAs as described previously [28,29]. For half-life determination, ADAMTS-13 was expressed in either HEK293S or CHO-K1 stable cell lines, and purified by fast protein liquid chromatography (FPLC) with zinc-coupled HiTrap chelating columns (GE Healthcare, Chicago, IL, USA). CHO-expressed ADAMTS-13, for use in the murine stroke model, was passed over a hydroxyapatite column to remove contaminating proteins, and the purified ADAMTS-13 was quantified with an in-house ELISA and dialyzed into 150 mM NaCl, 20 mM histidine, 2% sucrose, and 0.05% Tween-80 (pH 7.4). The dimeric VWF D4-CK domain fragment (VWF residues 1874–2813) in the vector pcDNA 3.1/His was transiently expressed in HEK293S, purified by FPLC, and quantified by ELISA as previously described [30].

**Parallel-flow assays**

For parallel-flow assays, performed in the absence of coagulation, Vena8 Fluoro+ biochips (Cellix, Dublin, Ireland) were coated with 200 μg mL−1 collagen type III (Southern Biotech, Birmingham, AL, USA) and blocked with 1% bovine serum albumin (BSA) and 1 mg mL−1 glucose in HEPES buffer. Whole donor blood was collected on PPACK (Sigma, Gillingham, UK) and enoxaparin (low molecular weight heparin from Sanofi-Aventis, Guildford, UK), and treated with 100 nM prostaglandin E1 (PGE1) (Sigma) and 75 mU mL−1 apyrase (Sigma), to prevent platelet activation. Platelets were labeled with 10 μM DiOC6 (Sigma), and perfused over the collagen surface at a constant shear rate of 1500 s−1 (at which rate platelet capture is VWF-dependent) for 3.5 min. Adhesion of labeled platelets was visualized by fluorescence imaging at 250-ms intervals with a ×20 objective, and analyzed with SLIDEBOOK software to determine platelet coverage (%) at 180 s. To determine the effect of ADAMTS-13 on platelet capture, the assay was also performed in the presence of WT or GoF ADAMTS-13 at a range of concentrations, and EC50 values were determined from dose–response curves with GRAPHPAD 7. To assess the effect of ADAMTS-13 on preformed platelet aggregates, the assay was altered to allow 5 min of perfusion in the absence of rADAMTS-13, before a further 5 min of perfusion in the presence of rADAMTS-13 (WT or GoF). For parallel-flow assays, performed in the presence of coagulation, Vena8 Fluoro+ biochips were coated with 200 μg mL−1 collagen type III and 100 pm tissue factor (Sigma) before being blocked with coagulation buffer (1% BSA, 75 mM CaCl2 and 37.5 mM MgCl2 in HEPES buffer). Whole human blood was collected in 129 mM trisodium citrate (1 : 10 dilution), and platelets were labeled with 10 μM DiOC6. Citrated blood was diluted 9 : 1 with coagulation buffer immediately before perfusion over the collagen/tissue factor-coated surface at 1500 s−1 for 3 min. This was
repeated three times to provide uninterrupted flow of coagulating blood for a sufficient time to allow the formation of stable pseudothrombi. This was followed by a further 5 min of perfusion with blood, collection on PPACK and enoxaparin, and supplementation with DiOC₆-labeled platelets and rADAMTS-13 (WT or GoF).

Platelet agglutination assay

Donor blood was collected in 85 mM sodium citrate, 111 mM glucose, and 71 mM citric acid (pH 4.5). Washed platelets were prepared and treated with 100 nM PGE1 (Sigma) and 75 mU mL⁻¹ apyrase (Sigma), to prevent platelet activation. Platelets were incubated with 10 µg mL⁻¹ VWF (purified from plasma), and agglutination was initiated by the addition of 0.6 mg mL⁻¹ ristocetin (Helena Bioscience, Gateshead, UK). Light transmission was recorded with a Chrono-Log dual channel aggregometer (Kordia BV, Leiden, the Netherlands). After 5 min, once stable agglutination was observed, ADAMTS-13 (WT or GoF) was administrated and light transmission was recorded for a further 30 min.

FRETS-VWF73 assay

The FRETS-VWF73 assays of ADAMTS-13 activity were performed as described previously [21,24]. Purified VWF D4-CK (20–60 nm) was added before a 45-min preincubation at 37°C. Results were normalized to WT ADAMTS-13 activity.

Determination of ADAMTS-13 half-life in murine plasma

All animal work was performed in compliance with animal ethics guidelines at Imperial College London according to the UK Home Office’s Animals (Scientific Procedures) Act 1986, in accordance with the local ethical law and local ethical committees (KU Leuven, Leuven, Belgium; act no. 87–848) and following guidelines for the care and use of laboratory animals. Age-matched and sex-matched C57BL/6 littermates, aged 6–8 weeks, were anesthetized with ketamine (75 mg kg⁻¹) and medetomidine (1 mg kg⁻¹). The injection of ADAMTS-13 was performed through the retro-orbital plexus, and blood samples were taken via the same route into 129 mM citrate. Plasma concentrations of ADAMTS-13 were determined with an in-house ELISA as previously described [28,29]. To derive t₁/₂ values, data were plotted, by use of a one-phase decay equation, in GRAPHPAD 7.

Murine focal ischemia stroke model

FeCl₃-induced occlusion of the middle cerebral artery (MCA) was performed with surgical techniques described previously [20]. Regional cerebral blood flow (rCBF) in the MCA territory was determined by laser Doppler flow monitoring, and infarct size was determined 24 h after occlusion of the MCA by staining brain sections with 2% 2,3,5-triphenyl-tetrazolium chloride, both as described previously [20].

Results

rADAMTS-13 requires conformational activation for full function in in vitro models of platelet clumping

In a previous study of GoF ADAMTS-13 [26], the variant showed increased efficacy in reducing the VWF-mediated capture of washed plaletets under parallel flow, as compared with WT ADAMTS-13. This was attributed to the variant’s preactivated state and the requirement of WT ADAMTS-13 for conformational activation. To investigate the dependence of WT ADAMTS-13 activity on conformational activation by VWF, and the hyperactivity of the GoF variant, in a physiological setting, we performed parallel-flow assays with whole human blood under arterial shear rates. In these assays, in which platelet capture is VWF-dependent, the addition of 2.5 nM WT rADAMTS13 elicited only a slight reduction in platelet capture over time (Fig. 1A). When WT ADAMTS-13 was preincubated with 100 nM VWF D4-CK prior to being added to the assay, there was a significant decrease (P < 0.005) in the platelet coverage recorded at the end of the assay (Fig. 1A,B). The extent of the decrease in platelet capture in the presence of conformationally activated WT ADAMTS-13 was similar to that observed in the presence of GoF ADAMTS-13 (Fig. 1A,B). The activity of GoF ADAMTS-13 was not further enhanced by the addition of VWF D4-CK. Dose–response curves were generated by titrating both WT and GoF ADAMTS-13 into the platelet capture assay and determining the platelet coverage after 270 s of blood perfusion (Fig. 1C,D). These plots were used to determine EC₅₀ values of 3.81 ± 0.97 nm and 0.73 ± 0.21 nm for WT and GoF ADAMTS-13, respectively.

Preactivated GoF ADAMTS-13 shows enhanced ability to dissolve VWF–platelet agglutinates/aggregates

Having established that WT ADAMTS-13 requires conformational activation for full activity against VWF in a physiological setting, we hypothesized that this may limit its ability to dissolve preformed VWF–platelet agglutinates, and that the GoF variant would not be limited in this manner. This was first investigated in an agglutination assay with washed platelets. In this assay, ristocetin-induced VWF unfolding triggers the formation of platelet agglutinates before the addition of ADAMTS-13. In the presence of WT ADAMTS-13, the agglutination of platelets continued to increase steadily over the course of 30 min (Fig. 2A,B). In samples treated with GoF
ADAMTS-13, there was initial partial dissolution of platelet agglutinates (Fig. 2A) and a 30–50% decrease in the final agglutination as compared with that in the WT ADAMTS-13-treated samples (Fig. 2B; \( P < 0.001 \)).

The ability of ADAMTS-13 to dissolve preformed VWF–platelet aggregates in whole blood was examined under arterial shear rates, under conditions in which platelet capture was VWF-dependent (Fig. 1A,B). After 5 min of perfusion of whole blood over a collagen-coated surface, approximately 60–70% of the surface was covered by labeled platelets. During a second 5-min period of perfusion with whole blood containing endogenous ADAMTS-13, this remained stable (Fig. 2C,D). When endogenous ADAMTS-13 was inhibited (AD13 NEG) by the addition of an inhibitory mAb (3H9), the platelet coverage continued to increase steadily throughout the second period of perfusion (Fig. 2C,D). With titration of WT and GoF ADAMTS-13 into this assay during the second perfusion period (Fig. 2C,D), \( EC_{50} \) values were determined for the dissolution of VWF–platelet aggregates, i.e. 10.2 ± 5.6 nM and 2.5 ± 1.1 nM, respectively (Fig. 2E).

**GoF ADAMTS-13 reduces the size of in vitro preformed pseudothrombi**

To examine the ability of WT and GoF ADAMTS-13 to dissolve preformed pseudothrombi, the parallel-flow assay with whole blood was adapted to monitor the formation of
of platelet aggregates in the presence of induced coagulation and fibrin deposition. Unlike the previous parallel-flow assays performed in the presence of anticoagulants, this assay generated large, stable platelet aggregates (Fig. 3), which have been shown previously to contain fibrin(ogen) [26]. When channels containing these pseudothrombi were further perfused with whole blood containing endogenous ADAMTS-13, there was some remodeling of the pseudothrombi (Fig. 3A), most of which were enlarged, resulting in a steady increase in the DiOC₆ fluorescence attributable to the ongoing incorporation of platelets into the thrombus (Fig. 3D). In the presence of added WT ADAMTS-13 (at the normal plasma concentration), there was still enlargement of the pseudothrombi (Fig. 3B); however, the extent of the fluorescence increase was somewhat reduced (Fig. 3D). When treated with GoF ADAMTS-13, the pseudothrombi underwent extensive remodeling (Fig. 3C), and, at the end of the second perfusion period, the size of the pseudothrombi (as measured by platelet fluorescence) was significantly reduced ($P < 0.005$) as compared with the fluorescence at $t = 0$ (Fig. 3D).

Characterization of ADAMTS-13 for in vivo administration

For in vivo studies, rADAMTS-13 (WT and GoF) was expressed in CHO-K1 stable cell lines, which have been shown in previous studies to generate ADAMTS-13 with a long half-life in murine experiments. This has been attributed to an altered pattern of glycosylation as compared with ADAMTS-13 expressed in HEK293 cell lines [14]. This material required extensive purification with a combination of techniques, designed to remove protein contaminants without affecting ADAMTS-13 activity. The results presented so far herein, and in all of our previous studies of GoF ADAMTS-13 [21,24,26], have been generated by the use of material expressed in HEK293S cell lines. Characterization of the purified, CHO-expressed ADAMTS-13 was therefore required. There was no significant difference in proteolytic activity (against FRETS-
VWF73) of WT ADAMTS-13 preparations expressed in HEK or CHO cell lines and no significant difference in the activity of CHO-expressed WT ADAMTS-13 before and after purification (Fig. 4A). The activity of CHO-expressed GoF ADAMTS-13 was enhanced approximately three-fold as compared with that of WT ADAMTS-13 (Fig. 4A). This was similar to the activity of the HEK-expressed GoF ADAMTS-13, here and in previous studies, and this was not altered upon purification. As expected, the activity of the CHO-expressed WT ADAMTS-13, but not that of the GoF variant, was dose-dependently enhanced by the addition of the VWF D4-CK domain fragment (Fig. 4A). A comparison of the half-lives of WT and GoF ADAMTS-13 confirmed the rapid clearance of HEK-expressed ADAMTS-13 from murine plasma (Fig. 4B,C), with $t_{1/2}$ values of 10.6 ± 0.45 min and 9.3 ± 0.58 min, respectively. Half-lives could not be determined for CHO-expressed ADAMTS-13, as the plasma concentrations (in mice injected with either WT or GoF ADAMTS-13) decreased by only 5–10% over the course of the experiment.

GoF ADAMTS-13 restores cerebral blood flow after experimental stroke and reduces cerebral ischemia at a lower dose than WT ADAMTS-13

As GoF ADAMTS-13 shows an increased ability to dissolve VWF/platelet-rich aggregates in vitro, we hypothesized that it may exert a more pronounced protective effect than WT ADAMTS-13 in an experimental model of stroke. Large, VWF-rich thrombotic occlusions were generated in the MCAs of C57BL/6 mice. Occlusion was defined by reduction of rCBF to <25% of baseline (determined by laser Doppler flowmetry) that remained stable for 5 min. In vehicle-only controls, rCBF remained stable for a period of 60 min, and no spontaneous recanalization, defined as a return of rCBF to >25% of baseline, was observed (Fig. 5A). Mice were injected with WT or GoF ADAMTS-13 at a final concentration of 200, 400 or 800 nm, and body weight was used to estimate the total blood volume. There was no restoration of blood flow in any of the mice injected ($n = 5$) with 200 nm WT ADAMTS-13, and there was minimal

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**Fig. 3.** Gain-of-function (GoF) ADAMTS-13 partially dissolves von Willebrand factor (VWF)/platelet-rich pseudothrombi under flow. (A) Whole human blood, collected on citrate and containing labeled platelets, was recalcified immediately prior to perfusion over a collagen/tissue factor-coated surface. After 9 min of continuous perfusion, large, VWF/platelet-rich pseudothrombs (shown previously to also contain fibrin (ogen) [26]) were formed. At the end of this period ($t = 0$ min) the channel was perfused for a further 4 min with labeled, recalcified blood, and platelet fluorescence was continuously monitored. (B, C) The assay was repeated with the addition of 5 nm ADAMTS-13 (wild-type [WT] or GoF) during the second perfusion period. Images are representative of three independent experiments. (D) Fluorescence readings for each sample were normalized to that at $t = 0$ min, and are expressed as mean ± SD, $n = 3$. [Color figure can be viewed at wileyonlinelibrary.com]

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restoration of blood flow in only one of the mice injected ($n = 5$) with 200 nM GoF ADAMTS-13 (Fig. 5C). The mean rCBF of the five mice injected with 400 nM WT ADAMTS-13 did not significantly increase above the recanalization threshold of 25% (Fig. 5A), with only two mice showing restored blood flow (Fig. 5C). Injection of 400 nM GoF ADAMTS-13 ($n = 5$) resulted in a mean rCBF that was significantly higher than the recanalization threshold (Fig. 5A), with two mice showing rCBF above 75% (Fig. 5C). At the highest dose tested (800 nM,
The ability of GoF ADAMTS-13 to reduce the size of preformed, stable platelet aggregates in vitro suggested that its protective effect in experimental stroke may be sustained even when administration is delayed. Delayed administration of both WT and GoF ADAMTS-13 at a single dose of 800 nM was evaluated. Although highly variable, and therefore not statistically significant, the injection of GoF ADAMTS-13 at 60 min after occlusion did result in a small increase in the mean rCBF above the recanalization threshold (Fig. 5E), with six of the seven mice in the group showing some level of blood flow restoration as compared with only two mice in the WT group (Fig. 5F). Despite these results, there was no observable improvement in terms of infarct volume 24 h after stroke (Fig. 5G).

**Discussion**

**Under physiological conditions (arterial flow rate in the presence of the plasma concentration of VWF): WT ADAMTS-13 is not fully active**

In previous studies of GoF ADAMTS-13, first described by Jian et al. [25], the hyperactivity of the variant was similar to that of conformationally activated WT ADAMTS-13 [21,24]. Therefore, it is described as being preactivated. In an assay of the VWF-mediated capture of platelets from plasma-free blood, performed under flow, the GoF variant showed a similar level of enhanced activity (approximately three-fold to four-fold decrease in EC50) as that observed in static assays [26]. This suggested that, under these conditions, WT ADAMTS-13 was not conformationally activated. We set out to establish whether or not WT ADAMTS-13 is conformationally active under conditions that better reflect the physiological setting of arterial thrombus formation, to allow us to establish whether or not preactivated GoF ADAMTS-13 would be a more potent thrombolytic agent in vivo.

In parallel-flow assays, in which platelet capture is dependent on the unfolding of VWF (present at the normal plasma concentration) by arterial shear, WT ADAMTS-13 was clearly less active than the GoF variant (Fig. 1A,B). However, preincubation of WT ADAMTS-13 with 100 nM VWF D4-CK (2.5 times the plasma concentration of monomeric VWF) results in an activity that is comparable to that of GoF ADAMTS-13. This strongly suggests that the physiological activity of WT ADAMTS-13 is, in part, dependent on the availability and/or binding affinity of the D4-CK domains within plasma VWF. The fact that the activity of GoF ADAMTS-13 in this assay cannot be further enhanced by VWF D4-CK is indicative of a preactivated state, as seen in static assays and in previous platelet capture assays [21,24,26]. This is further supported by the decrease in the EC50 determined for the GoF variant as compared with WT ADAMTS-13 (Fig. 1C,D). The fact that the activity of WT ADAMTS-13 is dependent, at least in part, on its conformational activation may be an important consideration, as the recombinant protein may be useful as a potential therapy for thrombotic thrombocytopenic purpura [31,32] and ischemic stroke [20].

**The preactivated state of GoF ADAMTS-13 increases its ability to dissolve platelet agglutinates/aggregates in vitro**

WT ADAMTS-13 requires activation by the D4-CK domains of VWF to attain full activity against VWF under flow. In a physiological setting, in which VWF binds to collagen and platelets as it is incorporated into the developing platelet plug, reduced access to the activating D4-CK domains may further limit the efficacy of WT ADAMTS-13. Therefore, we hypothesized that...
Efficacy of GoF ADAMTS-13 in experimental stroke

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preactivated GoF ADAMTS-13 may be better able to proteolyze VWF that is already incorporated into platelet aggregates, thereby reducing the size of preformed thrombi.

We first examined this in an assay of ristocetin-induced platelet agglutination, allowing the formation of VWF–platelet agglutinates before the addition of ADAMTS-13. The inability of WT ADAMTS-13 to dissolve the preformed agglutinates was clearly evident (Fig. 2A, B), in contrast to previous studies in which WT ADAMTS-13 reduced platelet agglutination [20]. This may be the result of differences in ADAMTS-13 concentration and/or activity between the ADAMTS-13 used here (prepared in-house) and the commercial ADAMTS-13 preparation used previously [20]. Comparatively, GoF ADAMTS-13 showed an ability to reduce the size of the agglutinates, which was evident from the 30–50% reduction in agglutination determined by light transmission measurements (Fig. 2A, B), and from visual inspection of the cuvette contents upon completion. The increased efficacy of GoF ADAMTS-13 against preformed platelet aggregates was also evident when it was examined under flow, where it showed an EC50 value four times lower than that of WT ADAMTS-13 (Fig. 2C–E). We also employed a more physiologically relevant model in which the complexity of the fibrin-containing pseudothrombi would be expected to further hinder the conformational activation, and therefore the efficacy, of WT ADAMTS-13. Indeed, WT ADAMTS-13, at least at physiological concentration, was unable to reduce the size of the thrombi, and only partially reduced the ongoing incorporation of platelets as compared with the control (Fig. 3A, B, D). The GoF variant, on the other hand, not only prevented ongoing platelet recruitment, but actively remodeled and reduced the size of the pseudothrombi (Fig. 3C, D).

GoF ADAMTS-13 shows enhanced thrombolytic activity and is protective in a murine model of AIS

Finally, we examined the efficacy of WT and GoF ADAMTS-13 in an established murine model of AIS. The formation of occlusive thrombi in this model, achieved through topical administration of FeCl3 to the MCA, has been shown previously to be highly dependent on VWF. Therefore, this is a relevant model of rt-PA-resistant thrombi, and is useful for demonstrating how targeting VWF can induce recanalization [20]. With its preactivated conformation, and enhanced proteolytic activity against VWF (Fig. 4A), it was hypothesized that GoF ADAMTS-13 would be more effective at dissolving these VWF-rich thrombi. This was true, to some extent, as GoF ADAMTS-13 remained protective at a lower dose than WT ADAMTS-13, in terms of both rCBF restoration (Fig. 5A–C) and infarct volume reduction (Fig. 5D). The fact that GoF ADAMTS-13 was effective at lower doses than WT ADAMTS-13 most likely reflects the enhanced activity of the preactivated ADAMTS-13 variant, which could become useful in the setting of stroke. The enhanced ability of GoF ADAMTS-13 to reduce the size of established pseudothrombi in vitro led us to examine whether or not it would remain effective at restoring blood flow in the AIS model when administration was delayed by 1 h. Again, this was observed to some extent, as a greater proportion of mice treated with GoF ADAMTS-13 showed partially restored rCBF as compared with those treated with WT ADAMTS-13 (Fig. 5E, F). However, this was not reflected in infarct volume measurements (Fig. 5G). Previous studies showed that WT ADAMTS-13 was able to reduce infarct size when administration was delayed by 1 h [20]. Although it is difficult to compare the concentrations used in this study, a difference in absolute concentration could explain this difference. The half-lives of the WT ADAMTS-13 and GoF ADAMTS-13 used in these experiments was not determined; however, there was negligible reduction in the plasma concentration of either protein over a similar time period (Fig. 4B, C). Furthermore, the half-lives of WT and GoF ADAMTS-13 (expressed in HEK293S cells) in murine plasma were comparable.

In summary, the enhanced activity of preactivated ADAMTS-13 (as compared with WT ADAMTS-13) in in vitro assays of platelet capture (Fig. 1B) suggests that, at plasma ADAMTS-13/VWF concentrations, the activity of ADAMTS-13 can be further increased. The fact that GoF ADAMTS-13 does not require conformational activation appears to result in increased efficacy in a model of AIS, which may reduce the required dose. Further studies of the therapeutic potential of GoF ADAMTS-13 in the setting of ischemic stroke, focusing particularly on thromboinflammatory stroke, are required, as is further investigation into the possibility of broadened substrate specificity and the possible impact of this on the variant’s safety and efficacy in vivo.

Addendum

K. South, I. I. Salles-Crawley, and F. Denorme performed research. K. South, F. Denorme, S. F. De Meyer, and D. A. Lane designed research, analyzed data, interpreted data, generated figures, and wrote the paper. All authors read and approved the final version of the manuscript.

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Disclosure of Conflict of Interests

F. Denorme and S. F. De Meyer have a patent WO2016191565 A1 for compositions comprising ADAMTS-13 for use in methods for the recanalization of occluded blood vessels in an infarction pending. The other authors state that they have no conflict of interest.

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