A disintegrin and metalloprotease with thrombospondin type 1 repeats-13 (ADAMTS13), a member of the “a disintegrin-like and metalloprotease with thrombospondin type 1 repeats” (ADAMTS) family,1,2 is synthesized primarily in the liver and is secreted into blood circulation. Plasma ADAMTS13 concentrations in healthy individuals range from 0.5 to 1.0 mg/L.3,4 Severe deficiency of plasma ADAMTS13 activity results in thrombotic thrombocytopenic purpura (TTP), a potentially fatal syndrome.5 Mild to moderate deficiency of plasma ADAMTS13 activity is associated with increased risk of other arterial thrombotic disorders, such as myocardial infarction6 and cerebral ischemic injury.7

Plasma ADAMTS13 is constitutively active in cleaving newly released ultralarge (UL) von Willebrand factor (VWF) from stimulated endothelial cells8 and thereby preventing an accumulation of platelets on injured vessel wall. In addition, ADAMTS13 cleaves released and soluble VWF or platelet-bound VWF in circulation under shear stress.9,10 Studies have shown that the cleavage of endothelial ULVWF occurs very rapidly with8 or without11,12 fluid shear stress, suggesting that newly released ULVWF on endothelial cell surface may be in its “open” conformation. However, the VWF cleaved from endothelial cells by ADAMTS13 remains UL by multimer analysis.11 This suggests that further proteolytic processing of these released VWF multimers, likely to occur in microvasculature, may be necessary to reduce the size. In blood, soluble VWF adopts a “closed” conformation,13 resistant to cleavage by ADAMTS13.

Exposure of the soluble VWF to high shear stress or denaturants may open up the binding and cleavage sites, which are normally buried under the β-sheet in the central A2 domain.14 Moreover, binding of coagulation factor VIII (FVIII),9 platelets,15 or both10 to the soluble VWF also increases its cleavage by ADAMTS13 under shear stress. These results suggest a cofactor-dependent mechanism regulating VWF proteolysis by ADAMTS13 under physiologically relevant conditions.

Human ADAMTS13 consists of a metalloprotease domain, a disintegrin domain, the first thrombospondin type 1 repeat (TSP1 repeat), a Cys-rich domain, and spacer domains. The more distal C terminus has 7 additional TSP1 repeats and 2 complement c1r/c1s, sea urchin epidural growth factor, and bone morphogenetic protein (CUB) domains.1,2 We16,17 and others18,19 have shown that the N-terminal half of
ADAMTS13 appears to be necessary and sufficient for proteolytic cleavage of VWF under various in vitro conditions. However, the role of the C-terminal domains of ADAMTS13 in vivo remains controversial. For instance, we reported that a C-terminally truncated ADAMTS13 variant after the spacer domain expressed by an in utero injection of lentiviral vector eliminated plasma ULVWF and inhibited ferric chloride (FeCl₃)-induced arterial occlusion in the carotid artery of ADAMTS13⁻ /⁻ mice.²⁰ Banno et al showed that a naturally occurring murine ADAMTS13 variant truncated after the sixth TSP1 repeat (ADAMTS13S⁵⁄₈) was less efficacious than full-length ADAMTS13 in inhibiting FeCl₃-induced thrombosis in the mesenteric arteriole.²¹ More recently, de Maeyer et al reported that a recombinant murine ADAMTS13 variant truncated after the eighth TSP1 repeat, infused into ADAMTS13⁻ /⁻ mice, was not able to cleave newly released ULVWF/VWF strings on the endothelial cells in the mesenteric arterioles/venules.²²

These discrepant results prompted us to systematically investigate the structure-function relationship of ADAMTS13 in vivo using a recombinant protein strategy and a murine thrombosis model. In addition, we hoped to determine the correlation between thrombus-inhibiting activity and VWF-cleavage activity under more physiologically relevant conditions. Our findings may shed light on the structure-function relationship of ADAMTS13 in vivo. The information obtained may be applicable for rational design of protein or gene-based therapies for TTP and other arterial thrombotic disorders.

Materials and Methods

Preparation of ADAMTS13 (or Variants), VWF, and Antisaccpe Antibody

Plasmids containing a full-length human ADAMTS13 (FL) and various truncated variants (truncated after the eighth thrombospondin type 1 repeat [T8], the spacer domain [S], and the first TSP1 repeat [T1]; and a mutant with residues Arg659 to Glu664 in the spacer domain deleted [d6a]) with a V5-His epitope tagged at their C termini are shown in Figure 1A. All plasmids were cloned into pcDNA3.1 V5-His TOPO vector (Invitrogen, Carlsbad, CA). Plasmids FL, T8, S, and d6a were stably transfected into human embryonic kidney (HEK293) cells, whereas plasmid T1 was stably transfected into Madin-Darby canine kidney (MDCK) cells using Lipofectamine 2000 reagent (Invitrogen). The reason for production of T1 in MDCK is purely technical because the expression level of T1 was higher in MDCK cells than in HEK293 cells. Full-length ADAMTS13 and variants were purified from Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium serum-free conditioned medium using the methods established previously.²³ Human VWF was purified from pooled normal human plasma using cryoprecipitation, followed by precipitation with polyethylene glycol 15 000 and Sephacryl-300 gel filtration as described previously.²⁴ Murine recombinant VWF was purified from Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium serum-free conditioned medium of stably transfected HEK293 cells using Q-fast flow ion exchange and Sephacryl-300 (GE Healthcare, Piscataway, NJ) gel filtration chromatography. The plasmid encoding full-length murine vwf was a kind gift from Dr David Motto (University of Iowa, Iowa City, IA). The purity of all proteins was determined by SDS-polyacrylamide gel electrophoresis with Coomassie Blue staining. The concentrations of those with greater than 95% purity (human VWF, murine recombinant VWF, FL, T8, and S) were determined by the optical density at 280 nm corrected with light scattering at 320 nm (1-cm cuvette) using a Nano-drop 2000c spectrophotometer (Thermo Scientific, San Diego, CA). The coefficients at optical density 280 (corrected) for human VWF, murine recombinant VWF, FL, T8, and S were 1.0, 1.0, 0.68, 0.71, and 0.91 mg/mL, respectively. The concentrations of those partially purified proteins (T1 and d6a) were determined by an in-house immunosorbent assay (ELISA) as described below using a purified FL as a standard. Human monoclonal antispacer antibody (monoclonal antibody [mAb] II-1) derived from B cells from a patient with acquired autoimmune TTP²⁵ have been described previously.

ELISA

A high-binding microtiter plate (Thermo Scientific, Rockford, IL) was coated with 100 µL of monoclonal antidisintegrin IgG in phosphate-buffered saline (PBS) (40 µg/mL) (Green Mountain Antibodies, Burlington VT). After being blocked with 2.5% bovine serum albumin in PBS, 100 µL of diluted samples containing ADAMTS13 or variants in 0.5% bovine serum albumin in PBS were added and incubated at 25°C for 2 hours. After 3 PBS washes, the bound ADAMTS13 and variants were incubated for 1 hour with 100 µL of biotinylated rabbit anti-V5 IgG (0.5 µg/mL) (Novus Biologicals, Littleton, CO), followed by a 30-minute incubation with streptavidin-peroxidase (1:2000) (Pierce, Burlingame, CA), 3,3’,5,5’-Tetramethylbenzidine substrate (100 µL) (Thermo Scientific, Rockford, IL) was added for color development. After stopping the reaction with 50 µL of sulfuric acid (H₂SO₄), the absorbance (450 nm) was determined on a SpectroMax microtiter plate reader ( Molecular Devices, Sunnyvale, CA). A purified recombinant FL at concentrations of 0.025, 0.05, 0.1, 0.2, and 0.4 µg/mL in 0.5% bovine serum albumin in PBS was used for calibration.

Inhibition of ADAMTS13 Activity by Human mAb II-1

Recombinant human ADAMTS13 (0.6 nmol/L) was incubated at 25°C for 30 minutes with human monoclonal antispacer IgG (mAb II-1) (0 to
35 nmol/L) or human monoclonal control antibody (mAb-c) (35 nmol/L). The residual ADAMTS13 activity was determined by the cleavage of a fluorescein-labeled recombinant human VWF73 peptide (rF-vWF73) (2 μmol/L) as described previously.26 Normal human plasma was used for calibration. The relative activity of residual ADAMTS13 (in percent) was plotted against the concentrations of mAb II-1 or mAb-c used in the reaction.

**Half-Life of ADAMTS13 and Variants in Mice**

ADAMTS13−/− mice (4 to 6 weeks old) were anesthetized with intraperitoneal injection of Nembutal (0.1 mg/g body weight). Recombinant ADAMTS13 or variants diluted with 100 μL of normal saline were injected into mice via a retroorbital sinus plexus. The amount of protein infused was dependent on the body weight and blood volume of mice to achieve physiological saline concentrations (~5 to 10 nmol/L). We used ~7 mL blood per 100 g of body weight in all of our calculations, as described.28 Blood samples (50 μL) were collected from a jugular vein at 5, 20, 40, and 70 minutes after protein injection and anticoagulated with 3.8% sodium citrate (9:1 blood:anticoagulant). Plasma was obtained after immediate centrifugation at 8000 rpm for 8 minutes and stored at −80°C in aliquots. The concentrations of ADAMTS13 and variants in murine plasma after 1:10 dilution with PBS at various time points were determined by the ELISA as described above.

**Carotid Arterial Occlusion Assay**

Mice at 2 to 4 months of age were anesthetized with Nembutal. The right carotid artery was exposed by blunt dissection. PBS (100 μL) alone or PBS containing recombinant human ADAMTS13 and variants (final concentration, 100 nmol/L) was injected into mice via the retroorbital plexus. The amount of protein injected was determined as described for the half-life assessment. In addition, purified human recombinant ADAMTS13 (100 nmol/L) was incubated with 3.5 μmol/L mAb II-1 or mAb-c in PBS for 30 minutes (total volume, 100 μL). The ADAMTS13-antibody mixtures or complexes were infused via a jugular vein into ADAMTS13−/− mice (~1 mL blood for ~14 g body weight). This resulted in final concentrations of ~10 nmol/L ADAMTS13 and 0.35 μmol/L mAb II-1 (or mAb-c) in mouse circulation, respectively. Three minutes after protein infusion, injury was induced by exposing the carotid artery to 10% ferric chloride (FeCl₃) (anhydrous) soaked on a piece of filter paper (1×2 mm) for 2 minutes. The filter paper was then removed, and the injured field was flushed with PBS. The blood flow was monitored using a Doppler flow probe (Transonic Systems, Ithaca, NY) until cessation or up to 30 minutes if no occlusion was observed. The times to the complete occlusion or cessation of blood flow were recorded. The differences among various means of the times to the complete occlusion were determined by 1-way ANOVA using Minitab statistical software (State College PA). All assays were performed in a blind fashion (the operator did not know the nature of the protein infused) to avoid bias.

**Thrombus Formation in Mesenteric Arteriole**

Before vessel injury, platelets were isolated from ADAMTS13−/− mice and labeled with calcine AM (Invitrogen) (2.5 μg/mL) for 15 minutes.29 The labeled murine platelets were washed with modified Tyrode buffer (10 mmol/L HEPES, pH 7.2, 137 mmol/L NaCl, 2.68 mmol/L KCl, 0.42 mmol/L NaH₂PO₄, 1.7 mmol/L MgCl₂, 11.9 mmol/L NaHCO₃, and 5 mmol/L glucose) containing 1 μg/mL PGE₂ (Sigma, St. Louis, MO) and then infused via a retroorbital plexus into the anesthetized mice. Five minutes after platelet infusion, recombinant ADAMTS13 proteins diluted into 100 μL of PBS were infused via the same route. The amount of protein infused was the same as that for the carotid arterial occlusion assay described above. The mesentery was gently exteriorized through a midline abdominal incision. The arterioles of 90 to 120 μm diameters (Supplemental Table I, available online at http://atvb.ahajournals.org) were visualized with an inverted fluorescence microscope (Nikon) and a charge-coupled device camera system. The arteriole was injured by topical application of 10% FeCl₃ saturated in a filter paper (1×2 mm) for 5 minutes. The filter paper was removed, and the injured site was flushed with PBS. The thrombus formation in the mesenteric arteriole was monitored for 30 minutes after injury or until its complete occlusion that lasted for 30 seconds. The times to initial thrombus formation (the diameter of the thrombus is >30 μm) and the completion occlusion (defined as the cessation of blood flow for at least 30 seconds) were determined from video clips using the NIH Elements software (Nikon, Melville, NY).26 The differences among various groups were determined by 1-way ANOVA. Again, the experiments were performed in a blind fashion to avoid the operator’s bias.

**Kinetic Cleavage of VWF by ADAMTS13 and Variants/Mutants Under Fluid Shear Stress**

For time-dependent cleavage, purified VWF (150 nmol/L) was incubated with purified recombinant ADAMTS13 proteins (25 nmol/L or 125 nmol/L) in the presence of lyophilized human platelets (150×10⁷ μL) and FVIII (1 nmol/L) at 25°C for various times (0 to 60 minutes) under constant vortex rotation (2500 rpm) on a MixMate PCR mixer (Eppendorf, Hauppaug, NY). The total volume was 20 μL in all reactions. For kinetic determination, purified VWF at various monomer concentrations (0, 187.5, 37.5, 75, 150, and 300 nmol/L) was incubated with 25 mmol/L ADAMTS13 or T8, S, and d6a or 125 mmol/L T1, at a physiological concentration of FVIII (1 nmol/L) and lyophilized platelets (150×10⁷/μL) at 25°C for 30 minutes with constant mixing at 2500 rpm. The proteolytic cleavage of multimeric VWF was determined by 1% agarose gel electrophoresis, followed by Western blotting as previously described.29 The increase in proteolytic cleavage products and reduction in high molecular weight VWF multimers was quantified by densitometry using the ImageJ software and plotted against time or VWF concentrations. The curve was fitted into the Michaelis-Menten equation to obtain the rate constants Km and kcat using SigmaPlot software.

**Results**

**Cleavage of Murine or Human VWF by Murine or Human ADAMTS13**

Species difference in proteolytic cleavage of VWF by ADAMTS13 had to be considered before a proper interpretation of in vivo data could be made. A previous study reported that human recombinant VWF was not efficiently cleaved by murine plasma ADAMTS13 under urea-denaturing conditions.31 However, recombinant human ADAMTS13 infused into ADAMTS13−/− mice rapidly cleaved newly released ULVWF on endothelial cells,29 suggesting that human ADAMTS13 does cleave murine VWF under physiological conditions. To further assess whether there was a species-dependent variation in proteolysis of VWF by ADAMTS13, we determined proteolytic cleavage of murine (or human) VWF by murine (or human) ADAMTS13 under 2 commonly used in vitro assay conditions. As shown, the cleavage of murine VWF by murine ADAMTS13 and human ADAMTS13 or the cleavage efficacy of human VWF by murine ADAMTS13 and human ADAMTS13 was quite similar if guanidine was used to denature VWF (Supplemental Figure I). However, if urea was used for denaturation, cleavage of murine VWF by human ADAMTS13 was reduced by ~5-fold compared with cleavage of human VWF by human ADAMTS13 (Supplemental Figure II). Such difference may be caused by higher urea concentration (2.0 mol/L) used for denaturation of murine VWF than human VWF (1.5 mol/L). Surprisingly, the cleavage of both murine and human VWF by murine ADAMTS13 was dramatically reduced compared with that by human ADAMTS13 (Supplemental Figure II), suggesting that murine ADAMTS13 is highly sensitive to urea denaturation under these assay conditions.

**Table 1**

<table>
<thead>
<tr>
<th>ADAMTS13 Variant</th>
<th>Cleavage Activity (%)</th>
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</thead>
<tbody>
<tr>
<td>ADAMTS13</td>
<td>100% (Control)</td>
</tr>
<tr>
<td>T8</td>
<td>90%</td>
</tr>
<tr>
<td>S</td>
<td>80%</td>
</tr>
<tr>
<td>d6a</td>
<td>70%</td>
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</tbody>
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**Figure 1**

Graph showing the cleavage of murine VWF by murine ADAMTS13 under urea-denaturing conditions. The data are presented as mean ± SEM. *p < 0.05 vs. control.
Half-Life of Recombinant ADAMTS13 Proteins in ADAMTS13−/− Mice

Full-length ADAMTS13 and variants with different truncations, mutations, or sources of production may exhibit different half-lives in vivo.20,32 To assess the half-life of our recombinant ADAMTS13 proteins used for murine studies, we injected, via a retroorbital sinus plexus, various recombinant ADAMTS13 proteins into ADAMTS13−/− mice (C57BL/6). The amount of injected recombinant proteins was determined by the body weight and blood volume of each mouse. By ELISA, we demonstrated that at 5 minutes after infusion, plasma concentrations of FL, T8, S, T1, and d6a were quite similar (∼6 to 10 nmol/L) (Figure 1B). The plasma concentrations of all infused ADAMTS13 proteins except for T1 reduced rapidly over time (Figure 1C). The estimated half-life (t1/2) for most infused recombinant ADAMTS13 proteins (FL, T8, S, and d6a) derived from HEK293 cells was between ∼15 and ∼18 minutes, consistent with those reported previously.20 However, the t1/2 of T1 that was purified from MDCK cells was ∼38 minutes, approximately twice as long as those produced in HEK293 cells (Figure 1C). Therefore, the average plasma concentration of T1 during 30 to 40 minutes of observation was almost twice as high as that of other variants (Figure 1C).

Effect of Full-Length ADAMTS13 and Variants on Carotid Arterial Occlusion

Ferric chloride is a widely used chemical for inducing arterial and venous thromboses in mice to study VWF-platelet functions.33 Tissue damage mediated by iron chemical oxidation predisposes the injured area to platelet adherence and aggregation, followed by coagulation activation and fibrin deposition. On topical application of a filter paper soaked with 10% FeCl3 (anhydrous) on an isolated carotid artery, blood flow was continuously monitored until cessation. Because of the short half-life of infused recombinant ADAMTS13 proteins, we started FeCl3 injury 3 minutes after protein injection. The entire experiment was completed within 35 minutes after protein infusion. We showed that the time to the complete vessel occlusion (or the cessation of blood flow) in carotid artery in ADAMTS13−/− mice (C57BL/6) was 5.4±0.3 minutes (n=14) (mean±SEM). This was significantly shorter than that in wild-type mice (10.4±0.9 minutes, n=13) with the same genetic background (P<0.0001) (Figure 2). These results suggest that FeCl3-induced arterial occlusion assay is sensitive for assessment of ADAMTS13 function in vivo.

We therefore used this assay to determine the function of various C-terminal-truncated ADAMTS13 variants using the same protocol. As shown, the time to the complete occlusion of carotid artery after infusion of recombinant FL, T8, and S was 13.4±1.3 minutes (n=16), 11.2±1.4 minutes (n=15), and 20.0±1.9 minutes (n=16), respectively (Figure 2). These results suggest that the amino-terminal half of ADAMTS13 (up to the spacer domain) is sufficient for inhibition of arterial thrombosis. An infusion of T1 and d6a into ADAMTS13−/− mice at the same concentrations (∼6 to 10 nmol/L) did not significantly prolong the time to complete occlusion of carotid artery. The time to the complete occlusion after T1 and d6a infusion was 6.4±0.7 minutes (n=14) and 5.9±0.6 minutes (n=11), respectively (Figure 2). The difference in the mean occlusion time between T1 or d6a-injected and PBS-injected was not statistically significant (P=0.156 or 0.411). These results suggest that the Cys-rich and spacer domains of ADAMTS13, particularly the amino acid residues between Arg659 and Glu664, are required for modulation of arterial thrombosis in vivo.

To further test this hypothesis, we infused a full-length ADAMTS13 pretreated with antiserum antibody (mAb II-1), which targets specifically at the variable region (Arg659 to Tyr665) of spacer domain, into ADAMTS13−/− mice. We found that the antibody-treated ADAMTS13 had dramatically reduced the ability to attenuate arterial thrombosis in carotid artery induced by FeCl3. As a control, an infusion of ADAMTS13 pretreated with a human control monoclonal antibody (mAb-c), into ADAMTS13−/− mice significantly attenuated the formation of arterial thrombosis under the same conditions. The difference in the time to complete occlusion between ADAMTS13−/− mice receiving ADAMTS13 pretreated with mAb II-1 (8.2±0.5, mean±SEM, n=10) and ADAMTS13 pretreated with mAb-c (19.5±3.5, n=10) was statistically highly significant (P=0.0058) (Figure 2B). However, the time to the complete occlusion in ADAMTS13−/− mice receiving mAb II-1–treated ADAMTS13 (8.2±0.5, n=10) was slightly longer than that in ADAMTS13−/− mice (6.6±0.4, n=9) (P=0.037) (Figure 2B), suggesting low residual activity of ADAMTS13-mAb II-1 complexes under these in vivo conditions.
Before infusion into mice, the inhibitory effect of purified mAb II-1 was confirmed by cleavage of rF-VWF peptide. As shown, mAb II-1 but not mAb-c inhibited proteolytic activity of recombinant full-length ADAMTS13 in a concentration-dependent manner (Supplemental Figure III). In the presence of 35 nmol/L mAb II-1, proteolytic activity of ADAMTS13 was completely inhibited. The half-maximal inhibitory concentration of mAb II-1 (EC$_{50}$) was estimated to be $\approx$4.5 nmol/L (Supplemental Figure III). In addition, the same mAb II-1 was shown to inhibit proteolytic cleavage of multimeric VWF under denaturing conditions and cell-bound ULVWF on endothelial cells under flow in the previous study.\textsuperscript{24}

### Effect of ADAMTS13 and Variants on Thrombus Formation in Mesenteric Arteriole

To assess the effect of ADAMTS13 proteins on modulation of thrombosis in smaller arteries with a shear rate of $\approx$1000 to $\approx$1500 s$^{-1}$, we determined the real-time thrombus formation in mesenteric arterioles. Mesenteric arterioles were injured for 5 minutes with topical application of 10% FeCl$_3$ soaked in a filter paper (1×2 mm). Thrombus formation was monitored every 10 seconds for 30 minutes or until complete occlusion under a Nikon inverted fluorescent microscope (with ×10 objective). A, The fluorescent signal was generated from the activated and accumulated platelets loaded with calcein AM at the site of injury. The dashed white lines indicate the boundary of vessel wall, whereas the red arrows point in the direction of blood flow in the mesenteric arteriole. B and C, The times to the initial thrombus formation (defined by the formation of thrombus $>30 \mu m$ in diameter) (B) and the complete occlusion (C) of the mesenteric arteriole were determined using NIS Elements software. The data are presented as means±SEM. The numbers in parentheses are the total numbers of mice in each group. Statistical analysis was performed by 1-way ANOVA with Tukey correction between the control (ADAMTS13$^{-/-}$ receiving PBS alone) and various groups. Probability values $<0.01$ were considered to be statistically highly significant.

### Figure 3

Effect of a disintegrin and metalloprotease with thrombospondin type 1 repeats-13 (ADAMTS13) and variants on mesenteric arteriolar thrombus formation. Wild-type (WT) and ADAMTS13$^{-/-}$ mice (C57BL/6) were injected with 100 $\mu$L of PBS alone or PBS containing full-length human ADAMTS13 (FL); variants truncated after the eighth thrombospondin type 1 (TSP1) repeat (T8), the spacer domain (S), and the first TSP1 repeat (T1); and a mutant with residues Arg659 to Glu664 in the spacer domain deleted (d6a). The amount of proteins was determined by body weight and blood volume of the mice. Mesenteric arterioles were injured for 5 minutes with topical application of 10% FeCl$_3$ soaked in a filter paper (1×2 mm). Thrombus formation was monitored every 10 seconds for 30 minutes or until complete occlusion under a Nikon inverted fluorescent microscope (with ×10 objective). A, The fluorescent signal was generated from the activated and accumulated platelets loaded with calcein AM at the site of injury. The dashed white lines indicate the boundary of vessel wall, whereas the red arrows point in the direction of blood flow in the mesenteric arteriole. B and C, The times to the initial thrombus formation (defined by the formation of thrombus $>30 \mu m$ in diameter) (B) and the complete occlusion (C) of the mesenteric arteriole were determined using NIS Elements software. The data are presented as means±SEM. The numbers in parentheses are the total numbers of mice in each group. Statistical analysis was performed by 1-way ANOVA with Tukey correction between the control (ADAMTS13$^{-/-}$ receiving PBS alone) and various groups. Probability values $<0.01$ were considered to be statistically highly significant.
(Supplemental Table I). After topical application of 10% FeCl₃ for 5 minutes, thrombus formation was monitored using time lapse under an inverted fluorescent microscope for accumulation of fluorescein-labeled murine platelets at the site of injury. Representative images (Figure 3A) and video clips (Supplemental Videos II to VIII) of the real-time thrombus formation (up to 10 minutes) in wild-type mice and ADAMTS13−/− mice receiving ADAMTS13 or variants are shown. Moreover, the time to initial thrombus formation and the time to complete occlusion of the mesenteric arterioles were determined. The results showed that the times to the initial thrombus formation and the complete occlusion in the mesenteric arteriole of ADAMTS13−/− mice were 5.2±0.6 and 9.6±0.8 minutes (mean±SEM, n=16), respectively. These values were significantly smaller than those in wild-type mice with the same genetic background (8.8±0.6 minutes and 13.8±1.0 minutes, n=12, respectively) (Figure 3B and 3C). The results imply that the FeCl₃-induced mesenteric arterial thrombus formation assay is also sensitive for assessing systemic antiarterial thrombotic function of ADAMTS13 in vivo. Similar to carotid arterial occlusion, an infusion of recombinant FL, T8, and S (∼6 to 10 nmol/L) into ADAMTS13−/− mice significantly prolonged the times to both the initial thrombus formation (mean±SEM) (FL: 9.7±0.9 minutes, n=10; T8: 10.1±1.5 minutes, n=10; S: 10.5±1.9 minutes, n=10) (Figure 3B and 3C) and the complete occlusion (FL: 15.3±1.4 minutes; T8: 21.9±2.3 minutes; S: 16.2±1.6 minutes) in these mice (Figure 3B and 3C). These results further confirmed that the N terminus of ADAMTS13 is sufficient for inhibition of arterial thrombosis in vivo. Similar to the results obtained from carotid arterial occlusion, an infusion of T1 or d6a into ADAMTS13−/− mice at the same concentrations did not prolong either the time to the initial thrombus formation (means±SEM) (T1: 5.5±0.37 minutes, n=12; d6a: 5.4±0.56 minutes, n=11) (Figure 3B) or the time to complete occlusion (means±SEM) (T1: 9.3±0.8 minutes, n=12; d6a: 10.0±1.1 minute, n=11) (Figure 3C). The differences in the time to the initial thrombus formation and that to the complete occlusion between T1 or d6a-injected and PBS-injected ADAMTS13−/− mice were not statistically significant (all probability values were greater than 0.5) (Figure 3B and 3C). These results further confirmed the importance of the Cys-rich and spacer domains, particularly the amino acid residues between the Arg659 and Glu664 in the spacer domain, in systemic antiarterial thrombosis under (patho)physiological conditions.

**Kinetic Cleavage of VWF by ADAMTS13 and Variants Under Fluid Shear Stress**

To correlate thrombosis inhibition function with VWF-cleavage activity under fluid shear stress, we performed kinetic analyses. Addition of FVIII,9 platelets,10,15 or both10 increased cleavage of VWF by ADAMTS13 under shear stress. Therefore, we performed all cleavage assays in the presence of FVIII (1 nmol/L) and lyophilized platelets (150×10⁶/µL). As shown, the formation of VWF cleavage product by FL, T8, and S increased as a function of vortexing time (Figure 4A to 4C), reaching a plateau after 30 minutes of incubation under constant vortexing at 2500 rpm on a MixMate PCR mixer. No cleavage products were observed when ADAMTS13 was omitted (not shown) or when 10 mmol/L EDTA was included in the reactions. There appeared to be no difference in terms of the cleavage efficiency among recombinant FL, T8, and S under these conditions (Figure 4A to 4C). However, little or no cleavage production was detected when VWF was incubated with 125 nmol/L T1 or d6a (5 times of FL) (Figure 4D and 4E).

Moreover, the VWF-cleavage product formation increased as a function of increasing concentrations of VWF substrate (Figure 5A and 5B). The proteolytic cleavage efficiencies (the ratios of kcat/Km), measured after 5 minutes of vortexing (the linear portion of the curve), for recombinant FL, T8, and S to cleave VWF were 1.0×10⁹, 0.9×10⁹, and 0.7×10⁹ M⁻¹s⁻¹, respectively (Table). The differences in kinetic parameters among FL, T8, and S were not statistically significant (P>0.05). When assessed after 30 minutes of vortexing, the ratios of kcat/Km for FL (0.3×10⁹ mol/L⁻¹s⁻¹), T8 (0.4×10⁹ mol/L⁻¹s⁻¹), and S (0.3×10⁹ mol/L⁻¹s⁻¹) to
cleave VWF remained the same. However, removal of the Cys-rich and spacer domains (T1) almost completely abol- ished proteolytic activity toward VWF under the same con- ditions (Figures 4D and 5A to 5D). No cleavage product was detected even with addition of 125 nmol/L T1 after 60 minutes of vortexing (Figure 4D). Furthermore, d6a exhibited dramatically reduced proteolytic cleavage of VWF under the same conditions (Figures 4E and 5). No cleavage products were detectable after 5 minutes of vortexing with both T1 and d6a; thus, no kinetic parameters were obtained. A small amount of cleavage product was detected after 30 minutes of vortexing with d6a (Figures 4D and 5), but not with T1 (Figures 4E and 5). These results suggest that the Cys-rich and spacer domains, particularly the spacer domain, are critical for substrate recognition. Together, our data demon- strate that thrombus-inhibition function of human ADAMTS13 and variants in mice is highly correlated to the VWF-cleavage activity under fluid shear stress.

### Discussion

ADAMTS13 limits platelet thrombogenesis presumably through proteolytic cleavage of newly released ULVWF anchored on endothelial cells,29 VWF in circulating blood, or VWF bound with platelets at the site of thrombus formation.37 The structural components of ADAMTS13 for modulation of arterial thrombosis are systemically investigated using recombinant ADAMTS13 proteins and murine models. We demonstrated that an infusion via retroorbital sinus plexus of recombinant FL, T8, and S (≈6 to 10 nmol/L) into ADAMTS13−/− mice (Figure 1) is able to inhibit FeCl3-induced thrombus formation in both carotid artery and mesenteric arteriole (Figures 2 and 3). Our data are consistent with those previously reported using a different model sys- tem,20,21 with one exception.22 For instance, ADAMTS13−/− mice expressing S via an in utero injection of lentiviral vector exhibited prolonged FeCl3-induced carotid arterial occlusion time.20 However, the relative efficacy of this variant to full-length ADAMTS13 was not assessed because of differ- ent plasma expression levels (0.5 to 0.7 U/mL versus 0.07 to 0.1 U/mL).20 Moreover, an effect from ectopic expression of the ADAMTS13 variant, such as in leukocytes and platelets, could not be excluded. An other study in congenic mice showed that an ADAMTS13 variant truncated after the sixth TSP1 repeat (ADAMTS133/4) was nearly as efficacious as wild-type ADAMTS13 (ADAMTS131/1) for inhibition of initial thrombus formation in mesenteric arterioles.21 How- ever, ADAMTS133/4 was found to be slightly less efficacious than ADAMTS131/1 in blocking the later phase of thrombus formation and collagen-induced platelet aggregation under shear stress greater than 5000 s−1.21 However, our results do not reveal significant difference regarding the effect on both early and later phases of thrombus formation among FL, T8, and S using either carotid arterial occlusion assay (Figure 2) or mesenteric arteriolar thrombus formation assay (Figure 3). Notably, our results are not fully in agreement with those by de Maeyer et al, in which an infusion of concentrated conditioned medium containing recombinant murine ADAMTS13 truncated after the eighth TSP1 repeat did not cleave the newly released ULVWF strings at all.22 The reason for this discrepancy is yet to be determined. One possibility is that cleavage of the ULVWF string requires the distal C-terminal

### Table. Kinetic Parameters of Proteolytic Cleavage of VWF by ADAMTS13 and Variants Under Fluid Shear Stresses

<table>
<thead>
<tr>
<th>Variant</th>
<th>kcat (s−1)</th>
<th>km (×10−9 mol/L)</th>
<th>kcat/km (×109 mol/L−1 s−1)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>55.9 ± 14.8</td>
<td>58.4 ± 14.1</td>
<td>1.0 ± 1.1</td>
<td>4</td>
</tr>
<tr>
<td>T8</td>
<td>44.1 ± 51.1</td>
<td>60.0 ± 55.5</td>
<td>0.9 ± 0.8</td>
<td>4</td>
</tr>
<tr>
<td>S</td>
<td>51.1 ± 37.6</td>
<td>73.5 ± 66.2</td>
<td>0.7 ± 0.6</td>
<td>4</td>
</tr>
<tr>
<td>T1</td>
<td>1.2 ± 0.7</td>
<td>ND</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>d6a</td>
<td>2.7 ± 0.5</td>
<td>ND</td>
<td>ND</td>
<td>3</td>
</tr>
</tbody>
</table>

Data are mean ± standard deviation. Constructs FL, T8, S, and T1 represent full-length ADAMTS13 and variants truncated after the eighth TSP1 repeat, the spacer domain, and the first TSP1 repeat, respectively; d6a indicates the full-length ADAMTS13 lacking amino acid residues Arg659 to Glu664. n indicates number of independent experiments; ND, not determined; VWF, von Willebrand factor.
domain, but inhibition of thrombus growth does not. Species-dependent variation may also play a role, although human ADAMTS13 cleaves murine VWF as efficiently as does murine ADAMTS13 under guanidine-denaturing conditions (Supplemental Figure I). Under urea-denaturization conditions, murine ADAMTS13 cleaves both murine and human VWF much less efficiently than human ADAMTS13 does (Supplemental Figure II), suggesting an inactivation of murine ADAMTS13 in the presence of 1.5 mol/L urea. Together, the data from our study and those of others suggest that the N-terminal half of ADAMTS13 (up to the spacer domain) is sufficient for substrate recognition and antithrombotic thrombosis under (patho)physiological conditions.

Additional analyses have demonstrated that the ADAMTS13 variant lacking the Cys-rich and spacer domains (T1) or bearing a small deletion of 6 amino acid residues within the spacer domain (d6a), which does not cleave VWF in vitro, also exhibits dramatically reduced efficacy in inhibiting thrombus formation in both carotid artery and mesenteric arteriole after FeCl₃ injury (Figures 2 and 3). Moreover, human monoclonal antibody against the variable region of β9 to β10 in the spacer domain (mAb II-1) significantly inhibits proteolytic cleavage of VWF- and FeCl₃-induced arterial thrombosis. These results suggest the critical role of the Cys-rich and spacer domains, particularly the amino acid residues between Arg659 and Glu664, for systemic antithrombotic function in vivo. The importance of the Cys-rich and spacer domains, particularly the variable region of β9 to β10 in the spacer domain, in substrate recognition and antithrombotic thrombosis function is highlighted by the fact that this region contains the core antigenic epitopes for IgG autoantibodies against ADAMTS13 in patients with autoimmune TTP. A substitution of a single or a cluster of residues in this region reduces substrate recognition and cleavage of VWF, as well as the binding of these autoantibodies against ADAMTS13.

Our data also demonstrate the direct correlation between antithrombotic thrombosis activity of ADAMTS13 (or variants) (Figures 2 and 3) and its VWF-cleavage activity under fluid shear stress (Figures 4 and 5 and the Table), suggesting the critical role of ADAMTS13-mediated VWF proteolysis in modulation of arterial thrombosis. However, it remains to be determined whether the VWF-cleavage-independent activity, such as the disulfide bond–reducing activity of ADAMTS13, plays a role in modulating arterial thrombosis. Recent studies suggest that both VWF and ADAMTS13 contain several surface-exposed free thiols. The interaction between VWF and ADAMTS13 via disulfide bond formation under shear stress may prevent shear-induced lateral association of VWF multimers to form bundles and strings, thereby modulating adhesion function of VWF. Further investigation of VWF-reducing activity of ADAMTS13 may shed light on the biological function of ADAMTS13 in vivo.

With growing interest in the therapeutic potential of recombinant ADAMTS13 protein° or gene transfer° in TTP and many other diseases, our findings may provide molecular basis for rational design of therapeutic targets. For instance, recombinant C-terminal-truncated ADAMTS13 variants may be used instead of full-length ADAMTS13 to prevent TTP or inhibit pathological thromboses such as myocardial infarction and ischemic cerebral infarction. The mouse model demonstrates that deletion of ADAMTS13 aggregates reperfusion injury in the brain, whereas infusion of recombinant ADAMTS13 into mice reduces infarct size and improves functional outcome without producing cerebral hemorrhage.° The truncated ADAMTS13 variants are easier to express in large quantity,°,°,°,° more resistant to proteolysis, and potentially less immunogenic than full-length ADAMTS13. In addition, supplementation of ADAMTS13 and variants may also be beneficial to patients with severe preeclampsia,° systemic inflammation,° or severe sepsis,° and multiorgan failure,° in which plasma ADAMTS13 activity was found to be severely reduced.

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The authors thank Dr Wolfgang Bergmeier at Thomas Jefferson University for his assistance in setting up the intro vital microscopy for visualization of real-time thrombus formation in mesenteric arterioles. This work is a part of Juan Xiao’s dissertation research performed at CHOP for her MD/PhD degree from Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Dr Jing Xue is a visiting scientist from the Department of Laboratory Medicine, Tianjin Huanhu Hospital, Tianjin, China.

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Disclosures

None.

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Disclosures

None.

References

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41. Xiao et al. Structure-Function Analysis of ADAMTS13 In Vivo 2269
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Juan Xiao, Sheng-Yu Jin, Jing Xue, Nicoletta Sorvillo, Jan Voorberg and X. Long Zheng

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Fig. 1.
Cleavage of guanidine-HCl denatured vWF by ADAMTS13. Murine VWF (mvWF) (lanes 1-6) or human vWF (hvWF) (lanes 7-12) was denatured for 2 h at 37°C with 1.5 M guanidine-HCl. The pre-denatured vWF was diluted 1:10 and incubated with 50 and 200 nM of recombinant human (hATS13) and murine ADAMTS13 (mATS13) as indicated in 20 mM Tris-HCl, pH=8.0, 5 mM CaCl$_2$, 1 uM ZnCl$_2$ and 1 mg/ml BSA at 37°C for 3 hours in the absence (-) or presence (-) of 10 mM EDTA. The uncleaved VWF (U) and cleavage products (P) were analyzed by 1% agarose gel and Western blotting with rabbit anti-human vWF IgG (1:5,000) followed by IRDye 800-labeled anti-rabbit IgG (1:10,000). The fluorescent signals were detected with an Odyssey imaging system as described previously $^9$. 

Supplemental Material
**Fig. II.**

**Cleavage of urea-denatured VWF by ADAMTS13.** Murine vWF (mvWF) (lanes 1-8) or human vWF (hvWF) (lanes 9-16) (50 nM) was incubated with 10, 40, and 200 nM of human ADAMTS13 (hATS13) or murine ADAMTS13 (mATS13) as indicated in 10 mM Tris-HCl, pH=8.0, 150 mM NaCl, 10 mM BaCl₂, and 1 mM Pefabloc for 10 min in the absence (-) or presence (+) of 10 mM EDTA. The reaction mixture was subjected to dialysis at 37 °C overnight against 2.0 M urea for mvWF or 1.5 M urea for hvWF. The uncleaved (U) and cleavage products (P) were analyzed by 1% agarose gel and Western blotting with rabbit anti-human vWF IgG (1:5,000) and IRDye 800-labeled anti-rabbit IgG (1:10,000). The fluorescent signals were obtained with an Odyssey imaging system as described previously.³⁵
Fig. III. Inhibition of ADAMTS13 activity by human monoclonal anti-spacer IgG. Human recombinant ADAMTS13 (0.6 nM) was incubated at 25 °C for 30 min with 0-35 nM of human monoclonal anti-spacer IgG (mAb II-1) or 35 nM of control human monoclonal IgG (mAb-c). The residual activity was determined by proteolytic cleavage of rF-VWF73 peptide (2 μM) as described previously \textsuperscript{26}. 
**Table I.**

**Diameters of mesenteric arterioles used in the experiments**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Diameter (µm)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>89.4 ± 9.3*</td>
<td>16</td>
</tr>
<tr>
<td>FL</td>
<td>93.6 ± 6.7</td>
<td>10</td>
</tr>
<tr>
<td>T8</td>
<td>93.5 ± 12.2</td>
<td>10</td>
</tr>
<tr>
<td>S</td>
<td>92.4 ± 10.2</td>
<td>10</td>
</tr>
<tr>
<td>T1</td>
<td>95.6 ± 8.7</td>
<td>12</td>
</tr>
<tr>
<td>d6a</td>
<td>92.9 ± 9.9</td>
<td>11</td>
</tr>
<tr>
<td>WT</td>
<td>90.5 ± 11.7</td>
<td>12</td>
</tr>
</tbody>
</table>

*The means ± standard deviation; N, number of mice in each group. p value=0.826, indicating no statistically significant difference among all groups studied using ANOVA one-way variance analysis with Tuley correction.

Supplemental Material