Essential Domains of A Disintegrin and Metalloprotease With Thrombospondin Type 1 Repeats-13 Metalloprotease Required for Modulation of Arterial Thrombosis

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Objective—A disintegrin and metalloprotease with thrombospondin type 1 repeats-13 (ADAMTS13) inhibits platelet aggregation and arterial thrombosis by cleavage of von Willebrand factor. However, the structural components of ADAMTS13 required for inhibition of arterial thrombosis are not fully defined.

Methods and Results—Using recombinant proteins and a murine model, we demonstrated that an ADAMTS13 variant truncated after either the eighth thrombospondin type 1 repeat or the spacer domain inhibits ferric chloride–induced arterial thrombosis in ADAMTS13−/− mice with efficacy similar to that of full-length ADAMTS13. The results obtained from monitoring thrombus formation in carotid and mesenteric arteries were highly concordant. Further analyses by site-directed mutagenesis and human monoclonal antibody inhibition assay revealed that the Cys-rich and spacer domains of ADAMTS13, particularly the amino acid residues between Arg559 and Glu664 in the spacer domain, may be critical for modulation of arterial thrombosis in vivo. Finally, the thrombosis-modulating function of ADAMTS13 and variants/mutants was highly correlated with the von Willebrand factor–cleavage activity under fluid shear stress.

Conclusion—Our results suggest that the amino terminus of ADAMTS13, specifically the variable region of the spacer domain, is crucial for modulation of arterial thromboses under (patho)physiological conditions. These findings shed more light on the structure-function relationship of ADAMTS13 in vivo and may be applicable for rational design of protein- or gene-based therapy of arterial thromboses. (Arterioscler Thromb Vasc Biol. 2011;31:2261-2269.)

Key Words: arterial thrombosis ■ ADAMTS13 ■ matrix metalloprotease ■ von Willebrand factor
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 in utero injection of lentiviral vector eliminated plasma ULVWF and inhibited ferric chloride (FeCl₃)-induced arterial occlusion in the carotid artery of ADAMTS13<sup>−/−</sup> mice.<sup>20</sup> Banno et al showed that a naturally occurring murine ADAMTS13 variant truncated after the sixth TSP1 repeat (ADAMTS13<sup>−/−</sup>) was less efficacious than full-length ADAMTS13 in inhibiting FeCl₃-induced thrombosis in the mesenteric arteriole.<sup>21</sup> More recently, de Maeyer et al reported that a recombinant murine ADAMTS13 variant truncated after the eighth TSP1 repeat, infused into ADAMTS13<sup>−/−</sup> mice, was not able to cleave newly released ULVWF/VWF strings on the endothelial cells in the mesenteric arterioles/venules.<sup>22</sup> 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 Antispacer 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.<sup>23</sup> Human VWF was purified from pooled normal human plasma using cryoprecipitation, Sephacryl-300 gel filtration as described previously.<sup>10</sup> Murine recombinant VWF was purified from pooled normal human plasma using cryoprecipitation, ferric chloride (FeCl₃)–induced arterial occlusion in the carotid artery of ADAMTS13<sup>−/−</sup> mice, was not able to cleave newly released ULVWF/VWF strings on the endothelial cells in the mesenteric arterioles/venules.<sup>22</sup>

**Figure 1.** Domain compositions and plasma half-lives of recombinant a disintegrin and metalloprotease with thrombospondin type 1 repeats-13 (ADAMTS13) and variants. A, Schematic representation of 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). All recombinant human ADAMTS13 proteins contain a V5-His tag at their C termini. B, Plasma concentrations of human ADAMTS13 and variants in ADAMTS13<sup>−/−</sup> mice at 5 minutes postinjection. C, The normalized plasma concentrations of human ADAMTS13 and variants in ADAMTS13<sup>−/−</sup> mice were plotted against time after injection. The half-life (t½) of each recombinant protein was determined from the curve. The data are the mean±SD of 3 independent experiments (n=3).

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 TTP<sup>24</sup> and control antibody against pneumococcus (mAb-c)<sup>25</sup> 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, Burlingham, 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 (fV-WF73) (2 μmol/L) as described previously. 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 inhaled halothane. Recombinant ADAMTS13 or variants diluted with 100 μL of normal saline were injected into mice via a retroorbital plexus. The amount of ADAMTS13 or variants injected was determined based on the body weight and blood volume of mice to achieve physiological plasma concentrations (~5 to 10 nmol/L). We used ~7 mL blood per 100 g of body weight in all of our calculations, as described. Blood samples (50 μL) were collected from a jugular vein at 5, 20, 40, and 70 minutes after protein injection and anticogulated 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, 10 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 SigmaPlot 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 calcein AM (Invitrogen) (2.5 μg/mL) for 15 minutes. 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 PGE1 (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 fluorescent 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). 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 lysophosphatidylcholine platelets (150×10^3 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, Hauppauge, NY). The total volume was 20 μL in all reactions. For kinetic determination, purified VWF at various monomer concentrations (0, 18.75, 37.5, 75, 150, and 300 nmol/L) was incubated with 25 nmol/L ADAMTS13 or T8, S, and d6a or 125 nmol/L T1, at a physiological concentration of FVIII (1 nmol/L) and lysophospholipid platelets (150×10^3/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. 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 K_m and k_cat 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-denaturation conditions. However, recombinant human ADAMTS13 infused into ADAMTS13−/− mice rapidly cleaved newly released ULVWF on endothelial cells, 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.
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 thrombosis 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 carotid 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), 11.1±2.1 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 antispacer 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-VWF73 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 \(\approx 4.5 \text{ nmol/L} \) (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.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 \(\approx 1500 \text{ s}^{-1}\), we determined the real-time thrombus formation in mesenteric arterioles. Mesenteric arterioles with diameters of 70 to 105 \(\mu\)m were chosen for the experiment.

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**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 \(\times 10\) 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, thrombosis 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 site of injury. Representative images (Figure 3A) and video accumulation of fluorescein-labeled murine platelets at the

Figure 4. Time-dependent cleavage of von Willebrand factor (VWF) by a disintegrin and metalloproteinase with thrombospondin type 1 repeats-13 (ADAMTS13) under fluid shear stress. Purified plasma VWF (150 nmol/L) was incubated for various times (0 to 60 minutes) with recombinant full-length human ADAMTS13 (FL) (25 nmol/L) (A); variants truncated after the eighth thrombospondin type 1 repeat (T8) (25 nmol/L) (B), the spacer domain (S) (25 nmol/L) (C); and the first TSP1 repeat (T1) (125 nmol/L) (D); and a mutant with residues Arg659 to Glu664 in the spacer domain deleted (d6a) (25 nmol/L) (E) in the presence of 1 mmol/L FVIII and 150×10³/µL lyophilized platelets under constant mixing at the rotation rate of 2500 rpm. The cleavage products (arrowheads) were determined by 1% agarose gel and Western blotting and quantified by ImageJ software. Uncleaved VWF is marked as U. In each blot, an FL was included for calibration (not shown). Right lanes are negative controls (with 10 mmol/L EDTA). T1 (125 nmol/L) was used because no cleavage product was observed with 25 mmol/L in the preliminary experiments. F represents the means±SEM from 3 independent experiments.

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 mmol/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 1.0×10⁶, 0.9×10⁵, and 0.7×10⁵ 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 abolished proteolytic activity toward VWF under the same conditions (Figures 4D and 5A to 5D). No cleavage product was detected even with addition of 125 nmol/L T1 after 60 minutes of vortexing. d6a, a mutant with residues Arg659 to Glu664, was detectable after 5 minutes of vortexing with both T1 and d6a at 25°C for 5 minutes (A and B) or 30 minutes (C and D) under a vortexing rotation rate of 2500 rpm in the presence of 1 mmol/L FVIII and 150×10^9/μL lyophilized platelets. (FL indicates full-length human ADAMTS13; T8, variant truncated after the eighth TSP1 repeat; S, variant truncated after the spacer domain; T1, variant truncated after the first TSP1 repeat; d6a, a mutant with residues Arg659 to Glu664 in the spacer domain deleted.) The proteolytic cleavage of VWF was determined by 1% agarose gel and Western blot as described in Materials and Methods. A and C show representative images of the Western blot for the formation of cleavage products (arrowheads) after 5 and 30 minutes of vortexing, respectively. Notice that although the VWF concentration in the reaction and the amount loaded into the gel for Western blotting in both A and B were the same, the contrast in A is higher than that in C for better visualization of the cleavage product. ImageJ was used to determine the intensity of cleavage product. The specific signal intensity (after subtraction of that with EDTA) normalized to that of FL cleavage in each blot (not shown) was plotted against the concentrations of VWF substrate (mean ± SEM) (n=3) (B and D). The quantitative data were fitted into a Michaelis-Menten equation to derive the kinetic constants, Km and kcat, for each ADAMTS13 variant. The molar concentration of VWF was assessed based on the monomer mass of 250 kDa.

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

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<th>kcat (s^-1)</th>
<th>Km (×10^-9 mol/L)</th>
<th>kcat/Km (×10^4 mol/L^-1 s^-1)</th>
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<tr>
<td>FL</td>
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<td>58.4±14.1</td>
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<td>60.0±55.5</td>
<td>0.9±0.8</td>
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<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>
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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.

### Discussion

ADAMTS13 limits platelet thrombogenesis presumably through proteolytic cleavage of newly released ULVWF anchored on endothelial cells, VWF in circulating blood, or VWF bound with platelets at the site of thrombus formation. 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 mmol/L) into ADAMTS13S/-/ 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 system,20,21 with one exception. For instance, ADAMTS13S/-/ 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 different 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 (ADAMTS13S/S) was nearly as efficacious as wild-type ADAMTS13 (ADAMTS13L/L) for inhibition of initial thrombus formation in mesenteric arterioles.21 However, ADAMTS13S/S was found to be slightly less efficacious than ADAMTS13L/L 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 strands 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...
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 antiatherial 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 antiatherial thrombotic 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 antiatherial thrombotic 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-cleaving 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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**Disclosures**

None.

**References**


Essential Domains of A Disintegrin and Metalloprotease With Thrombospondin Type 1 Repeats-13 Metalloprotease Required for Modulation of Arterial Thrombosis
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**Fig. I.**

**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.
**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 BaCl$_2$, 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 $^{35}$. 

![Image of gel electrophoresis](image-url)
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.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.