ADAMTS-13 Metalloprotease Interacts with the Endothelial Cell-derived Ultra-large von Willebrand Factor*  

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Thrombotic thrombocytopenic purpura is caused by congenital or acquired deficiency of ADAMTS-13, a metalloprotease that cleaves the endothelial-derived ultra-large multimers of von Willebrand factor (ULVWF). The proteolysis converts hyper-reactive and thrombogenic ULVWF into smaller and less adhesive plasma forms. Activity of ADAMTS-13 is usually measured in a static system under non-physiological conditions that require protein denaturation and prolonged incubation. We have demonstrated previously that ULVWF multimers, upon release from endothelial cells, form platelet-decorated string-like structures that are rapidly cleaved by ADAMTS-13. Here we report the direct interaction between ADAMTS-13 and VWF under both static and flowing conditions. ADAMTS-13-coated beads adhered to both immobilized VWF and ULVWF strings presented by stimulated endothelial cells. These beads adhered to VWF under both venous (2.5 dynes/cm²) and arterial (30 dynes/cm²) shear stresses. We then demonstrated that ADAMTS-13 beads adhered to immobilized recombinant VWF-A1 and -A3 domains, but soluble metalloprotease bound preferentially to the A3 domain, suggesting that the VWF A3 domain may be the primary docking site for the metalloprotease. We suggest that tensile stresses imposed by fluid shear stretch endothelial bound ULVWF multimers to expose binding sites within the A domains for circulating ADAMTS-13. The bound enzyme then cleaves within the A2 domain that lies in close proximity and releases smaller VWF multimers into the plasma. Once released, these cleaved VWF fragments become inaccessible for the metalloprotease to prevent further cleavage.

The hemostatic function of von Willebrand factor (VWF)¹ affixed to the subendothelium is to capture circulating platelets to the site of vessel injury by binding the platelet GP Ib-IX-V complex (1–3). This interaction results in bleeding arrest at the injury site. The similar interaction can also occur in circulation, leading to pathological platelet aggregation and thrombus formation, but it only occurs under extremely high fluid shear stress (2–5). VWF is synthesized and stored in only two places: α-granules of megakaryocytes/platelets and the Weibel-Palade bodies of endothelial cells (2, 6, 7). The latter, which constitute the main source of plasma VWF, release VWF either constitutively or upon stimulation of endothelial cells (2, 8). The newly released VWF is rich in the ultra-large and hyper-reactive forms (3, 8–11), which form high strength bonds with the platelet GP Ib-IX-V complex in the absence of any modulators (12, 13).

The direct release of these hyper-reactive ULVWF multimers into plasma is normally prevented by limited proteolysis of ULVWF by a VWF-cleaving enzyme present in plasma. This enzyme has recently been purified and cloned and found to be the 13th member of the ADAMTS (A Disintegrin and Metalloprotease with Thrombospondin motif) metalloprotease family (ADAMTS-13) (14–16). This metalloprotease cleaves in vitro at a single peptide bond between Tyr-842 and Met-843 in the VWF-A1 and -A3 domains, but soluble metalloprotease bound preferentially to the A3 domain, suggesting that the VWF A3 domain may be the primary docking site for the metalloprotease. We suggest that tensile stresses imposed by fluid shear stretch endothelial bound ULVWF multimers to expose binding sites within the A domains for circulating ADAMTS-13. The bound enzyme then cleaves within the A2 domain that lies in close proximity and releases smaller VWF multimers into the plasma. Once released, these cleaved VWF fragments become inaccessible for the metalloprotease to prevent further cleavage.

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§ The abbreviations used are: VWF, von Willebrand factor; HUVEC, human umbilical veins; BSA, bovine serum albumin; ULVWF, ultra-large von Willebrand factor; PBS, phosphate-buffered saline.
face of endothelial cells, providing an efficient mechanism that prevents the release of the hyper-reactive ULVWF into plasma. This mechanism is consistent with a recent report by Andre et al. (25) showing that GP Ibα-mediated platelet adhesion in vivo to stimulated mouse venular endothelium is dependent upon the endothelial cell-derived VWF. An interesting observation of this study is that platelet adhesion to endothelium begins within 15 s of stimulation and peaks after about 1 min, with a subsequent decrease in the number of adherent platelets. This time sequence coincides with our real time observation of ADAMTS-13 cleavage of ULVWF strings on the surface of stimulated human endothelial cells (13).

Because ADAMTS-13 is a secreted protein lacking a trans-membrane domain, it has to be captured to ULVWF from rapidly circulating plasma. Here we report that ADAMTS-13 interacts with VWF under both static and flowing conditions, and the interaction is predominantly through the A3 domain of VWF. These results may provide a means for ADAMTS-13 to form an enzyme-substrate complex and to cleave VWF strings on endothelial cells in blood flow.

EXPERIMENTAL PROCEDURES

Platelet and Plasma Preparations—Freshly drawn blood from 28 healthy donors was used as the source of plasma and platelets under a protocol approved by the Institutional Review Board of the Baylor College of Medicine. All donors signed consent forms before blood was drawn. The donor pool consisted of 16 females and 12 males, ranging in age from 24 to 48 years.

Whole blood drawn into 10% acid-citrate dextrose buffer (ACD, 85 mM sodium citrate, 111 mM glucose, and 71 mM citric acid) and was centrifuged at 1500 × g for 15 min at 24 °C to obtain platelet-rich plasma (PRP), which was then centrifuged at 900 × g for 10 min to separate the platelets from the plasma. Platelet pellets were washed once with a Ca2+ and Mg2+-free Tyrode’s buffer (138 mM sodium chloride, 5.5 mM glucose, 12 mM sodium bicarbonate, 2.9 mM potassium chloride, and 0.36 mM dibasic sodium phosphate, pH 7.4).

Plasma obtained using D-Phe-Pro-Arg-chloromethyl ketone, HCl (75 μM final concentration) as anticoagulant was used as the source of ADAMTS-13.

Endothelial Culture—Endothelial cells were obtained from human umbilical veins (HUVEC) under a protocol approved by the Institutional Review Board of the Baylor College of Medicine, as described previously (13, 26). The umbilical cords were washed with phosphate
buffer (140 mM NaCl, 0.4 mM KCl, 1.3 mM NaH₂PO₄, 1.0 mM Na₂HPO₄, 0.2% glucose, pH 7.4) and then infused with a collagenase solution (0.02%, Invitrogen). After 30 min of incubation at room temperature, the cords were rinsed with 100 ml of the phosphate buffer. Elutes containing endothelial cells were centrifuged at 250 × g for 10 min. The cell pellets were resuspended in Medium 199 (Invitrogen) containing 20% heat-inactivated fetal calf serum and 0.2 mM of L-glutamine and kept at 37 °C with a thermostatic air bath during the experiments.

Endothelial cells were activated with 25 μM histamine (Sigma) for 10 min at room temperature before the perfusion experiments.

**Parallel-Plate Flow Chamber**—We induced secretion and formation of ULVWF strings on endothelial cells using a previously described method with slight modifications (13, 27). Briefly, HUVECs growing in a 35-mm cell culture dish were stimulated with histamine and then assembled to form the bottom of the parallel plate flow chamber (Glycotech, Rockville, MD). The assembled chamber was connected to a syringe pump to draw the Tyrode’s buffer containing washed platelets through the chamber at defined flow rates to generate specific wall shear stresses. The chamber was mounted onto an inverted stage microscope (Nikon, Eclipse TE300, Garden City, NY) equipped with a high speed digital camera (Photometrics, Model Quantix, Tucson, AZ) and kept at 37 °C with a thermostatic air bath during the experiments. Acquired images were analyzed offline using MetaMorph software (Universal Images, West Chester, PA). Under this experimental condition, ULVWF released from endothelial cells formed string-like structures that were quantitated by counting individual strings in 20 continuous ×400 view fields after 2-min perfusion of the platelet suspension.

For cell-free flow experiments with polystyrene beads coated with test proteins, a glass coverslip coated with proteins under evaluation was assembled as the bottom of the flow chamber. ADAMTS-13 beads in Tyrode’s buffer were perfused over the immobilized protein under either 2.5 or 30 dynes/cm² shear stress. The number of adherent beads were counted after 2 or 5 min of perfusion.

**Polystyrene Beads and Protein Coating**—Polystyrene beads with green fluorescence (0.5 μm in diameter, fluoresbrite YG microspheres, Polysciences, Inc., Warrington, PA) were coated with purified ADAMTS-13 according to the manufacturer’s instructions. Briefly, beads were incubated with 200 μl of purified ADAMTS-13 (146 μg of total protein) overnight at room temperature with gentle shaking. Coated beads were washed twice with 0.5 ml of borate buffer, pH 8.5, and then incubated with 1 ml of 1% bovine serum albumin (BSA) for 30 min at room temperature. The beads were washed again with borate buffer and resuspended in PBS buffer containing 1% BSA. Control beads were coated with 10% BSA alone.

VWF-coated coverslips were prepared as described previously (27, 28). Briefly, glass coverslips were incubated with either UUVWF (2 μg/ml), plasma VWF (20 μg/ml), or recombinant VWF A domains (100 μg/ml) for 2 h at room temperature. Non-absorbed proteins were then removed by washing the coverslips with PBS buffer immediately before use. Coverslips coated with 5% of BSA were used as controls.

**Purification of Plasma VWF and Endothelium-derived ULVWF**—Plasma VWF was purified from human cryoprecipitate by glycine and NaCl precipitation and chromatography on a Sepharose 4B column (2.5 × 50 cm, a bed volume of 3000 ml, Amersham Biosciences) as described previously (26). UUVWF multimers were produced from HUVECs as described previously (10, 26, 29). For this, the confluent HUVECs were washed with PBS and incubated with a serum-free medium (insulin 5–10 μg/ml, transferrin 5 μg/ml, M199, 1% glutamine) for 48–72 h. The cultured cells were then treated with 100 μM histamine for 30 min at 37 °C to induce the release of UUVWF. After incubation, the conditioned medium was centrifuged at 150 × g for 10 min to remove cell debris, and the supernatant was used as the source of UUVWF multimers. The multimeric composition of purified plasma VWF and UUVWF was evaluated by SDS, 1% agarose gel electrophoresis and chemiluminescence.

**Purification of ADAMTS-13**—ADAMTS-13 was purified to the DEAE column step from factor VIII/VWF concentrate by the method described earlier (30). Purified ADAMTS-13 cleaved VWF in overnight incubation in the presence of 1 M urea and BaCl₂, yielding 176- and 140-kDa fragments on reduced SDS-PAGE gels.

**Purification of Human Glycocalcin**—Human glycocalcin was purified from platelets by a method described previously (31). Briefly, 10 liters of outdated PRP was centrifuged at 150 × g for 15 min. The platelet pellet was suspended in 500 ml of buffer B (10 mM Tris-HCl, 150 mM NaCl, and 2 mM CaCl₂, pH 7.4) and sonicated. The resultant suspension was incubated at 37 °C for 30 min to allow the calpain released from the platelets to cleave glycocalcin from membrane-bound
Expression and Purification of the Recombinant A Domains of VWF—

The recombinant VWF A domains (A1, A2, and A3) were expressed as histidine-tagged fusion proteins (HisA) and purified by a method described previously (32–34). Briefly, for the VWF-A1, the washed bacterial pellet was solubilized by 6.5 mg guanidine hydrochloride in 50 mM Tris-HCl, pH 7.5. The solubilized proteins, diluted 40-fold in 50 mM Tris-HCl, 500 mM NaCl, 0.2% Tween 20, pH 7.8, were eluted with 350 mM imidazole from a Ni²⁺-chelated Sepharose column (Amersham Biosciences) equilibrated with 25 mM Tris-HCl, 200 mM NaCl, pH 7.8 buffer). The isolated protein was adsorbed to and eluted from a heparin-Sepharose column (Amersham Biosciences). The purified polypeptide was dialyzed against 25 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.8.

For the VWF-A3, the washed bacterial pellet was solubilized by 7.5 mM urea in 25 mM Tris-HCl, pH 8.8. The solubilized protein was first dialyzed against 25 mM Tris-HCl, pH 8.2, and then eluted from a Q-Sepharose column (Amersham Biosciences) with sodium chloride.

For the purification of VWF-A2 (amino acids 718–905), the washed pellet was solubilized by 7.5 mM urea in 25 mM Tris-HCl, pH 8.8, and the solubilized protein was eluted from a Ni²⁺-chelated Sepharose column (Amersham Biosciences) equilibrated with 5 mM urea, 25 mM Tris-HCl, 200 mM NaCl, pH 7.8 buffer. The purified VWF-A2 was dialyzed against 1 mM urea, 25 mM Tris-Cl, 150 mM NaCl, pH 7.4. The buffer was rapidly changed to TBS, by using a desalting column.

Soluble ADAMTS-13 Interaction with VWF A Domains—To evaluate the interaction of ADAMTS-13 with the VWF A domains in fluid phase, we took advantage of the C-terminal histidine tag to capture the recombinant A domains to the CM5 sensor chip (Uppsala, Sweden) using a monoclonal anti-His antibody. For this, each recombinant VWF-A domain was perfused over the anti-His antibody immobilized onto the CM5 sensor chip for 3 min at 25 °C in the BIACore Biosensor (BIAcoreTM 2000, Biacore AB, Uppsala, Sweden). Uncaptured protein was then washed with 2.5% acetyl-D-glucosamine and 20 mM Tris-HCl, pH 7.4. The solubilized protein was removed by washing the chip with 200 µl of PBS buffer for 3 min. The purified ADAMTS-13 was then perfused over the surface at a flow rate of 20 µl/min at 25 °C. Binding was monitored in real time for 3 min and expressed as resonance units. The recombinant I domain of the α₂ chain of integrins was used as a negative control. The kinetics of the ADAMTS-13-A3 interaction was also determined by perfusing purified ADAMTS-13 (at 25, 50, 100, and 200 nM) over captured VWF A3.

Results—All experimental data are presented as mean ± S.E. The unpaired two-tailed Student’s t test was used for data analysis, and a p value less than 0.05 was considered to be statistically significant.

Adhesion of ADAMTS-13 beads to immobilized VWF under Static Conditions—

The unique pattern of adhesion of ADAMTS-13 beads strongly suggests that ADAMTS-13 directly interacts with VWF strings, but it may also be possible that ADAMTS-13 interacts with endothelium-derived molecules adjacent to VWF strings. To distinguish between these two possibilities, we examined adhesion of ADAMTS-13 beads to purified VWF in a cell-free system. The ADAMTS-13 beads were incubated with immobilized VWF for 10 min at room temperature, and the adherent beads were then counted after removal of unbound beads by PBS washing. ADAMTS-13 beads adhered specifically to immobilized VWF but not to a BSA-coated surface (Fig. 3A). The adhesion was blocked by a synthetic peptide derived from the CUB domain of ADAMTS-13 (HLEPTGTG, Sigma Genosys, St. Louis, MO), but not by a scrambled peptide with the same amino acid composition (PELHTTG, Sigma Genosys) (Fig. 3B).

In reverse experiments, polystyrene beads coated with ULVWF adhered to ADAMTS-13-coated surfaces but not to BSA surfaces (data not shown).

Adhesion of ADAMTS-13 beads to immobilized VWF was also examined at 30 dynes/cm², a shear stress that is often encountered in small arteries. After a 5-min perfusion at room temperature, adherent beads were counted and found to be significantly higher than those to the BSA surface (Fig. 5, Student’s t test, n = 4, *p < 0.001). There was no difference in the numbers of adherent beads at the two shear stresses tested. This experiment is designed to answer two questions. First, can ADAMTS-13 form a bond with VWF quickly enough to attach the beads to immobilized VWF under flow conditions? Second, once formed, is the ADAMTS-13-VWF bond strong enough to

![Fig. 5. ADAMTS-13 beads directly adhere to immobilized VWF at both low and high shear stresses.](http://www.jbc.org/Downloaded from http://www.jbc.org/ by guest on July 24, 2018)
ADAMTS-13 Interaction with VWF

ADAMTS-13 Interact with the A Domains of VWF under Flow—Having demonstrated a direct interaction between ADAMTS-13 and VWF, we investigated the regions of VWF that may interact with ADAMTS-13. Because ADAMTS-13 cleaves within the A2 domain of VWF, we first focused on the A domains of VWF as potential ADAMTS-13 docking site(s). ADAMTS-13 beads were incubated with each of the three immobilized recombinant A domains for 5 min and then exposed to a fluid shear stress of 2.5 dynes/cm² for 2 min. A significantly higher percentage of ADAMTS-13 beads remained adherent to the A1 and A3 surfaces, whereas the adhesion to A2 surface was minimal (Fig. 6, A and B). BSA beads failed to adhere to any of the A domain-coated surfaces. Similarly, ADAMTS-13 beads did not adhere to a BSA-coated surface.

Adhesion of ADAMTS-13 beads to the A domains was prevented by pretreatment of these beads with soluble A domains (Fig. 6, C and D). In contrast, glycocalcin, the extracellular portion of GP Ibα¹, failed to block binding of ADAMTS-13 to the immobilized A1 domain (Fig. 6C).

ADAMTS-13-VWF Interaction Determined by Surface Plasmon Resonance—The previous section demonstrated the interaction between ADAMTS-13 and VWF with both proteins immobilized on a surface. Because immobilization is known to activate VWF (36) and the interaction between ADAMTS-13 and VWF in vivo likely occurs in solution, we tested by surface plasmon resonance whether soluble ADAMTS-13 would interact with the A domains in the fluid phase. For this, the A domain proteins were first captured through their His tags to the chips coated with a monoclonal anti-His antibody. Soluble ADAMTS-13 metalloprotease was then perfused over the chips. As shown in Fig. 7, ADAMTS-13 predominantly bound to the captured A3 domain and, to a lesser extent, to the A1 and A2 domains. By comparison, it did not bind to the surface coated with the immobilized I domain of the α₂ integrin (Student’s t test, n = 3). The kinetics of the ADAMTS-13-A3 interaction was determined by perfusion of ADAMTS-13 at concentrations of 25, 50, 100, and 200 nM over the captured A3 under a constant flow of 20 μl/min. The apparent dissociation constant (Kd) was calculated to be 58.7 nM.

DISCUSSION

In the current study, we demonstrated by several means that ADAMTS-13 interacts with VWF primarily through the VWF A domains. First, polystyrene beads coated with ADAMTS-13 adhere to ULVWF strings formed on histamine-activated endothelial cells. Second, ADAMTS-13 adhered to immobilized VWF under both static and flowing conditions. Third, ADAMTS-13 beads adhered to the immobilized A1 and A3 domains but significantly less to A2 domain. The finding that glycocalcin failed to block adhesion of ADAMTS-13 beads to the immobilized A1 domain suggests that the ADAMTS-13-binding site on the A1 domain is likely to be different from that of the platelet GP Ib-IX-V complex. In contrast to results obtained with immobilized ADAMTS-13, soluble ADAMTS-13 bound predominantly to the A3 domain and much less to the A1 domain in fluid phase as demonstrated by the surface plasmon resonance experiments. These results, combined with our previous finding that ULVWF forms string-like structures on endothelial cells under flow (13), provide a mechanism through which ADAMTS-13 cleaves ULVWF multimers that are newly released from endothelial cells.

We hypothesize that, upon release from the Weibel-Palade bodies of stimulated endothelial cells through a triggering event, ULVWF multimers are anchored to the surface of endothelial cells and capture circulating platelets. The ULVWF anchorage and wall shear stress as well as torque forces applied to platelets adhered to VWF strings may stretch the ULVWF multimers into the elongated string-like structures to expose either the cleavage site or the anchorage site for ADAMTS-13. As a result, circulating ADAMTS-13 is able to bind to ULVWF strings to form enzyme-substrate complexes to cleave VWF. Once VWF is cleaved at selected sites, the fragments are released into plasma where they no longer experience the tensile stress as they do when tethered to the endothelial surface, and therefore are able to adopt a conformation...
that is no longer accessible to the metalloprotease. This is consistent with previous studies (37) showing that VWF multimers change from a globular structure to an extended linear one after being exposed to shear stress. This proposed mechanism of cleavage offers several advantages over other mechanisms. First, the cleavage occurs very rapidly under flowing conditions to satisfy the in vivo requirements. Second, it could explain the source of the size heterogeneity of plasma VWF. For example, VWF sites for ADAMTS-13 may not always be available, either because of an unfavorable conformation of the docking site or because of steric hindrance by platelets that adhere to the VWF strings first. In either case, the selective adhesion of ADAMTS-13 to VWF strings likely results in the release of VWF fragments of various sizes, yielding the size heterogeneity observed for plasma VWF. This suggestion is supported by our observation that the adhesion of ADAMTS-13 to the VWF substrate in plasma does not completely cleave the plasma VWF to its minimal form. One possibility is that the endothelium-derived ULVWF and plasma VWF interact differently with ADAMTS-13, possibly because of the different conformation of the A domains. We have observed such a difference for the VWF-GP Ib interaction (12). We found that ULVWF forms spontaneous high strength bonds with the GP Ib-IX-V complex, with similar bond strengths to those formed between GP Ib-IX-V and the isolated A1 domain. The plasma form of VWF, on the other hand, does not spontaneously bind GP Ib-IX-V, requiring the modulators ristocetin or botrocetin to do so. When considered in the light of these findings, our current observations lend weight to the notion that the A domain region of VWF exists in an inherently different conformation in ULVWF than in the processed, plasma forms of VWF. Finally, this putative mechanism safeguards against the direct release of hyper-reactive ULVWF into plasma by allowing for its cleavage right at the site of its release.

Because the cleavage site on VWF is in the A2 domain, it is logical for ADAMTS-13 to be anchored to the same domain. However, as shown in Fig. 6B, ADAMTS-13 bound equally well to the immobilized A1 and A3 domain but minimally to the A2 domain. Furthermore, when the interaction was induced in fluid phase as demonstrated by the surface plasmon resonance experiments, ADAMTS-13 bound predominantly to the A3 domain (Fig. 7). Because the cleavage of ULVWF in vivo occurs in the fluid phase (the VWF strings are anchored to only a few sites and appeared to wave in the flowing fluid rather than being attached firmly to the endothelial cells (13)), the ADAMTS-13-A3 interaction may be more relevant for docking the metalloprotease to VWF strings.

In summary, we have demonstrated in this study that the VWF cleaving metalloprotease ADAMTS-13 binds to ULVWF under both static and flow conditions. The interaction is strong enough to resist considerable shear stress and has a fast on rate, meeting the critical requirements for the interaction to occur in rapidly flowing blood. Furthermore, the adhesion occurs randomly and discontinuously along the VWF strings with spaces of various lengths between the adhesion sites for ADAMTS-13, a phenomenon that may explain the heterogeneity of the size of plasma VWF. Finally, both A1 and A3 may contain the binding sites for ADAMTS-13, but the A3 domain appears to be more important in vivo for docking the metalloprotease to VWF strings on the surface of endothelial cells in flowing blood.

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