Factor VIII and Platelets Synergistically Accelerate Cleavage of von Willebrand Factor by ADAMTS13 under Fluid Shear Stress*

Christopher G. Skipwith§, Wenjing Cao§, and X. Long Zheng†§

From the Biochemistry and Molecular Biophysics Graduate Program and the Department of Pathology and Laboratory Medicine, the Children’s Hospital of Philadelphia and the University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104

Previous studies have demonstrated that factor VIII (FVIII) or platelets alone increase cleavage of von Willebrand factor (VWF) by ADAMTS13 under mechanically induced shear stresses. We show in this study that the combination of FVIII and platelets at the physiological concentrations is more effective than either one alone. In the absence of FVIII, lyophilized platelets increase the formation of cleavage product by 2–3-fold. However, in the presence of physiological concentration of FVIII (1 nM), the formation of VWF cleavage product increases dramatically as a function of increasing platelets with the maximal rate enhancement of ~8-fold. Conversely, in the presence of a physiological concentration of lyophilized platelets (150 × 10^3/μl), the half-maximal concentration of FVIII required to accelerate VWF proteolysis by ADAMTS13 reduces by ~10-fold (to ~0.3 nM) compared with that in the absence of platelets (~3.0 nM). Further studies using the FVIII derivative that lacks an acidic region (a3), an antiplatelet glycoprotein Ibα IgG, and a purified recombinant VWF-A1 domain or glycoprotein Ibα-striped platelets demonstrate that the synergistic rate-enhancing effect of FVIII and platelets depends on their specific binding interactions with VWF. Our findings suggest that FVIII and platelets are cofactors that regulate proteolysis of multimeric VWF by ADAMTS13 under physiological conditions.

ADAMTS13, a member of the A Disintegrin And Metallopeptase with Thrombospondin type repeats (ADAMTS)2 family (1, 2), controls the sizes of von Willebrand factor (VWF) by cleaving VWF at the Tyr1605–Met1606 bond in the central A2 domain (3, 4). This proteolytic cleavage appears to be critical for regulating VWF adhesive function and maintaining normal hemostasis (5). The inability to cleave the ultralarge (UL) VWF into the smaller forms due to a hereditary (1, 6–8) or acquired deficiency (9–11) of plasma ADAMTS13 results in thrombocytopenic purpura, a potentially fatal thrombotic microangiopathy (5, 12, 13). Conversely, excessive proteolytic cleavage of plasma multimeric VWF by ADAMTS13 leads to a certain subtype of von Willebrand disease (5, 14), the most common bleeding disorder seen in the hematology clinic.

Proteolytic processing of UL-VWF by ADAMTS13 appears to occur at least at two different sites: one is on the endothelial cells where UL-VWF is newly released from Weibel-Palade bodies upon stimulation (15, 16), and the other may occur in solution (or blood) (3, 10). The cleavage of the cell membrane-anchored UL-VWF by ADAMTS13 occurs very rapidly and requires low (15–17) or almost no shear stress (18, 19). However, the cleavage of UL-VWF on the endothelial cell membrane does not appear to be sufficient to reduce the VWF multimer sizes that have been observed in plasma. This is inferred from our study on cultured endothelial cells. The soluble VWF cleaved from the endothelial cell membrane by ADAMTS13 remains ultralarge in size, and the multimer distribution is not different from that released from Weibel-Palade bodies upon histamine stimulation (18). Thus, further proteolytic cleavage of the released or soluble UL-VWF in circulation may be necessary to reduce VWF multimer sizes consistent with normal distribution. This is likely accomplished in small arterioles and capillaries, where high fluid shear stress is present. Patients with aortic stenosis, which generates high flow shear stress in circulation, do exhibit increased VWF proteolysis and reduced VWF multimer size compared with healthy individuals (20, 21), suggesting that increased VWF proteolysis is correlated with increased fluid shear stress in vivo. However, under in vitro assay conditions, fluid shear stress alone (22, 23) has only a modest effect on proteolytic cleavage of soluble multimeric VWF by ADAMTS13, suggesting that other proteins or non-protein cofactors or cellular components may be required for efficient proteolytic processing of soluble multimeric VWF by ADAMTS13.

Shim et al. (24) and we (22) have demonstrated that lyophilized platelets or coagulation factor VIII (FVIII) alone at physiological concentrations can increase the proteolytic cleavage of VWF by ADAMTS13 by ~2–3-fold under mechanically induced fluid shear stresses (22, 24). In this study, we show that the combination of the two (FVIII and platelets) at physiological concentrations synergistically accelerates the proteolytic cleavage of VWF by ADAMTS13 under similar conditions. This rate-enhancing effect of both platelets and FVIII on ADAMTS13-mediated VWF proteolysis depends on the
specific high affinity interactions between FVIII/platelets and VWF. These findings suggest that binding of the platelet GP1b receptor and FVIII to the specific regions of VWF may accelerate the conformational alterations under fluid shear stress, thereby allowing ADAMTS13 to access the binding site and cleavage bond. Our study provides a novel insight into how proteolytic cleavage of soluble multimeric VWF by ADAMTS13 may be regulated under physiologically relevant conditions.

EXPERIMENTAL PROCEDURES

Preparation of Fresh and Recombinant Proteins—Multimeric VWF was prepared from plasma using the method developed in the laboratory. All studies involving human materials were approved by the Institutional Review Board, the Children’s Hospital of Philadelphia. Briefly, citrated human plasma (2.5 liters) yet to be discarded in the Apheresis Unit, the University of Pennsylvania Hospital, was frozen at −80 °C and thawed at 4 °C overnight. The thawed plasma was centrifuged at 4 °C for 30 min at 15,000 × g. The supernatant was decanted, and the cryoprecipitate was dissolved with ∼80 ml of PBS in the presence of 20 mM EDTA, 1 mM PMSF, and 5 mM benzamidine at 37 °C. After removal of the insoluble materials by centrifugation, the supernatant was further precipitated with 10% polyethylene glycol (PEG) 8,000 to reduce the volume. The precipitate was then redissolved with 10 ml of PBS. Only 5 ml of clear supernatant (∼1% of the column volume) was loaded onto a Sephacryl-300 gel filtration column (2.5 cm × 100 cm, ~490 ml) (GE Healthcare). The purity of VWF was determined by 8% SDS-PAGE and Coomassie Blue staining. The multimer distribution was determined by 1% mini-agarose gel electrophoresis and Western blotting as described previously (22, 25). The amount of purified VWF was determined by A\text{280 nm} corrected by A\text{320 nm} (1 corrected A\text{280 nm} = approximately 1 mg/ml) (26). The purified plasma VWF is free of detectable FVIII, assessed by an ELISA established in the laboratory (data not shown). Recombinant ADAMTS13 was expressed in stably transfected HEK293 cells and purified according to the protocol described previously (23, 27). The contaminated proteins were removed by gel filtration on a Superose-6 column (GE Healthcare) with 20 mM HEPES, pH 7.5, 150 mM NaCl, and 5 mM CaCl\text{2}. The amount of the purified proteins was determined by absorbance at 280 nm (corrected with light scattering at 320 nm) (1 corrected A\text{280 nm} = 0.68 mg/ml) as described previously (23, 27). Recombinant human FVIII and FVIII derivative (FVIII-2RKR) were prepared as previously described (22). The final products were exchanged into 20 mM HEPES, 0.15 M NaCl, and 5 mM CaCl\text{2}, pH 7.5, and stored at −80 °C.

Preparation of Fresh and Lyophilized Human Platelets—Whole blood (10 ml) from a healthy volunteer was drawn to a blue top tube containing 3.8% sodium citrate and 1 μg/ml prostaglandin E1. Platelet-rich plasma was prepared as previously described (22). The final products were exchanged into 20 mM HEPES, pH 7.4, 137 mM NaCl, 5.6 mM glucose, 1 g/liter BSA, 1 mM MgCl\text{2}, 2.7 mM KCl, 3.3 mM NaH\text{2}PO\text{4}. The fractions containing platelets were pooled, and the platelet concentration was determined by manual counting using a hemacytometer under a light microscope.

Formalin-fixed and lyophilized human platelets were purchased from Helena Laboratories (Beaumont, TX). The platelets were reconstituted with 10 ml of 20 mM Tris-HCl, pH 7.4, and 100 mM NaCl and centrifuged at 10,000 rpm for 5 min. The pellets were resuspended in the same buffer to a platelet concentration of 20 × 10\text{6}/μl, determined by counting in the hemacytometer.

Removal of GP1b Receptor from Fresh Platelets by O-Sialoglycoprotein Endopeptidase—Freshly isolated human platelets (3 × 10\text{9}/μl) were treated with or without various concentrations (0–400 μg/ml) of O-sialoglycoprotein endopeptidase (OSE) isolated from Pasteurella hemolytica (28–30) (Cedarlane Laboratories, Burlington, NC) at 25 °C for 4 h in modified Tyrode’s buffer. OSE specifically cleaves proteins containing O-linked glycans but spares N-linked glycoproteins or unglycosylated proteins (43–45). This metalloendopeptidase has been reported specifically to cleave the 25-kDa N-terminal fragment of platelet GPIbα, which is heavily O-glycosylated, while not affecting other receptors such as GPIIb-IIIa (31). The enzyme digested fresh platelets were washed with modified Tyrode’s buffer to remove the excessive protease, soluble GPIb, and VWF prior to addition into the reactions.

Ristocetin-induced Platelet Agglutination Assay—Freshly isolated platelets that were treated with the modified Tyrode’s buffer alone or various concentrations of OSE (0, 25, 50, 100, 200, and 400 μg/ml) in modified Tyrode’s buffer for 4 h were mixed with purified plasma VWF (15 μg/ml, final concentration) and ristocetin (1 mg/ml) (Helena Laboratories) in a glass tube with stirring at 1,100 rpm. The light transmission was recorded simultaneously in all the channels for 4 min at 37 °C with a PAP-8E aggregation module (Bio/Data Corporation, Horsham, PA). The relative difference in light transmission before and after addition of ristocetin was used to determine the percentage of platelet agglutination. The light transmission prior to addition of ristocetin was defined as 0%, whereas the maximal light transmission was 100%.

Cleavage of Multimeric VWF by ADAMTS13 under Fluid Shear Stress—Purified plasma VWF (37.5 μg/ml or 150 nM VWF subunits) was incubated with recombinant ADAMTS13 (25 nM) in the presence or absence of various other components (such as FVIII, FVIII-2RKR, and platelets) in 20 mM HEPES, pH 7.5, containing 1 mg/ml BSA, 150 mM NaCl, and 5 mM CaCl\text{2} in a 0.2-ml thin walled PCR tube with dome caps (total volume 20 μl) for 10 min. The reaction mixtures were then subjected to mixing in a 96-well Eppendorf MixMate vortexer (Fisher-Thermo Scientific) at a rotation rate of 2,500 rpm for 10 min. The reaction mixtures were then denatured without reducing agents at 100 °C for 5 min with a sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, and 0.01% bromphenol blue) for the SDS-PAGE or at 60 °C for 20 min with a sample buffer (70 mM Tris-HCl, pH 6.5, containing 2.4% SDS, 4% urea, and 4 mM EDTA) for the agarose gel. The denatured VWF substrate was fractionated either in a 5% Tris-glycine SDS-polyacrylamide gel at 25 °C, 120 V for 150 min or in a 1% agarose gel at 4 °C, 15 mA for 100 min in a mini-protein III gel cassette (Bio-Rad). The proteins were then transferred onto a nitrocel-
lulose membrane (Millipore) at 100 mA for 60 min from the SDS-polyacrylamide gel and at 15 V for 30 min from the agarose gel, respectively. The proteolytic cleavage products of VWF were detected by rabbit anti-VWF IgG (1:5,000) (Dako, Carpinteria, CA) and IRDye 800CW-labeled goat anti-rabbit IgG (1:12,500–20,000) (LI-COR Bioscience, Lincoln, NE) in TBS containing 1% casein and 0.05% Tween 20. The fluorescent signals were collected by an Odyssey infrared imaging system (LI-COR Bioscience) (22). The cleavage products were quantified by ImageJ software (National Institutes of Health) after the fluorescent intensities were converted into the gray image signals.

RESULTS

Factor VIII and Platelets Synergistically Increase Cleavage of VWF by ADAMTS13 under Fluid Shear Stress—Previous studies have shown that FVIII (22) or platelets (24) separately increase proteolytic cleavage of VWF by ADAMTS13 under mechanically induced fluid shear stress. FVIII and platelets both bind VWF with high affinity at two distinct regions, i.e. the D’D3 domain and A1 domain, respectively (14). We sought to determine whether binding of FVIII and platelets to VWF could synergistically enhance proteolytic cleavage of VWF by ADAMTS13 under fluid shear stress conditions. In these experiments, purified plasma-derived VWF (~150 nM or 37.5 μg/ml) was incubated with purified recombinant ADAMTS13 (~25 nM) in the absence or in the presence of a fixed concentration of purified recombinant FVIII (1 nM) or lyophilized platelets (150 × 10^3/μl) or both at 25 °C for 10 min in assay buffer (20 mM HEPES, pH 7.5, 1 mg/ml BSA, 150 mM NaCl, and 5 mM CaCl_2) under constant vortexing (2,500 rpm) in a PCR tube mixer. Under the current conditions, the control reactions in the presence of EDTA (10 mM) or in the absence of ADAMTS13 or without vortexing exhibited no detectable cleavage product by Western blotting (data not shown). The proteolytic cleavage of VWF by ADAMTS13 in the presence of 1 nM FVIII or 150–600 × 10^3/μl lyophilized platelets alone increased by 2–3-fold after 10 min of incubation on the PCR tube mixer (2,500 rpm) (Fig. 1). However, when both FVIII (1 nM) and lyophilized platelets (150 × 10^3/μl) were added into the reaction, a more substantial increase in the formation of cleavage product was detected than in the presence of either FVIII or platelets alone (Fig. 1). The amount of cleavage product formed when both FVIII and platelets were present was ~8-fold (Fig. 1), greater than the sum of the product generated in the presence of either FVIII or platelets alone (not shown), suggesting a synergistic effect of FVIII and platelets in enhancing VWF proteolysis by ADAMTS13 under fluid shear stress.

Conversely, in the absence of platelets FVIII alone, increased the formation of cleavage product in a concentration-dependent manner (Fig. 2). However, in the presence of a physiological concentration of platelets (~150 × 10^3/μl), FVIII more dramatically increased the formation of the proteolytic cleavage product, which shifted the FVIII dose-response curve to the left. Therefore, the concentration of FVIII required to achieve the half-maximal cleavage of VWF by ADAMTS13 (C_{50}) was reduced by 10-fold (from 3.0 nM in the absence of platelets to 0.3 nM in the presence of platelets) (Fig. 2), which falls into the physiological range of human plasma FVIII concentrations.

Binding of FVIII to VWF Is Required for Their Synergistic Effect on VWF Proteolysis by ADAMTS13 under Shear Stress—FVIII binds VWF with high affinity (K_D 0.3~0.5 nM) through its interaction with the D’D3 domain of VWF (32, 33). This high affinity binding appears to be necessary for the enhancing effect of FVIII on VWF proteolysis by ADAMTS13 under shear stress (22). To determine whether the binding of FVIII to VWF is also required for synergistic rate enhancement with platelets, we incubated plasma VWF (150 nM) with ADAMTS13 (25 nM) in the presence of 1 nM B-domainless FVIII (150 nM) increased the formation of proteolytic cleavage of VWF by ADAMTS13 by ~2–3-fold (Fig. 3), similar to that observed in the absence of FVIII. These results suggest that the high affinity binding interaction...
between FVIII and VWF is required for the synergistic effect of FVIII with platelets accelerating VWF proteolysis by ADAMTS13 under fluid shear stress.

**Binding of Platelet Receptor GP1bα to VWF Is Also Required for the Synergistic Effect of Platelets with FVIII on VWF Proteolysis by ADAMTS13 under Shear Stress**—Platelets bind VWF with high affinity through their interactions of the surface receptor GP1b with the A1 domain of VWF (14). To determine whether binding of platelet receptor GP1bα to the VWF-A1 domain may be required for the synergistic effect of platelets with FVIII on VWF proteolysis by ADAMTS13, we incubated VWF and ADAMTS13 in the absence and presence of increasing concentrations of monoclonal antibody 6D1 (Fig. 4, A and C), provided by Dr. Barry Coller at Rockefeller University, New York, NY (34) or purified A1 domain (Fig. 4, B and D), provided by Dr. Miguel Cruz at Baylor College of Medicine, Houston, TX, under constant vortexing at 2,500 rpm for 10 min. We showed that an addition of purified A1 domain or 6D1 to the reaction dramatically inhibited the formation of the proteolytic cleavage product in a concentration-dependent manner.

**FIGURE 4. Disruption of VWF-platelet interactions inhibits cleavage of VWF by ADAMTS13 under shear stress.** Purified plasma VWF (150 nM) was incubated with recombinant ADAMTS13 (25 nM), lyophilized platelets (150 × 10^3/μl), and FVIII (1 nM) in the absence or presence of increasing concentrations of 6D1, an anti-GP1bα monoclonal antibody (0–3.3 μM) (A) or purified recombinant A1 domain (0–100 μM) (B). The proteolytic cleavage product (350 kDa) (arrowhead) was detected by 5% SDS-PAGE and Western blotting as described in the Fig. 1 after 10 min of constant mixing on the MixMate PCR mixer. The relative cleavage product formation was quantified by densitometry using ImageJ software and plotted against the concentrations of 6D1 (C) and A1 domain (D), respectively.
ADAMTS13 Cleavage of von Willebrand Factor

**FIGURE 5.** Removal of surface GP1bα receptor reduced the synergistic effect of platelets with FVIII on proteolytic cleavage of VWF by ADAMTS13. Purified VWF (150 nM) was incubated for 10 min under constant vortexing (2,500 rpm) in a MixMate mixer with ADAMTS13 (25 nM), and FVIII (1 nM) in the absence or the presence of various concentrations of freshly purified platelets (50–150 × 10^3/μl) that were pretreated with a buffer alone (lanes 1–5) or with 200 μg/ml OSE (lanes 7–11) for 4 h and washed. The proteolytic cleavage of VWF was assessed by 1% agarose gel electrophoresis and Western blotting using rabbit anti-VWF IgG and IRDye 800CW-labeled goat anti-rabbit IgG. Arrowhead indicates the cleavage product ~350 kDa (A), which was quantified by densitometry using ImageJ and plotted against final concentrations of platelets (B). The removal of GP1bα receptor from the platelet surface after treatment with various concentrations of OSE (0–400 μg/ml) was assessed by the ristocetin-induced light-scattering platelet agglutination assay (C).

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**C**

![Time vs. Light Transmission](image)

Light Transmission (%)

- 0 ug/ml
- 12.5 ug/ml
- 25 ug/ml
- 50 ug/ml
- 100 ug/ml
- 200 ug/ml
- 400 ug/ml

Time (sec)

20 to 220

- 0 to 100

As a control, fresh platelets treated with a buffer alone worked as well as or appeared to be better than the lyophilized platelets in accelerating VWF proteolysis by ADAMTS13 under fluid shear stress (Fig. 5, A and B), consistent with that previously reported (24). The more pronounced rate enhancing effect of fresh platelets than lyophilized platelets may be partially attributed to the platelet-bound VWF, which increased VWF substrate concentration in the reactions as demonstrated in the multimer analysis (Fig. 5A). Such a result was not observed using a 5% SDS-polyacrylamide gel by Shim et al. (24) and us (data not shown), as most of the undigested VWF could not get into the gel for accurate assessment of VWF antigen. Treatment of freshly isolated platelets with OSE removed platelet-bound VWF as a result of the removal of GP1bα receptor on the platelet surface (Fig. 5A).

To assess whether the GP1bα receptor was removed or not, we tested the ability of these treated platelets to agglutinate upon induction with ristocetin. As shown in Fig. 5C, OSE treatment dramatically reduced ristocetin-induced platelet agglutination in a concentration-dependent manner.

Assessing the Amount of Fluid Shear Stress Generated in Our Assay System—The described in vitro system, which contains only plasma VWF, ADAMTS13, FVIII, platelets, and a physiologic buffer, is not subject to the changes in fluid viscosity that would be observed in blood. The system could, thus, be considered a Newtonian system in which the relationship between shear stress and shear rate is linear. Using a cone-plate viscometer we showed that water (Fig. 6A) and a reaction system (Fig. 6B) containing purified plasma VWF (~150 nM), recombinant ADAMTS13 (25 nM), recombinant FVIII (2 nM), and lyophilized platelets (150 × 10^3/μl) exhibited a Newtonian behavior. This indicates that the observed increase in cleavage product can be directly correlated with the increase in fluid shear stress generated in the system, but not with the changes in the fluid viscosity of the reaction mixture. To assess the wall shear stress generated in our vortex assay reactions, we compared the fold increase of the VWF cleavage product in the presence and in the absence of FVIII and platelets on a cone-plate viscometer with that on two different vortexers. We found that the fold increase in the formation of VWF cleavage product dramatically increased as a function of increasing fluid shear stress on the cone-plate viscometer in the presence of physiologic concentrations of FVIII and platelets, but modestly increased in the absence of added FVIII and platelets (Fig. 6C). Curve fitting of the data obtained from three independent experiments revealed a linear relationship between the shear stress and the fold enhancement in the VWF cleavage (Fig. 6D). Using this standard function, the fluid shear stress generated in the MixMate PCR tube mixer and mini-vortexer was estimated.
ADAMTS13 Cleavage of von Willebrand Factor

FIGURE 6. Assessing the fluid shear stress generated by various vortexing assays. A and B, linear relationships between the averaged fluid shear stress and shear rate when water and a reaction mixture containing VWF (150 nM), ADAMTS13 (25 nM), FVIII (2 nM), and lyophilized platelets (150 × 10^7/μl) was placed on the cone-plate viscometer, respectively. The linear relationship between the shear stress and shear rate in the assay mixture suggests a laminar, but not turbulent flow generated similar to that generated in the water. C, shear-dependent increase in proteolytic cleavage product in the absence and the presence of FVIII/platelets. In this experiment, purified plasma VWF (~150 nM) was incubated with recombinant ADAMTS13 (25 nM) in the absence (−) or the presence (+) of recombinant FVIII (2 nM) and lyophilized platelets (150 × 10^7/μl) for 10 min under increasing shear rates on a cone-plate viscometer (cone diameter 35 mm, 0.5° angle) (HAAKE Roto-Visco1) (lanes 1–8) or mini-vortexer (Fisher Scientific) (lanes 9 and 10) or MixMate PCR mixer (Eppendorf) (lanes 11 and 12) for 10 min at rotation rate of 2,500 rpm. The samples were denatured and separated with a 5% SDS-PAGE. The proteolytic cleavage product (350 kDa) (arrowhead) was determined by Western blotting as described in Fig. 1. D, ratio of the cleavage product (mean ± S.D.) (y axis) in the presence of FVIII/platelets to that in the absence of FVIII/platelets obtained from the cone-plate viscometer experiments (n = 3) plotted against fluid shear stress (x axis). As seen, the mini-vortexer and the MixMate PCR mixer generate ~75 dynes/cm² and 32 dynes/cm² of shear stress, respectively.

to be ~32 dynes/cm² and ~75 dynes/cm², respectively (Fig. 6, C and D). The difference in fluid shear stress generated among the PCR tube mixer, the mini-vortexer and cone plate viscometer may offer some explanation for the formation of various amounts of proteolytic cleavage product in different assays.

DISCUSSION

This study demonstrates the synergistic effect of FVIII and platelets, accelerating proteolytic cleavage of soluble VWF by ADAMTS13 under mechanically induced fluid shear stress. Unlike cleavage of newly released and membrane-anchored UL-VWF on stimulated endothelial cells, which requires little or no shear stress (15, 16, 18, 19) or cofactors (18), the proteolytic cleavage of soluble VWF by ADAMTS13 requires drastic manipulations, such as denaturization of VWF substrate with 1.5 M urea/guanidine (3, 27, 35) or exposure of soluble VWF to high fluid shear stress (22, 23). In vitro studies have demonstrated that even under high shear stress, the proteolytic cleavage of multimeric VWF in solution by ADAMTS13 is rather slow and inefficient. This is because VWF in solution adopts a globular conformation with various subunits held together by weak interactions (36). Such a globular VWF is neither active for ligand binding (such as interacting with platelet GP1b) (37) nor sensitive to ADAMTS13 proteolysis (23, 38), although the C terminus of globular VWF (the D4-CK domain) is able to interact with the C-terminal TSP1 5–8 repeats of ADAMTS13 in the absence of denaturants or shear stress (38, 39). Such an initial contact may be critical for the subsequent interaction between the exposed VWF-A2 domain and the proximal C-terminal domains of ADAMTS13, leading to proteolytic cleavage of multimeric VWF under fluid shear stress. A disruption of these C-terminal VWF and C-terminal ADAMTS13 interactions inhibits VWF proteolysis under these conditions (38, 39). High fluid shear stress results in conformational changes of VWF multimers and opens up the additional binding sites and cleavage bond in the central A2 domain (23, 38–40) normally buried in the hydrophobic core of the native VWF-A2 domain (41). However, in a diluted proteinaceous solution, the shear stress that an individual VWF molecule experiences may be quite small relative to the shear stress that has been applied to the reactions in our current assay system. Under such conditions, little or no detectable cleavage product was formed when no FVIII or platelets was added (Figs. 1–3). Addition of FVIII or platelets to VWF alone can increase the formation of cleavage product (350 kDa) (Figs. 1–3), suggesting that binding of FVIII or platelets to VWF facilitates the deformation of VWF substrate by altering the domain-domain interactions among the various VWF subunits. This global conformational change appears to be sufficient to increase VWF proteolysis. There appear to be some differences in the fold enhancement by either FVIII or platelets on ADAMTS13-mediated VWF proteolysis between this report and those previously published (22, 24). These differ-
ences may result from the amount of shear stress applied and the concentrations of VWF and ADAMTS13 used in the different assays. As shown in Fig. 6, vortexing at the rotation rate of 2,500 rpm on a mini-vortexer generates ~75 dynes/cm² of fluid shear stress, whereas vortexing at the same rotation rate on a MixMate PCR mixer generates only ~32 dynes/cm² of fluid shear stress.

Further analyses demonstrate that FVIII and platelets act synergistically to accelerate VWF proteolysis by ADAMTS13 under shear stress (Fig. 1–3). The enhancing effect of FVIII and platelets on VWF proteolysis appears to depend on the specific binding interactions between the light chain of FVIII and the VWF-D’D3 domain and the interactions between platelet GP1bα receptor and the VWF-A1 domain. No synergistic effect is observed when the FVIII variant, FVIII-2RKR, which fails to bind VWF, is added into the reactions (Fig. 3). Furthermore, the synergistic effect of platelets with FVIII is inhibited dramatically by the addition of an increasing concentration of 6D1 (Fig. 4, A and C) or purified VWF-A1 domain (Fig. 4, B and D), which disrupts the specific platelet-VWF interactions. The requirement of such specific VWF-A1-GP1bα interactions for the synergistic rate enhancing effect of platelets with FVIII has been further supported by the lack of an enhancing effect of fresh platelets with FVIII after the N-terminal 25-kDa portion of the platelet GP1bα receptor has been removed by treatment with a metalloprotease (OSE) isolated from P. hemolytica, although the platelet-bound VWF may partially contribute to the increase in formation of the cleavage products (Fig. 5).

It remains to be determined how binding of FVIII and platelets to soluble VWF results in an increased cleavage of VWF by ADAMTS13 under fluid shear stress. The binding of FVIII to the VWF-D’D3 domain may cause a large scale conformational change of VWF multimers, such as pulling away the D’D3 domain from its neighboring A1 or A2 domain in the presence of fluid shear stress. Under physiological conditions, plasma FVIII concentration is quite low, ~0.5–1 nM (33, 42), whereas plasma VWF (subunit) concentration is ~50 nm (42), suggesting that approximately one FVIII is bound to every 50 VWF subunits (equal to ½ to one UL-VWF multimer). Similarly, fewer than three platelets may be bound to every 1,000 VWF subunits (24). The binding of platelets or even soluble GP1bα to the VWF-A1 domain may further alter the domain-domain interactions of multimeric VWF. This was shown to occur even after VWF or A1A2A3 or A1A2 has been pre-denatured under static conditions (43). Moreover, the binding of two or more platelets on either side of the cleavage bond of VWF multimers may dramatically increase the peak force exerted on the central A2 domain. For instance, in the presence of 20 dyne/cm² shear force, the predicted peak force soluble VWF multimers experience ranges from 0.2 to 0.8 piconewtons (44) in the absence of platelets. The peak force increases to ~390 piconewtons when two or more platelets are bound at either side of the sessile bond (24, 44). This is the amount of shear stress if applied directly to a VWF fragment such as A1A2A3 or A2 domain in which both ends are attached to a solid surface (40, 45). However, this does not appear to be the case in our assay system because only a modest increase of VWF cleavage is observed with addition of physiological concentrations of lyophilized platelets alone (Figs. 1–3). The combination of low occupancy of VWF by FVIII and platelets may increase the chances of being proteolyzed under fluid shear stress, which may be sufficient to eliminate circulating UL-VWF multimers under physiological conditions. However, our in vitro assay may underestimate the degree of VWF proteolysis because we rely on the appearance of the 350-kDa product as a measure of proteolysis, which requires a simultaneous cleavage of two immediately adjacent Tyr–Met bonds.

More studies are needed to address the in vivo function of FVIII and platelet in regulation of VWF proteolysis. For instance, the effect of FVIII deficiency or infusion of a large dose of FVIII in patients with hemophilia A on VWF multimer distribution is largely unknown. In addition, how platelet counts affect VWF proteolysis in vivo has not been fully investigated. Limited clinical reports suggest that patients with thrombocytopenia (or increased platelet counts) (46) or patients with type 2B von Willebrand disease (or increased binding of plasma VWF to platelets) (47) or recombinant murine type 2B-VWF (R1306Q and V1316M) expressed in mice (48) exhibit dramatically reduced high molecular weight VWF multimers with a concurrent accumulation of low molecular weight VWF multimers, indicative of increased proteolytic cleavage. Moreover, the sizes of plasma VWF multimer fluctuate with patient platelet counts (i.e. VWF multimers are smaller when platelet count is normal and larger when platelet count is low), suggesting a physiological link between platelet count and VWF multimer distribution. Together, our findings and clinical observations support a role of FVIII and platelets as physiological cofactors regulating plasma VWF multimer distribution under dynamic flow conditions.

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