Light Chain of Factor VIII Is Sufficient for Accelerating Cleavage of von Willebrand Factor by ADAMTS13 Metalloprotease

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Background: The structural component of FVIII required for regulating VWF proteolysis is not fully understood.
Results: A light chain of FVIII appears to be sufficient for accelerating the cleavage of VWF by ADAMTS13 in vitro and in vivo.
Conclusion: Proteolytic cleavage of VWF can be regulated by the FVIII light chain.
Significance: The findings provide insight into the structure-function relationship of FVIII in maintaining VWF homeostasis.

We previously demonstrated that coagulation factor VIII (FVIII) accelerates proteolytic cleavage of von Willebrand factor (VWF) by a disintegrin and metalloprotease with thrombospondin type 1 repeats (ADAMTS13) under fluid shear stress. In this study, the structural elements of FVIII required for the rate-enhancing effect and the biological relevance of this cofactor activity are determined using a murine model. An isolated light chain of human FVIII (hFVIII-LC) increases proteolytic cleavage of VWF by ADAMTS13 under shear in a concentration-dependent manner. The maximal rate-enhancing effect of hFVIII-LC is ~8-fold, which is comparable with human full-length FVIII and B-domain deleted FVIII (hFVIII-BDD). The heavy chain (hFVIII-HC) and the light chain lacking the acidic (a3) region (hFVIII-LCAa3) have no effect in accelerating VWF proteolysis by ADAMTS13 under the same conditions. Although recombinant hFVIII-HC and hFVIII-LCAa3 do not detectably bind immobilized VWF, recombinant hFVIII-LC binds VWF with high affinity ($K_D \sim 15$ nM). Moreover, ultra-large VWF multimers accumulate in the plasma of FVIII⁻/⁻ mice after hydrodynamic challenge but not in those reconstituted with either hFVIII-BDD or hFVIII-LC. These results suggest that the light chain of FVIII, which is not biologically active for clot formation, is sufficient for accelerating proteolytic cleavage of VWF by ADAMTS13 under fluid shear stress and (patho) physiological conditions. Our findings provide novel insight into the molecular mechanism of how FVIII regulates VWF homeostasis.

Proteolytic cleavage of ultra-large von Willebrand factor (VWF) on endothelial cells (1, 2) and in flowing blood (3, 4) by a plasma metalloprotease, ADAMTS13, is crucial for normal hemostasis. ADAMTS13 cleaves VWF at the specific Tyr\(^{1605}\)–Met\(^{1606}\) bond in the central A2 domain (5, 6). This cleavage is dramatically accelerated by fluid shear stress (4, 6, 7) or mild denaturation with urea (5, 8) or guanidine (6, 9), which alters VWF conformation and exposes binding and cleavage sites. Inability to cleave VWF as a result of a severe deficiency of plasma ADAMTS13 leads to an accumulation of ultra-large VWF on endothelial cells or in blood (10). This triggers spontaneous platelet aggregation and disseminated microvascular thrombosis, characteristic of thrombotic thrombocytopenic purpura (11, 12). Moreover, mild to moderate deficiency of plasma ADAMTS13 has been shown to be a risk factor for cardiovascular events such as myocardial infarction (13–16) and ischemic cerebral stroke (17, 18).

In addition to fluid shear stress, we and others have shown that cleavage of soluble multimeric VWF by ADAMTS13 under fluid shear stress is dramatically accelerated by coagulation factor VIII (FVIII) (19), platelets (20, 21), and glycoprotein 1b\(\alpha\) (21, 22). FVIII and platelets appear to synergistically accelerate cleavage of VWF by ADAMTS13 under these conditions (21). We have previously shown that the FVIII a3 domain is not required, but the a3 in the context of two chain B-domainless FVIII, which binds VWF with high affinity, is required for the rate-enhancing effect on proteolytic cleavage of VWF by ADAMTS13 under shear stress (19).

Here, we show that an isolated light chain of FVIII, which is biologically inactive for clot formation, is sufficient for accelerating proteolytic cleavage of VWF by ADAMTS13 in vitro using a fluid shear-based assay and \(\text{in vivo}\) using FVIII⁻/⁻ mice expressing FVIII variants via a hydrodynamic approach. This rate-enhancing effect by FVIII light chain also depends on its high affinity binding with VWF; a light chain of FVIII lacking the a3 region and a heavy chain of FVIII, which do not bind...
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VWF detectably, exhibit no effect on ADAMTS13-mediated VWF proteolysis under the same conditions. Our findings may shed more light on the structure-function relationship of FVIII in regulation of the VWF-ADAMTS13 axis, which helps in understanding the clinical heterogeneity of patients with severe hemophilia A.

EXPERIMENTAL PROCEDURES

Constructs—The cDNA fragments encoding a heavy chain (hFVIII-HC), a light chain (hFVIII-LC), and a light chain lacking α3 (hFVIII-LCα3) of human FVIII were amplified by PCR using hFVIII-BDD as a template (19). The primer pairs were as follows: hFVIII-HC (5'-GAGTACTCTCCTCTCAAAAGCGG-GCATG-3' and 5'-GGAGAAGCTTCTTGTGTTCAAT-3'); hFVIII-LC (5'-GAATAACTCTGACTACCTT-3' and 5'-GTAAGGTTCTTGTGTCCCTGCA-3'); and hFVIII-LCα3 (5'-AGCTTCTAAAAAGACAGA-3' and 5'-GTAAGAGGTCCCTGCGCA-3'). The amplified hFVIII-LC and hFVIII-LCα3 fragments were cloned into pcDNA3.1 V5-His TOPO vector (Invitrogen) according to the manufacturer's recommendation. All three constructs were tagged at their C termini with a V5-His epitope (Fig. 1A). Plasmids were sequenced at the Nucleic Acid Core Facility at the Children's Hospital of Philadelphia to confirm the accuracy. Plasmids encoding a B-domain deleted human factor VIII (hFVIII-BDD) (19) or canine FVIII (cFVIII-BDD) (23) were described in other studies.

Preparation of Recombinant FVIII Variants—Baby hamster kidney cells were used to express human recombinant hFVIII-BDD and cFVIII-BDD, as described previously (19, 23, 26). Human embryonic kidney (HEK293) cells were used to express recombinant hFVIII-LC, hFVIII-LCα3, and hFVIII-HC. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% of FetalPlex (Gemini BioProducts, West Sacramento, CA). The cells were transfected with LipofectAMINE2000 and plasmid DNA in serum-reduced OptiMEM. For those vectors without the G418-resistant gene, co-transfection with pcDNA3.1 (10:1 ratio) containing the neomycin resistance gene (Invitrogen) was carried out. Stable clones were selected after culturing cells for 10 days in the presence of 0.5–1.0 mg/ml of G418 (Invitrogen). The positive clones were selected after culturing cells for 10 days in the presence of 0.5–1.0 mg/ml of G418 (Invitrogen). The positive clones were selected after culturing cells for 10 days in the presence of 0.5–1.0 mg/ml of G418 (Invitrogen).

Binding of FVIII Variants to Immobilized VWF—A microtiter plate was coated with 100 μl of human VWF (2 μg/ml) and blocked with 1% casein in PBS, pH 7.4. Recombinant FVIII variants at various concentrations diluted with PBS containing 0.2% casein were added and incubated at 25 °C for 1 h. After being washed with PBS, bound FVIII variants were detected by peroxidase-conjugated mouse anti-human monoclonal anti-FVIII IgG (ESH-8HR) (1:3,000) that recognizes the C2 domain in the light chain of FVIII (American Diagnostica Inc., Stamford, CT) or peroxidase-conjugated monoclonal anti-V5 (1:1,000) (Invitrogen). A pre-mixed chromogenic substrate, 3,3',5,5'-tetrathenylbenzidine (TMB) (Thermo Fisher Scientific, Rockford, IL), was added for color reaction. The absorb-
ance was determined at 450 nm in a SpectroMax microtiter plate reader (Molecular Device, Sunnyvale, CA).

Hydrodynamic Injection—The Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania and the Children’s Hospital of Philadelphia approved the protocol for the mouse study. FVIII-deficient mice (fVIII−/−) in a C57BL6/129 strain with exon-16 deletion were described previously (27). Mice at the age of 6–8 weeks were injected with 2 ml of saline alone or saline containing 100 µg of plasmid DNA (endotoxin-free) via a tail vein within 5 s as described previously (28). 48 h after injection, whole blood (200 µl) was collected after tail clip and anti-coagulated with sodium citrate (3.8%). Platelet-poor plasma was obtained after centrifugation at 10,000 rpm for 10 min and stored in small aliquots at −80 °C until use.

Plasma FVIII Antigen in fVIII−/− Mice after Reconstitution—Plasma antigen levels of FVIII variants expressed after hydrodynamic injection were determined by a home grown ELISA. A microtiter plate was coated with polyclonal goat anti-FVIII IgG (1:2,000) at 25 °C for 1 h. The plate was blocked with 1% (w/v) casein in PBST for 30 min. Purified recombinant hFVIII-BDD or cFVIII-BDD (0, 5, 10, 20, 40, 80, 160, and 320 ng/ml) or mouse plasma (1:20 or 1:40) in PBS was incubated in antibody-coated wells for 2 h. After being washed with PBS, bound FVIII variants (hFVIII-BDD, cFVIII-BDD, hFVIII-LC, and hFVIII-LCa3) were detected by peroxidase-conjugated monoclonal anti-FVIII IgG (ESH-8HR) (1:1,000) (American Diagnostica, Stamford, CT) for 1 h. For quantification of plasma hFVIII-HC antigen, mouse plasma was incubated in a nickel-coated microtiter plate for 2 h. The bound hFVIII-HC was detected by monoclonal anti-V5 IgG (Invitrogen), followed by a horseradish peroxidase-conjugated rabbit anti-mouse IgG (DAKO, Carpinteria, CA). A pre-mixed TMB solution was added for color development, and absorbance at 450 nm was determined by a SpectroMax microtiter plate reader as described above.

Plasma VWF Antigen in fVIII−/− Mice before and after Reconstitution with FVIII Variants—Plasma VWF antigen was quantified by an ELISA as described previously (29). A microtiter plate was coated with rabbit anti-vWF IgG (Dako, Carpinteria, CA) (1:2,000) and blocked with 1% casein in PBS containing 0.05% Tween 20 (TBST). Mouse plasma diluted (1:20 and 1:40) with 0.2% casein in TBST was added and incubated for 1 h. After being washed with PBS, bound VWF was detected by peroxidase-conjugated rabbit anti-VWF IgG (1:2,000) (Dako, Carpinteria, CA). Pooled murine plasma from C57BL6 was used as a reference. A pre-mixed TMB solution was used for color development. The absorbance at 450 nm was determined by a SpectroMax microtiter plate reader (Molecular Device, Sunnyvale, CA).

Plasma VWF Multimer Distribution in fVIII−/− Mice before and after Reconstitution with FVIII Variants—Mouse plasma (1 µl) was denatured at 60 °C for 20 min with 10 µl of sample buffer (70 mM Tris, pH 6.8, 2.4% SDS, 0.67 M urea, and 4 mM EDTA, 10% glycerol, 0.01% bromphenol blue). The proteins were fractionated on a 1% agarose gel (7 × 8 cm) on ice. After being transferred to a nitrocellular membrane, VWF multimers were detected by incubation with rabbit anti-VWF IgG (1:5,000) (Dako, Carpinteria, CA), followed by IRDye 800CW-labeled goat anti-rabbit IgG (1:20,000) in 1% casein/TBST. Odyssey imaging analysis (LI-COR Bioscience, Lincoln, NE) was used to scan the membrane (19, 30). Densitometry analysis using ImageJ software determined the formation of cleavage product or the ratio of high to low molecular weight VWF multimers.

Statistical Analysis—The difference in means between the control and experimental groups was determined by one-way analysis of variance using Minitab16 software. p values less than 0.05 and 0.01 are considered to be statistically significant and highly significant, respectively.

RESULTS

Biochemical Characterization of Recombinant FVIII Variants—We previously found that full-length FVIII accelerates proteolytic cleavage of VWF by ADAMTS13 under fluid shear stress (19). However, the domain components of FVIII required for the cofactor activity to enhance VWF proteolysis are not fully understood. We therefore prepared various recombinant FVIII variants, including hFVIII-HC, hFVIII-LC, and hFVIII-LCa3, in addition to hFVIII-BDD and cFVIII-BDD (Fig. 1A). All variants except for hFVIII-BDD and cFVIII-BDD contained a V5-His epitope at their C-terminal end to facilitate purifica-
tion and detection (Fig. 1A). All variants except for hFVIII-HC were purified to homogeneity as demonstrated by SDS-PAGE and Coomassie Blue staining (Fig. 1B, 1st lane). The hFVIII-HC was only partially purified due to low secretion of this variant from stably transfected cells (data not shown). Therefore, a purified preparation of hFVIII-HC (without V5-His) was obtained from Dr. Phillip J. Fay, University of Rochester, School of Medicine and Dentistry, Rochester, NY.

As shown, cFVIII-BDD was purified as a single chain protein (Mr ~160,000) (Fig. 1B, 1st lane), whereas hFVIII-BDD was purified as a two-chain protein (Mr ~90,000 and ~80,000) (Fig. 1B, 2nd lane). The hFVIII-HC and hFVIII-LC were secreted and purified as a single chain protein with Mr of ~95,000 and ~84,000, respectively (Fig. 1B, 2nd and 3rd lanes, and C, 6th and 7th lanes). Interestingly, purified hFVIII-LCΔa3 exhibited two different sizes (~80,000 and ~75,000) (Fig. 1B, 5th lane), both of which were recognized by monoclonal anti-V5 IgG on a Western blot (Fig. 1C, 8th lane), suggesting potentially aberrant N-terminal processing of hFVIII-LCΔa3 during biosynthesis.

Moreover, using a one-stage clotting assay, we had previously shown that hFVIII-BDD exhibited specific clotting activity, comparable with full-length FVIII (19), whereas cFVIII-BDD exhibited an ~3-fold increase in specific activity compared with hFVIII-BDD (23). No clotting activity was detected with hFVIII-LC, hFVIII-HC, and hFVIII-LCΔa3 (data not shown).

**Light Chain of FVIII Accelerates Cleavage of VWF by ADAMTS13 under Fluid Shear Stress**—To determine whether the isolated light chain of FVIII, a biologically inactive FVIII variant for clot formation, accelerates VWF proteolysis by ADAMTS13, we incubated a fixed concentration of VWF (150 nM) with ADAMTS13 (50 nM) in the presence of various concentrations of recombinant hFVIII-LC (0, 0.5, 1.0, 2.5, 5.0, and 10 nM) for 10 min under constant vortexing (2,500 rpm). The proteolytic cleavage of VWF was determined by SDS-PAGE and Western blotting as described under “Experimental Procedures.” Recombinant hFVIII-LC increased the formation of the proteolytic cleavage product (350 kDa) in a concentration-dependent manner (Fig. 2, A and B). The maximal rate-enhancing effect was ~8-fold (Fig. 2B). Similar fold of rate-enhancing effect by hFVIII-LC without a V5-His epitope on the cleavage of VWF by ADAMTS13 under the same conditions was observed (data not shown). The concentration of hFVIII-LC achieving 50% of the maximal enhancing effect (C50) was ~1.0 nM (Fig. 2B), quite similar to that of full-length FVIII and hFVIII-BDD that we previously reported (19). Addition of EDTA (15 mM) into the reaction completely inhibited proteolytic cleavage of VWF by ADAMTS13 under fluid shear stress.
ADAMTS13 even in the presence of 10 nM hFVIII-LC (Fig. 2, A, 2nd lane, and B). These results demonstrate for the first time that the isolated light chain of FVIII is sufficient for accelerating proteolytic cleavage of VWF by ADAMTS13 under fluid shear stress.

**Light Chain of FVIII Lacking the a3 or the Heavy Chain Has No Effect on Cleavage of VWF by ADAMTS13 under Fluid Shear Stress**—To determine whether the light chain of FVIII or the heavy chain, both lacking the high affinity binding site for VWF, was able to enhance VWF proteolysis by ADAMTS13 under the same conditions, a fixed concentration of human VWF (150 nM) and human ADAMTS13 (50 nM) was incubated with various concentrations of recombinant hFVIII-LCα3 or hFVIII-HC (0, 0.5, 1.0, 2.5, 5.0, and 10 nM) for 10 min under constant vortexing at a rotation rate of 2,500 rpm. The proteolytic cleavage of VWF was determined by SDS-polyacrylamide gel and Western blotting. As shown, recombinant hFVIII-LCα3 (Fig. 2, C and D) or hFVIII-HC (Fig. 2, E and F) at any given concentration (up to 10 nM) did not exhibit a rate-enhancing effect on cleavage of VWF by ADAMTS13 under the same conditions. Recombinant hFVIII-BDD (20 nM) used as a positive control dramatically increased the formation of the proteolytic cleavage product (Fig. 2, 1st lane). These results suggest that the acidic a3 region in the light chain of FVIII is required for accelerating VWF proteolysis by ADAMTS13 under shear stress.

**Binding of FVIII Variants to Immobilized VWF**—To assess the binding affinity between FVIII variants and VWF, increasing concentrations of recombinant cFVIII-BDD, hFVIII-BDD, hFVIII-LC, hFVIII-HC, and hFVIII-LCα3 (0, 1.56, 3.1, 6.25, 12.5, 25, 50, and 75 nM) were incubated with human VWF immobilized on a microtiter plate (1.0 μg/well). The bound FVIII variants were determined by anti-FVIII IgG or anti-V5 IgG (if V5-His tagged) as described under “Experimental Procedures.” We showed that cFVIII-BDD (Fig. 3A), hFVIII-BDD (Fig. 3B), and hFVIII-LC (Fig. 3C) bound human VWF in a concentration-dependent manner. The dissociation constants (K_d) for cFVIII-BDD, hFVIII-BDD, and hFVIII-LC binding to immobilized human VWF were ~1.1, ~1.3, and ~15.6 nM, respectively (Table 1). These results indicate that the heavy chain may contribute to the overall binding affinity of FVIII to immobilized VWF. Moreover, the K_d values for cFVIII-BDD, hFVIII-BDD, and hFVIII-LC to bind immobilized murine VWF was 1.9, 1.7, and 26 nM, respectively. These results suggest that there is little species difference between VWF and FVIII binding. As predicted, no detectable binding was observed between hFVIII-LCα3 (or hFVIII-HC) and immobilized human VWF and murine VWF under the same conditions (data not shown). These results indicate that the light chain, particularly the a3 region in the light chain of FVIII, contains the major binding site for VWF.

**Plasma VWF Multimer Distribution in fVIII<sup>−/−</sup> Mice Expressing FVIII Variants**—Although full-length FVIII, B-domainless FVIII variant, and the isolated light chain of FVIII are able to accelerate proteolytic cleavage of VWF by ADAMTS13 under mechanically induced shear stress in vitro, the physiological relevance of such an enhancing effect has never not been determined in vivo. Using a hydrodynamic approach, we were able to reconstitute plasma FVIII in fVIII<sup>−/−</sup> mice with various FVIII variants, including cFVIII-BDD, hFVIII-BDD, hFVIII-LC, and hFVIII-LCα3 at levels between 0.7 and 2.8 μg/ml measured 48 h after injection (Table 2). Plasma VWF antigen and multimer distribution in fVIII<sup>−/−</sup> mice were determined after 48 h of post-reconstitution. We showed that although plasma VWF antigen did not change dramatically in different

**FIGURE 3. Binding of FVIII variants to immobilized VWF.** Purified cFVIII-BDD (A), hFVIII-BDD (B), and hFVIII-LC (C) at various concentrations were incubated with immobilized human (●) and murine (○) VWF (10 μg/ml) on a microtiter plate. The bound FVIII variants were detected by peroxidase-conjugated monoclonal anti-FVIII IgG (ESH-8HR) as described under “Experimental Procedures.” The specific binding was obtained after subtracting from the total the nonspecific binding in the wells without VWF immobilized but with same concentrations of FVIII variants added. The K_d value(s) was obtained by fitting the binding data (n = 3) into a nonlinear binding equation using the SigmaPlot software.
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**TABLE 1**

Binding of recombinant FVIII variants to immobilized human (h) and murine (m) VWF

| Variants    | hVWF $K_D$ (nM) | mVWF $K_D$ (nM) |
|-------------|-----------------|-----------------|
| cFVIII-BDD  | 1.1 ± 0.2*      | 1.9 ± 0.3       |
| hFVIII-BDD  | 1.3 ± 0.4       | 1.7 ± 0.4       |
| hFVIII-LC   | 15.6 ± 4.5      | 26.0 ± 6.0      |
| hFVIII-LCΔα3| Undetectable    | ND              |
| hFVIII-HC   | Undetectable    | ND              |

$K_D$ is the dissociation constant; ND means not determined.

* The means and standard deviation are from three independent experiments. BDD means B-domain deleted FVIII variant; LC and HC mean light chain and heavy chain of FVIII.

**TABLE 2**

Plasma FVIII antigen (Ag), VWF antigen (Ag), and VWF multimer distribution in FVIII~−/− mice before and after hydrodynamic challenge

| Groups                  | FVIII Ag (μg/ml) | VWF Ag (μg/ml) | VWF multimer ratio of H/L |
|-------------------------|-----------------|-----------------|--------------------------|
| fVIII~−/− (unchallenged) | ND              | 2.2 ± 0.7       | 1.7 ± 0.5                |
| fVIII~−/− + saline      | 1.2 ± 0.8       | 1.7 ± 0.8       | 0.4 ± 0.4**              |
| fVIII~−/− + cFVIII-BDD  | 0.6 ± 0.2       | 1.7 ± 0.6       | 0.9 ± 0.2**              |
| fVIII~−/− + hFVIII-HC/LC| 0.5 ± 0.1       | 2.0 ± 0.3       | 0.4 ± 0.2**              |
| fVIII~−/− + hFVIII-LC   | 0.6 ± 0.2       | 2.7 ± 0.3       | 1.0 ± 0.3*              |
| fVIII~−/− + hFVIII-LCΔα3| 0.2 ± 0.1**     | 3.0 ± 0.4       | 1.4 ± 0.4               |

Plasma FVIII antigen (Ag), VWF antigen (Ag), and VWF multimer distribution in FVIII~−/− mice before and after hydrodynamic challenge. The following abbreviations have been used: ND, not determined; n, number of mice in each group; Ag, antigen; H/L, high to low molecular weight VWF multimers; HC and LC are heavy chain and light chain of FVIII. * and ** indicate p values <0.05 and <0.01, respectively. The plasma FVIII Ag levels in the fVIII~−/− mice receiving hFVIII-BDD (as in column 2) or plasma H/L VWF multimer ratios in the fVIII~−/− mice receiving saline alone (as in column 4) were used for comparison with other groups. All results are expressed as the means ± S.D.

**FIGURE 4.** Plasma VWF multimer distribution in FVIII~−/− mice expressing various FVIII variants. A, plasma multimer distribution determined by agarose gel electrophoresis and Western blotting. Representative images of plasma VWF multimers are shown in FVIII~−/− mice before (unchallenged) and after hydrodynamic injection of saline or saline containing a plasmid encoding cFVIII-BDD, hFVIII-BDD, hFVIII-LCΔα3, hFVIII-LC, and hFVIII-HC/LC. B, ratios of high to low molecular weight VWF multimers, which were determined by densitometry using ImageJ software, are shown for each group of mice before (unchallenged) and after hydrodynamic injection of saline or saline containing various FVIII variants. Statistical analysis was performed using one-way analysis of variance with Tukey correction. p values < 0.05 and 0.01 are considered to be statistically significant and highly significant as compared with the saline control.

In this study, we demonstrate both in vitro and in vivo that an isolated light chain of FVIII, which is biologically inactive for enhancing clot formation, appears to be sufficient for accelerating proteolytic cleavage of VWF by ADAMTS13 and the maximal rate enhancing effect with 5 nM of hFVIII-LC was ~8-fold, comparable with that of hFVIII-BDD (Fig. 2, A and B). The $C_{50}$ is estimated to be ~1.0 nM (Fig. 2, A and B), which is within the physiological ranges of FVIII in human plasma. In contrast, a light chain of FVIII lacking the acidic a3 region (hFVIII-LCΔα3) and hFVIII-HC, both of which do not bind VWF, exhibit no enhancing effect under the same conditions (Fig. 2, C–F). These results indicate that high affinity binding of FVIII to VWF through the acidic a3 region may be critical for accelerating VWF proteolysis.

However, the rate-enhancing effect of FVIII on VWF proteolysis does not appear to be in a linear relationship with its VWF binding affinity. For instance, although hFVIII-LC binds human VWF ($K_D = 15$ nM) and murine VWF ($K_D = 26$ nM) ~10 times less than binding of hFVIII-BDD and full-length FVIII to human VWF ($K_D = 1.3$ nM) and murine VWF ($K_D = 1.9$ nM) (Table 1), the rate-enhancing activity is quite similar to that of hFVIII-BDD (Fig. 2) and full-length FVIII (19).
a murine model. At the steady state, plasma levels of VWF antigen in hFVIII−/− mice are increased by ~2-fold when compared with wild-type mice in the same genetic background C57BL6/129 (data not shown), whereas the ratio of high to low molecular weight VWF multimers was not statistically different between the two groups of mice. Similar increases in plasma levels of VWF antigen and ristocetin-cofactor activity were observed in patients with severe hemophilia A compared with healthy controls (32). These findings suggest that FVIII may be involved in regulating VWF homeostasis under physiological conditions. However, the interpretation of these data may be complicated by the fact that plasma VWF antigen levels differ significantly among various strains of mice or human individuals.

Although hydrodynamic challenge is considered to be a non-physiological transfection method, it activates endothelial cells and triggers the release of ultra-large VWF from endothelial cells, resulting in an accumulation of ultra-large VWF multimers in plasma of the fVIII−/− mice receiving saline alone. However, these ultra-large VWF multimers were not observed in the same mice receiving plasmids encoding cFVIII-BDD, hFVIII-BDD, and hFVIII-LC or hFVIII-LC+HC (Fig. 4 and Table 2). These results demonstrate for the first time that reconstitution of functional and nonfunctional FVIII variants restores the distribution of plasma VWF multimers in severe hemophilia A mice.

Consistent with the in vitro data, reconstitution in the fVIII−/− mice with a plasmid encoding hFVIII-LCΔa3 (Fig. 4 and Table 2) has no effect on plasma VWF multimer distribution. However, the in vivo effect of hFVIII-HC alone remains to be determined because plasma levels of the expressed hFVIII-HC were low, only 1/10 of the plasma levels of other constructs after the hydrodynamic injection (data not shown). The low expression of the heavy chain is due to inefficient secretion of this chain when it is expressed alone (24, 26). Interestingly, co-expression of hFVIII-LC dramatically improves the secretion of the heavy chain, thereby synergistically enhancing proteolytic cleavage of VWF by ADAMTS13 in vivo. The efficacy of the co-expressed hFVIII-LC and hFVIII-HC appears to be similar to that of hFVIII-BDD but better than that of hFVIII-LC alone (Table 2), suggesting that the heavy chain is able to stabilize the light chain to improve the light chain function.

The implication of these findings is not clear. Patients with severe hemophilia A (with FVIII activity <1%) are heterogeneous in their clinical presentations (31). Further investigation of plasma VWF multimer distribution in correlation with the genetic basis that results in severe FVIII deficiency may shed new light on how FVIII-dependent proteolysis of VWF may play a role in the modification of the clinical phenotype in patients with severe hemophilia A.

In conclusion, our findings provide novel insight into the structure-function relationship of FVIII in the regulation of ADAMTS13-mediated proteolysis in vitro under fluid shear stress and in vivo under (patho) physiological conditions. This may help explain the heterogeneity of VWF multimer distribution and clinical phenotype in patients with hemophilia A.

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