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Compromised Shear-Dependent Cleavage of Type 2N von Willebrand Factor Variants by ADAMTS13 in the Presence of Factor VIII

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Our studies have demonstrated that binding of coagulation factor VIII (FVIII) and/or platelets to von Willebrand factor (VWF) dramatically enhances its proteolytic cleavage by ADAMTS13 metalloprotease under fluid shear stress (1;2). The rate enhancing effect of FVIII on proteolysis of ultra large VWF by ADAMTS13 has also been demonstrated in *fVIII*^{-/-} mice after hydrodynamic reconstitution with various FVIII constructs that retain the binding capacity toward VWF (3). Here, we sought to determine whether mutations in VWF, found in patients with type 2N von Willebrand disease (VWD) that result in reduced FVIII-binding capacity, would affect its shear-dependent proteolysis by ADAMTS13 in the presence of FVIII.

Wild type and several naturally occurring type 2N VWF variants (H817Q, R854Q+T789A, R782W, and R782W+H817Q) or a hereditary persistence of propeptide (HPP) variant (**Fig. 1A**) were constructed and expressed transiently in human embryonic kidney 293T cells. Multimer analysis revealed a similar multimer distribution of all type 2N variants on a low resolution (1%) agarose gel (not shown) but significantly increased molecular weight of each ladder of the HPP variant on a high resolution (2%) agarose gel compared with that of WT and type 2N variants (**Fig. 1B**, lane 6), consistent with the presence of propeptide. These results indicate that the type 2N and HPP VWF variants chosen for the study have a normal (or expected) multimer distribution.

Type 2N mutations are primarily localized to the D'D3 domains of VWF that contain a high-affinity binding site for FVIII (4-6). The HPP variant also exhibited severely defective FVIII-binding capability, owing to the blocking effect of the propeptide (7). The FVIII-binding capacity of type 2N and HPP variants was determined by a microtiter plate-based assay described previously (8;9). WT-VWF bound FVIII in a concentration-dependent manner, whereas type 2N and HPP variants bound FVIII with a progressively reduced capacity in the following order: H817Q>R854Q+T789A>R782W>R782W+H817Q>HPP VWF (**Fig. 1C**), consistent with the clinical phenotypes of these patients (10;11).

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Because three body interactions among VWF, ADAMTS13, and FVIII may be necessary for the efficient proteolysis of VWF by ADAMTS13 under fluid shear stress (12), the binding of immobilized WT, type 2N, and HPP VWF to soluble ADAMTS13 at various concentrations was also determined. As shown, ADAMTS13 bound WT, type 2N, and HPP VWF variants with similar capacity under these conditions (**Fig. 1D**). These results demonstrate that type 2N and HPP mutations in VWF only affect its FVIII binding but not ADAMTS13 binding.

To rule out the possibility of any global effect in type 2N and HPP variants, proteolytic cleavage of these variants by ADAMTS13 under denaturing conditions was determined by the method described previously (13). As shown, proteolytic cleavage of type 2N (H817Q, R854Q+T789A, R782W, and R872W/H817Q) and HPP variants was quite similar to that of WT under these conditions (**Fig. 1E**). These results indicate that there is no intrinsic defect in the susceptibility of these VWF variants to ADAMTS13 when the central A2 domain of the VWF variants is sufficiently unfolded by denaturants.

In this study, we report the novel demonstration that proteolytic cleavage of type 2N and HPP VWF variants by ADAMTS13 in the presence of FVIII was variably reduced as compared with that of WT. By a cleaved A2-specific ELISA that detected the N-terminal peptide after the Tyr residue (**Fig. 1F**) or by Western blotting that detected the cleavage product of 350 kDa (the dimer of two C-terminal fragments) (**Fig. 1G and 1H**), we observed significantly reduced cleavage in two type 2N variants (R782W+H817Q and HPP) that had a severe defect in FVIII-binding (**Fig. 1C**). Even in the presence of 20 nM FVIII, little or no cleavage was detected for R782W+H817Q and HPP variants under fluid shear stress (**Fig. 1F, 1G, and 1H**). The reduction in the proteolytic cleavage of type 2N variants was proportional to the severity of the FVIII-binding defect (**Fig. 1F, G, and H**). These results suggest that type 2N and HPP VWF variants that are defective in their FVIII binding are compromised in cofactor- and shear-dependent proteolysis by ADAMTS13.

Binding of the propeptide to the D'D3 domains of VWF has been shown to attenuate its function in agglutinating platelets (14). Therefore, ristocetin-induced platelet agglutination and proteolysis of type 2N and HPP VWF variants were assessed as described previously (2). Our results demonstrated that all type 2N variants agglutinate lyophilized platelets normally in the presence of ristocetin (data not shown). Moreover, the proteolytic cleavage of type 2N variants by ADAMTS13 in the presence of both lyophilized platelets ($150 \times 10^3/\mu\text{l}$) and ristocetin (1 mg/ml) under fluid shear stress was also similar to that of WT (not shown). These results indicate that type 2N mutations in the D'D3 domains do not affect platelet binding and ristocetin/platelet-mediated proteolysis. Interestingly, a statistically significant reduction (~20%) in the ristocetin-induced platelet aggregation and proteolytic cleavage by ADAMTS13 was observed for the HPP variant compared with WT under the same conditions ($p < 0.01$) (not shown), consistent with the detrimental effect of binding of the propeptide to the D'D3 domains of VWF on both FVIII and platelet-dependent proteolysis by ADAMTS13 under fluid shear stress.

We conclude that the expressed type 2N VWF variants have normal patterns of multimerization, normal binding to ADAMTS13, and normal ability to agglutinate platelets in the presence of ristocetin. However, these variants have variably reduced FVIII-binding capability and reduced cofactor- and shear-dependent proteolysis by ADAMTS13. The findings may suggest an evolutionary advantage by limiting the proteolytic cleavage of a hemostatic protein like VWF in cases of a bleeding disorder.

Acknowledgments

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Figure 1.

Panel A. Schematic representation of a full-length human pro-VWF with type 2N mutations indicated below the structure. Mutations affect either the furin cleavage site (HPP) or the D'D3 domain (H817Q, R854Q+T789A, and R782W). **Panel B.** Multimer distribution of the expressed WT, type 2N, and HPP VWF variants by 2% agarose gel electrophoresis, followed by Western blotting. **Panel C.** Concentration-dependent binding of WT, type 2N, and HPP VWF variants to the immobilized FVIII on a microtiter plate. **Panel D.** Concentration-dependent binding of ADAMTS13 to the immobilized WT, type 2N, HPP variants on a microtiter plate. **Panel E.** Degradation of urea-denatured WT, type 2N, and

HPP variants by ADAMTS13 after 4 hours of incubation on a dialysis membrane, which was determined by 1% agarose gel electrophoresis and Western blotting. *Panels F, G, and H.* The proteolytic cleavage of WT, type 2N, and HPP variants by ADAMTS13 under vortexing-induced fluid shear stress (2,500 rpm for 2.5 min). The cleavage of VWF was determined by a cleaved A2-specific ELISA (**F**) or Western blotting (**G**). EDTA (20 mM) was included in **G**-lane 1 as a negative control. The relative signal intensity of the cleavage product (350 kDa) in **G** was determined by ImageJ. The data are the means and standard error of the means from three independent experiments (**H**).