Regulation of Adamts13 Function in Hemostasis by Cofactor and Substrate Exosite interactions

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Abstract

ADAMTS13 (A Disintegrin And Metalloprotease with Thrombospodin type1 repeats-13) is an enzyme that is mainly synthesized in the liver and secreted into the bloodstream. In plasma, ADAMTS13 cleaves ultra large (UL) von Willebrand factor (VWF) newly released from stimulated and/or injured endothelial cells. It also cleaves soluble UL-VWF and VWF in flowing blood or at the site of growing thrombi under high shear stress. The basic enzymology and structure-function of the ADAMTS13-VWF interaction have presented many interesting questions, particularly in the context of physiological cofactors and fluid shear stress. The relatively poor understanding of this system reflects the complex assessment of VWF proteolysis, as VWF in solution exhibits a conformation that does not actively engage with ADAMTS13. It has been unclear whether force-induced unfolding of VWF is the only mechanism to enhance proteolysis by ADAMTS13, or if interactions of ADAMTS13 and VWF with cofactors ideally position ADAMTS13 on VWF multimers for enhanced cleavage. We demonstrated that coagulation factor VIII (FVIII) and blood platelets cooperatively accelerate proteolytic cleavage of soluble VWF by ADAMTS13 through an alteration of VWF substrate conformation under physiologically relevant fluid shear stresses. In addition, we have established the critical role of the VWF propeptide and FVIII- and platelet-binding domains of VWF in regulating proteolysis of VWF by ADAMTS13 under physiologically relevant shear stress. Through site-directed mutagenesis, kinetic analyses, and peptide inhibition assays we have identified a substrate-binding exosite containing ADAMTS13 residues Arg659, Arg660 and Tyr661 that exhibits an important role in proteolytic cleavage of VWF under both non-physiological and physiological conditions. In addition, modification of this exosite region of ADAMTS13 yielded ADAMTS13 variants with reduced inhibition by autoantibodies and enhanced specific activity. Finally, we have demonstrated that infusion of ADAMTS13 and a truncated variant into ADAMTS13/-/- mice can restore the thrombus composition and kinetics of fibrin and platelet accumulation in an arterial thrombosis model. Together, these results suggest an important physiological role of cofactor binding to VWF and VWF interactions with ADAMTS13 exosites in regulating ADAMTS13 function in hemostasis.

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REGULATION OF ADAMTS13 FUNCTION IN HEMOSTASIS BY

COFACTOR AND SUBSTRATE EXOSITE INTERACTIONS

Christopher Gemil Skipwith

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REGULATION OF ADAMTS13 FUNCTION IN HEMOSTASIS BY
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"It must be borne in mind that the tragedy in life doesn't lie in not reaching your goal. The tragedy lies in having no goal to reach. It isn't a calamity to die with dreams unfulfilled, but it is a calamity not to dream. It is not a disaster to be unable to capture your ideal, but it is a disaster to have no ideal to capture. It is not a disgrace not to reach the stars, but it is a disgrace to have no stars to reach for. Not failure, but low aim is sin."

- Dr. Benjamin Elijah Mays

I am nothing without those who have come before me, nor do I amount to anything without reaching back to those who come behind me.
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ABSTRACT

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Christopher Gemil Skipwith
X. Long Zheng, M.D., Ph.D.

ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin type1 repeats-13) is an enzyme that is mainly synthesized in the liver and secreted into the bloodstream. In plasma, ADAMTS13 cleaves ultralarge (UL) von Willebrand factor (VWF) newly released from stimulated and/or injured endothelial cells. It also cleaves soluble UL-VWF and VWF in flowing blood or at the site of growing thrombi under high shear stress. The basic enzymology and structure-function of the ADAMTS13-VWF interaction have presented many interesting questions, particularly in the context of physiological cofactors and fluid shear stress. The relatively poor understanding of this system reflects the complex assessment of VWF proteolysis, as VWF in solution exhibits a conformation that does not actively engage with ADAMTS13. It has been unclear whether force-induced unfolding of VWF is the only mechanism to enhance proteolysis by ADAMTS13, or if interactions of ADAMTS13 and VWF with cofactors ideally position ADAMTS13 on VWF multimers for enhanced cleavage. We demonstrated that coagulation factor VIII (FVIII) and blood platelets cooperatively accelerate proteolytic cleavage of soluble VWF by ADAMTS13 through an alteration of VWF substrate conformation under physiologically relevant fluid shear stresses. In addition, we have established the critical role of the VWF propeptide and FVIII- and platelet-binding
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CHAPTER 1: INTRODUCTION

1.1 Overview

The coagulation system includes soluble and cellular factors that operate in concert to prevent hemorrhage. For the coagulation system to serve the needs of the organism, the fluidity of blood must be maintained under physiologic situations and clotting must be promoted only when and where there is a threat of vascular interruption by trauma. This process of maintenance of normal blood flow is termed hemostasis, whereby the system is maintained in a constant anticoagulant state.\(^1\)

1.2 Maintenance of Hemostasis

1.2.1 The vascular endothelium

The vascular endothelium is strategically located at the interface between the tissues and blood. It is pivotal for protecting against vascular injury and maintaining blood fluidity. Disruption of the vascular endothelium results in loss of the anticoagulant state. In addition to the loss of anticoagulant function, disruption of the endothelium exposes the subendothelial cell matrix, which contains collagen, von Willebrand factor (VWF) and other substances to which circulating platelets bind.\(^1,2,3\) Trauma also directly stimulates the expression of procoagulant activity by endothelial cells.\(^3\)

1.2.2 Platelet aggregation as a hemostatic mechanism

Platelet aggregates bound to the subendothelium serve to reinforce the effect of vasoconstriction by interrupting the loss of blood at sites of trauma.\(^4\) This “platelet plug”
is physically unstable and is often insufficient by itself to permanently arrest bleeding\textsuperscript{4}, which is why the process of clot formation is critical. Activated platelets accelerate the rate at which clotting occurs compared with reaction rates in plasma by provide binding sites for the clotting proteins, concentrating and orienting them on the cell surface in a way that enhances their efficient interaction\textsuperscript{4,5}. Activated platelets also promote redistribution of anionic phospholipids from the inner to the outer cell membrane where they serve as important cofactors for several clotting reactions\textsuperscript{5}.

\textit{1.2.3 The coagulation system}

The coagulation system is composed of a series of proteins, most of which are synthesized in the liver and circulate in plasma primarily as single chain, inactive precursors (Figure 1.1)\textsuperscript{6}. Once the coagulation system has been activated, several of these proteins are converted into two-chain, active enzymes. In turn, these enzymes act upon other specific coagulation protein precursors converting them into active enzymes or into active cofactors. Ultimately, the enzyme thrombin is generated, which cleaves fibrinogen to fibrin\textsuperscript{6,7}. Polymerized fibrin is the predominant constituent of the visible clot. Cell surfaces accelerate coagulation reactions dramatically by binding and orienting enzymes, substrates and cofactors\textsuperscript{7}. These cell surfaces may include injured or activated endothelium, activated monocytes/macrophages, exposed smooth muscle cells, or activated platelets.
Figure 1.1. The Coagulation Cascade. The coagulation cascade can follow alternative routes depending on the initiating factor. The *extrinsic* pathway is initiated by tissue factor and involves calcium ions and factor VII. In the *intrinsic* pathway, factors XII, XI, IX and VIII are activated by exposure to subendothelial collagen or foreign surfaces. Both pathways lead to the activation of factor X and proceed along the *common* pathway, involving factors V, II, I and XIII, to the formation of a fibrin clot.

1.3 von Willebrand Factor Plays a Central Role in Hemostasis

1.3.1 von Willebrand Factor synthesis

VWF is synthesized in all vascular endothelial cells, and in developing megakaryocytes. A 275 kDa monomer of pro-VWF, consisting of the D1-D2-D’-D3-A1-A2-A3-B-C1-C2-CK domains (Figure 1.2), is initially synthesized in the endoplasmic reticulum (pH ~7.4). N-linked glycans are added in the endoplasmic reticulum, most disulfide bonds are formed, and pro-VWF is dimerized through formation of inter-
monomer disulfide bonds in the C-terminal cysteine-knot (CK) domain\(^9\). After transport to the Golgi (pH \(\sim 6.2\)), pro-VWF dimers form homo-polymers or multimers through an additional disulfide bond near the amino terminus of the mature subunit, and the propeptide is then cleaved from pro-VWF by furin\(^7,8\). It is also in the Golgi that N-linked glycans are processed and O-linked glycans are added\(^9\).

![Figure 1.2. Schematic representation of pro-VWF.](image)

**Figure 1.2. Schematic representation of pro-VWF.** From Zheng and Sadler\(^{79}\). Binding sites are indicated for collagen, FVIII, GPIb and integrin \(\alpha\)IIb\(\beta\)3. The cleavage site (Tyr1605-Met1606) and inter-subunit disulfide bonds (S-S) are also shown.

1.3.2 Storage and secretion of von Willebrand Factor

The multimeric VWF is stored in the Weibel-Palade bodies of endothelial cells and \(\alpha\)-granules of platelets. A small fraction of VWF multimers is constitutively secreted from endothelial cells\(^9\). It has been shown *in vitro* (with Ca\(^{2+}\) and pH 6.2) that D1-D2 monomers and D’D3 dimers can assemble into helices with the same dimensions and three-dimensional shape as VWF tubules in Weibel-Palade bodies\(^{10,11}\). The ultimate purpose of this method of assembly is likely to overcome the challenges of synthesis of a long disulfide-linked polymer that can be coiled into a compact form for storage.
Figure 1.3. Biosynthesis, helical assembly, and secretion of VWF multimers. Modified from Springer\textsuperscript{12}. Panel A. Primary structure and domain organization of VWF. Cysteines are vertical lines and are connected for chemically determined disulphide bonds. N and O-linked glycans are closed and open circles, respectively. Panels B-E. Scheme for biosynthesis, helical assembly, and secretion.
VWF dimers linked by disulfide bonds near the N-terminal end in the D3 domain are assembled onto the ends of growing helical tubules in the Weibel-Palade bodies. Pro-VWF dimers that are nearby have growing tubule ends that place one D’D3 domain adjacent to the D’D3 domain of the previously assembled pro-VWF dimer (Figure 1.3). The role of this helical assembly is to encourage inter-dimer disulfide bond formation and enable furin cleavage between the D2 and D’D3 domains\textsuperscript{12}. It has been shown that the organization of VWF dimers in the Weibel-Palade bodies is quite peculiar (Figure 1.4)\textsuperscript{11}. Whereas DNA forms structures with two molecules per helix, the VWF helices only contain one multimer\textsuperscript{11}. Similar to DNA, however, there is 2-fold symmetry, which occurs because of a two-fold axis about the D1, D2, D’D3 dimer (Figure 1.4)\textsuperscript{11,12}. 
Upon stimulation, VWF is secreted from endothelial cells as ultra-large (UL)-VWF multimers that form string-like structures attached to the endothelial cell surface\textsuperscript{13}, perhaps through interaction with P-selectin\textsuperscript{14}. Orderly coiling of VWF in Weibel-Palade bodies is paramount to proper unraveling of VWF during secretion\textsuperscript{15}. The first step in secretion involves formation of a narrow constriction between the plasma membrane and the Weibel-Palade bodies. Electron microscopy indicates that secretion occurs from one end of a Weibel-Palade body\textsuperscript{10}. After fusion with the plasma membrane, there is an
expansion in Weibel-Palade body width and an increase in spacing between the tubules\textsuperscript{10}. A reasonable conclusion is that dissociation of the D1-D2 pro-domain, coupled with unraveling of VWF multimers, proceeds at the VWF tubules closest to the secretion pore\textsuperscript{12}. Given this method of VWF secretion, the process may proceed such that multiple VWF multimers are released in parallel. It has been shown that VWF molecules can self-associate\textsuperscript{16}, and VWF fibrils extend from stimulated endothelial cells in the flow direction\textsuperscript{17}.

\textit{1.3.3 von Willebrand Factor conformation and length}

Electron microscopy of VWF at pH 7.4 reveals either compact or extended structures\textsuperscript{18,19}. Atomic force microscopy (AFM) has demonstrated that, under shear flow, VWF adopts an extended conformation\textsuperscript{20}. The domain organization can be described as variably-sized beads spaced close together on a flexible string. This structure is interrupted by thin interdomain segments, such as O-glycosylated segments on either side of the A1 domain (Figure 1.3)\textsuperscript{12}. An estimate of the monomer size is about 60-70 nm based on the average spacing observed between domains\textsuperscript{18}. VWF is highly flexible at pH 7.4\textsuperscript{19}, permitting dynamic, rapid changes in multimer shape under flow conditions.

SDS-agarose of VWF suggests that larger multimers consist of 14-20 dimers\textsuperscript{18}. Electron microscopy shows longer VWF multimers that can consist of 30 dimers\textsuperscript{18}. Under shear flow, fluorescent-labeled VWF molecules can extend up to 15 µm\textsuperscript{21}. Combining these measurements with the previously-mentioned monomer size of 60-70 nm, one can estimate that there are 60–250 monomers per multimer\textsuperscript{12}. It has been reported that there is a 50-fold expansion in length between stored VWF and the extended form seen by
electron microscopy\textsuperscript{11}. Thus, a 5 µm long Weibel-Palade body tubule would yield a 250 µm long molecule of VWF, which is highly concordant with the observation of 100-1000 µm VWF strings after secretion from endothelial cells \textit{in vitro}\textsuperscript{13}.

\subsection*{1.3.4 The effect of flow on von Willebrand Factor conformation}

The conformation of VWF after release is governed by fluid dynamics in vascular systems. The most common type of flow encountered by VWF is shear flow. In shear flow, the rate of fluid flow increases from the wall toward the center of the vessel (Figure 1.5). This system exhibits laminar flow, which dictates that the velocity of each lamina increases from negligible at the wall to maximal at the center of the vessel (Figure 1.5)\textsuperscript{22,23}. Conversely, the shear rate is highest at the wall. The shear flow experienced by VWF is a combination of rotational flow and elongational flow\textsuperscript{23}. Rotational flow causes particles to tumble, whereas elongational flow causes particles to adapt elongated conformations\textsuperscript{22}. VWF multimers have a compact shape\textsuperscript{18,19,21,26} however in flow VWF elongates and tumbles\textsuperscript{20,21,26}. 

9
In shear flow, the rate of fluid flow increases from the wall toward the center of the vessel. This system exhibits laminar flow, where the velocity of each lamina increases from negligible at the wall to maximal at the center of the vessel. The shear rate is highest at the wall.

This remarkable behavior of VWF under flow makes it such that VWF is most extended where its hemostatic functions are required. Because shear stress is greatest at the vessel wall, VWF which has been recruited to the sub-endothelial matrix experiences forces that are much greater than free-flowing VWF\textsuperscript{12,14}, suggesting that the forces experienced are solely elongational\textsuperscript{26}.

1.3.5 The interaction of von Willebrand Factor with other molecules

Coagulation Factor VIII

VWF binds factor VIII (FVIII), a clotting cofactor, and stabilizes it in the circulation. Factor VIII, which is synthesized as a 2,351 amino acid single-chain glycoprotein, binds VWF with high affinity ($K_D<0.5$ nM) through its D'D3 domain\textsuperscript{27}. The domains of FVIII
are organized as A1-A2-B-A3-C1-C2 (Figure 1.6). The mature protein has a molecular weight of 280 kDa composed of a light chain and a heavy chain\textsuperscript{28}. The light chain has a molecular weight of 80 kDa and is made up of domains A3-C1-C2, while the heavy chain is composed of domains A1-A2-B and has a more heterogeneous composition with molecular weights varying from 90 – 200 kDa\textsuperscript{28}.

![Molecular Structure of human factor VIII and thrombin cleavage](image)

**Figure 1.6. Molecular Structure of human factor VIII and thrombin cleavage.** Schematic representation of the domain structure of FVIII. The heavy chain composed of A1–A2 domains is linked to a heterogeneously processed B domain of variable length. The light chain is composed of A3–C1–C2 domains. The three acidic regions are denoted as a1, a2, and a3. Upon activation by thrombin, FVIII is released from VWF, after which thrombin cleaves FVIII after Arg residues 372, 740, and 1689. Dissociation of FVIII from VWF is facilitated by the cleavage after Arg-1689. Cleavage after Arg-740 releases the dispensable B chain while cleavage after Arg-372 allows the A1 domain to separate from the A2 domain.

FVIII circulates in plasma at levels of approximately 200 ng/ml\textsuperscript{27,28}. *In vivo*, a 1:50 ratio of FVIII to VWF is constantly and rigorously maintained. VWF primarily serves to stabilize the FVIII and promote the association of the light and heavy chains of FVIII.
through a non-covalent complex which persists until the FVIII is activated by thrombin. The VWF-bound FVIII is protected from being inactivated by activated factor IX, activated factor X and activated protein C and antibodies against FVIII.

Upon activation by thrombin, FVIII is released from VWF, after which thrombin cleaves FVIII after Arg residues 372, 740, and 1689. Dissociation of FVIII from VWF is facilitated by the cleavage after Arg-1689. Cleavage after Arg-740 releases the dispensable B chain while cleavage after Arg-372 allows the A1 domain to separate from the A2 domain. Activated FVIII (FVIIIa) accelerates the rate of FX activation by FIXa and eventually leads to the formation of a blood clot.

Platelets

VWF also binds platelets (Figure 1.7) through interaction with the platelet receptor glycoprotein Iba of the GP1b-IX-V receptor complex in the VWF A1 domain, and interactions of the VWF C-domains with integrins αIIbβ3 and αVβ3. The ligand-binding site for VWF is located within the 45-kDa N-terminal region of GP1ba. The GP1bα receptor is expressed on the platelet surface in a functional state that requires no prior activation to bind VWF. After initial platelet arrest due to GP1ba-VWF interaction, intracellular signaling occurs and the platelets become activated. This leads to conformational change and engagement of further receptors and the presentation of phosphatidylserine (PS) on the platelet membrane. Upon ligand binding, the GP1b-IX-V receptor complex can induce signals leading to calcium mobilization, the rearrangement of the cytoskeleton, granule release, and activation of αIIbβ3 integrins.
Normal hemostasis in response to vascular injury is initiated by exposure of subendothelial matrix, allowing VWF and collagen access to their platelet receptors, GPIb-IX-V and GPVI. These interactions lead to rapid elevation of cytosolic Ca\(^{2+}\), cytoskeletal rearrangements, and activation of \(\alpha_{\text{IIb}}\beta_3\) that binds VWF or fibrinogen and mediates platelet aggregation.

There is a strong correlation between the effects of hydrodynamic flow on VWF elongation and platelet aggregation. Studies have shown that VWF adsorbed to the wall of a flow chamber adheres to platelets through GPIb\(\alpha\)\(^{37}\). The binding site for GP1b\(\alpha\) in the A1 domain appears to be hidden or cryptic under static conditions, and exposed by flow\(^{12,33,34}\). The D’D3 domain has been shown to shield recognition of platelets by the A1 domain\(^{40}\). VWF length may potentiate binding to GP1b\(\alpha\) on platelets by enhancing shear or elongational flow-dependent extension of VWF. The avidity of GP1b\(\alpha\) to VWF is also modulated by flow\(^{37}\). Multivalent binding sites are better exposed in the extended conformation of VWF, as opposed to its compacted conformation. Furthermore, platelets have a greater hydrodynamic surface area than VWF\(^{39}\), which effectively increases the
elongational force on VWF. This results in a positive feedback mechanism for amplifying hemostasis.

**Collagen**

VWF has also been shown to bind sub-endothelial collagens through the VWF A1 and A3 domains\(^4^1\). VWF mediates the adhesion of platelets to exposed subendothelium by forming a bridge between collagen, heparin-like glycosaminoglycans and other components of the subendothelium. Several types of fibrillar collagen are bound by VWF in vitro\(^4^2\), and the major collagen binding site is within VWF domain A3\(^4^1\), while a second site may be present in the A1 domain\(^4^3\). Non-fibrillar collagen type VI binds domain A1 rather than domain A3\(^4^2\). In addition, monoclonal antibodies can prevent VWF binding to fibrillar collagen or subendothelial connective tissue, but not both\(^4^1,4^3\). VWF can be deposited on collagen substrates under static conditions\(^4^1\), however it has also been shown that the shear thresholds for VWF extension and deposition on collagen substrates are superimposable\(^2^1\), indicating that, unlike platelet binding, VWF binding to collagen may not be dependent on hydrodynamic flow.

**1.3.6 Molecular structure of von Willebrand Factor**

The current understanding of the organization and boundaries of domains in VWF remains imperfect. Only the 3 A domains (A1, A2, A3) are well characterized with determined crystal structures\(^4^4,4^5,4^6\). The VWF A domains are the defining members of the VWA protein fold and family\(^4^7\). They have a central hydrophobic β-sheet with 6 β-strands, surrounded typically by 6 amphipathic α-helices (Figure 1.8). VWF A-like
domains are also found in integrins as ligand-binding αI and βI domains\cite{44,45}, in complement components\cite{47}, and in intracellular proteins with diverse functions\cite{47}. Some VWF A-like domains contain a metal-ion dependent adhesion site, but those in VWF do not\cite{12,47}. VWF A-like domains in integrins and complement undergo substantial conformational changes that regulate affinity for ligand\cite{47}. Conformational change in VWF A domains has not yet been observed. VWF A1 and integrins differ substantially in the location of ligand binding sites on the surfaces of the VWF A-like domains. Most modules in extracellular proteins are all-β\cite{44,45}, and have their N- and C-terminal ends at opposite ends of the domain. In contrast, VWF A-like domains are α/β, and their N- and C-termini are adjacent to one another\cite{44,46,47}, enabling greater flexibility when arranged as beads on a string, as in VWF.
Figure 1.8. Crystal structures of the VWF A domains. Panels A-C. Ribbon diagrams for VWF-A2, VWF-A2, and VWF-A3, respectively. α-helices (green, blue, cyan), β-strands (yellow) and loops (grey) are shown. The primary sequence (denoting secondary structure by the Dictionary of protein secondary structure (DSSP) algorithm\(^2^4\)) is displayed, showing the similarity in the placement of α-helices, β-strands, and 3/10 helices.
Unique among VWF domains, A2 lacks a long-range disulfide bond (Figure 1.7) and, therefore, is unprotected from unfolding by tensile force applied along the length of VWF multimers. Two 1.9 Å crystal structures of A2 have revealed surprising evolutionary adaptations to the function of the VWF A2 domain as a “shear bolt” domain (Figure 1.9), and how mutations destabilize A2 in von Willebrand Disease (VWD). The wild-type mammalian A2 structure reveals two N-glycosylation sites, as well as a vicinal disulfide bond whereas the mutant E. coli A2 structure reveals a Ca\(^{2+}\)-binding loop.

**Figure 1.9. The VWF A2 domain.** Adapted from Emsley et. al. and Zhang et. al. Ribbon diagrams show α-helices (cyan), β-strands (yellow) and loops (grey). The α4-less loop is in magenta. Key side chains are shown as sticks, and coordinated Ca\(^{2+}\) is shown as a sphere. Carbons of the ADAMTS13 cleavage site residues Tyr\(^{1605}\) and Met\(^{1606}\) are black. The vicinal disulfide is in gold. Hydrogen and metal coordination bonds are dashed.
1.4 von Willebrand Factor Pathophysiology

1.4.1 The deficiency or abnormality of VWF causes von Willebrand Disease

von Willebrand Disease (VWD) is an inherited bleeding disorder that is caused by deficiency or dysfunction of VWF. Defects in VWF can cause bleeding by impairing platelet adhesion or by reducing the concentration of FVIII. VWD is a relatively common cause of bleeding, but the prevalence varies considerably among studies and depends strongly on the case definition that is used. VWD prevalence has been estimated in several countries on the basis of the number of symptomatic patients seen, and the values range from roughly 23 to 110 per million population (0.0023–0.01%)\textsuperscript{49}.

The patient who led to the discovery of VWD was a 5-year-old girl who was seen by Dr. Erik von Willebrand\textsuperscript{50}. He ultimately assessed 66 members of her family and reported, in 1926, that this was a previously undescribed bleeding disorder that differed from hemophilia and exhibited mucocutaneous bleeding, autosomal inheritance, prolonged bleeding times, and normal clotting time\textsuperscript{50}. In the 1950s, it became clear that a “plasma factor” was decreased in these patients, and the factor was called “von Willebrand factor”\textsuperscript{49}. As cryoprecipitate and commercial FVIII concentrates were developed, it was recognized that both VWF and antihemophilic factor (FVIII) purified together.

When immunoassays were developed, persons who had VWD were found to have reduced factor VIII-related antigen (FVIIIR:Ag) (which we now refer to as VWF antigen (VWF:Ag))\textsuperscript{51}. Characterization of the proteins revealed that FVIII was the clotting protein deficient in hemophilia A (HA), and VWF was a separate FVIII carrier protein that resulted in the fractionation of both proteins in commercial concentrates\textsuperscript{51}. 

Furthermore, a deficiency of VWF resulted in increased FVIII clearance because of the reduced VWF carrier protein.

1.4.2 Genetics of von Willebrand Disease

Since the 1980s, molecular and cellular studies have defined HA and VWD more precisely. Persons who had VWD had a normal FVIII gene on the X chromosome, but some were found to have an abnormal VWF gene on chromosome 12\textsuperscript{51}. Variant forms of VWF were recognized in the 1970s, and these variations are now recognized as the result of synthesis of an abnormal protein\textsuperscript{51}. Gene sequencing identified many of these persons as having a VWF gene mutation. The genetic causes of milder forms of low VWF are still under investigation, and these forms may not always be caused by an abnormal VWF gene. In addition, acquired disorders may result in reduced or dysfunctional VWF.

1.4.3 Classifications of von Willebrand Disease

VWD is classified into three major categories: partial quantitative deficiency (type 1), qualitative deficiency (type 2) and total deficiency (type 3) (Table 1.1)\textsuperscript{52,53}. Type 2 VWD is divided further into four variants (2A, 2B, 2M and 2N) on the basis of details of the phenotype. Before publication of the 1994 revised classification of VWD\textsuperscript{53}, VWD subtypes were classified using roman numerals (types I, II, and III), generally corresponding to types 1, 2 and 3 in the 1994 classification, and within type II several subtypes existed (designated by adding sequential letters of the alphabet, i.e. II-A through II-I). Most of the latter VWD variants were amalgamated as type 2A in the 1994 classification, with the exception of type 2B, for which a separate new classification was
created. In addition, a new subtype (2M, with “M” representing “multimer”) was created to include variants with decreased platelet-dependent function but no significant decrease of higher molecular weight VWF multimers. Subtype 2N VWD was defined, with “N” representing “Normandy”, where the first individuals were identified, with decreased FVIII because of VWF defects of FVIII binding.

Table 1.1. Classification of von Willebrand Disease

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>Partial quantitative deficiency of VWF</td>
</tr>
<tr>
<td>2</td>
<td>Qualitative VWF defect</td>
</tr>
<tr>
<td>2A</td>
<td>Decreased VWF-dependent platelet adhesion with selective deficiency of high-molecular-weight multimers</td>
</tr>
<tr>
<td>2B</td>
<td>Increased affinity for platelet GPIbα</td>
</tr>
<tr>
<td>2M</td>
<td>Decreased VWF-dependent platelet adhesion without selective deficiency of high-molecular-weight multimers</td>
</tr>
<tr>
<td>2N</td>
<td>Markedly decreased binding affinity for FVIII</td>
</tr>
<tr>
<td>3</td>
<td>Complete deficiency of VWF</td>
</tr>
</tbody>
</table>

1.4 Structure, Evolution, and Cellular Functions of ADAMTS13

1.4.1 ADAMTS13 is the von Willebrand Factor-cleaving protease

Newly released UL-VWF is not only large in size, but also functionally hyperactive. It binds platelets with increased affinity and causes abnormal platelet adhesion and aggregation on endothelial cell surface. Therefore, proteolytic processing of UL-VWF multimers into normal sizes of VWF (from 500 kDa to 2 million Da) circulating in blood is critical for preventing arterial thrombosis. This trimming process is primarily
achieved by proteolytic cleavage of VWF by a plasma metalloprotease, ADAMTS13 (A Disintegrin and Metalloprotease with Thrombospondin type1 repeats-13)\textsuperscript{13}. The translated product consists of 1,427 amino acid residues with an estimated molecular weight of 145 kDa\textsuperscript{58}. It comprises a signal peptide, a propeptide, a metalloprotease domain (Met), a disintegrin domain (Dis), first thrombospondin type 1 repeat, a Cys-rich domain and a spacer domain (Figure 1.10). The C-terminus of ADAMTS13 has seven additional TSP1 repeats and two CUB domains (Figure 1.9)\textsuperscript{58}.

![Figure 1.10. Schematic structures of the ADAMTS13 gene and protein. Modified from Zheng et. al.\textsuperscript{58} ADAMTS13 consists of a signal peptide (S), propeptide (P) and a metalloprotease domain, followed by a proximal C-terminal region including disintegrin (Dis), first TSP1 (1), Cys-rich (Cys) and spacer domains. The middle and distal C-terminal regions have the 2\textsuperscript{nd} to 8\textsuperscript{th} TSP1 repeats and two CUB domains, respectively.]

1.4.2 Specificity of ADAMTS13

The catalytic subunit, known as the metalloprotease domain (Met), contains a characteristic HEXXHXXGXXHD sequence that coordinates Zn\textsuperscript{2+} or Ca\textsuperscript{2+} binding\textsuperscript{58}. However, Met alone is not sufficient for recognition and cleavage of multimeric VWF or peptidyl substrates that consist of 73 amino acid residues from the A2 domain (such
as GST-VWF73\textsuperscript{59,60} and FRETS-VWF73\textsuperscript{59,61}). The ancillary domains within the proximal C-terminal region of ADAMTS13 including the disintegrin domain (Dis), the first TSP1, the Cys-rich domain (Cys) and the spacer domain (Spa) appear to be required for recognition and cleavage of VWF under static/denaturing conditions\textsuperscript{59,61}. Removal of either domain within the proximal C-terminal region significantly reduces the ability of ADAMTS13 to cleave VWF and reduces the specificity\textsuperscript{59,61}.

Cleavage of peptide substrates, such as GST-VWF73 and FRETS-VWF73, requires even fewer domains of ADAMTS13\textsuperscript{59}, suggesting that the interaction between ADAMTS13 and full-length VWF is quite different from that between ADAMTS13 and peptide substrates. Regardless of the kind of substrate used, it has been demonstrated that the Cys/Spa region of ADAMTS13 is critical for recognition of VWF, partly through its binding to the amino acid residues (Glu\textsuperscript{1660}-Arg\textsuperscript{1668}) in the VWF-A2 domain\textsuperscript{60}.

The role of the more distal C-terminal domains (such as TSP1 repeats and CUB domains) of ADAMTS13 in recognition of VWF, however, remains controversial. Peptides or the fragment derived from the first CUB domain of ADAMTS13 inhibited cleavage of endothelial cell bound UL-VWF multimers\textsuperscript{62}, but another study showed that an ADAMTS13 mutant truncated after Spa was active in cleavage of endothelial cell bound UL-VWF using the same assay\textsuperscript{63}. Under physiologically relevant conditions, the cooperative activity between the TSP1 5-8 and CUB domains appeared to be critical for recognition of soluble VWF under fluid shear stress\textsuperscript{64}, suggesting that binding of the middle and distal C-terminal domains of ADAMTS13 may be required for further interactions between VWF-A2 and the proximal C-terminal domains such as Cys and
Spa. Homozygous mutations found in hereditary TTP resulted in truncation of ADAMTS13 four amino acid residues after Spa\textsuperscript{65}, further supporting the role of the middle and distal C-terminal domains in ADAMTS13 function \emph{in vivo}.

\subsubsection*{1.4.3 Molecular structure of ADAMTS13}

To date, crystal structures of the M and D domains of three human ADAMTS-family proteins, ADAMTS1\textsuperscript{66}, ADAMTS4\textsuperscript{67} and ADAMTS5\textsuperscript{67}, have been reported. The first crystal structure of exosite-containing fragments of ADAMTS13 was reported by Akiyama et. al\textsuperscript{68}. The structures of the ADAMTS13-DTCS fragment, obtained at 2.6-Å and 2.8-Å resolution, revealed linearly distributed exosites that recognize distinct substrate regions (Figure 1.11)\textsuperscript{68}.

The ADAMTS13 cleavage site at residues Tyr\textsuperscript{1605} and Met\textsuperscript{1606} lies in a very inaccessible site in the folded A2 domain, situated in the center of the β4-strand, sandwiched between amphipathic α-helices and loops on either side (Figure 1.9)\textsuperscript{46}. The regions around the cleavage site, as well as between the cleavage site and the C-terminus, are poorly packed\textsuperscript{46}. Multiple side chain conformations are visible for cleavage site residues Met\textsuperscript{1606} and Leu\textsuperscript{1603}, whereas disordered side chains are not seen in similar buried positions in the crystal structure of the VWF A1 and A3 domains (Figure 1.8)\textsuperscript{44,45,46}. Cys\textsuperscript{1669} and Cys\textsuperscript{1670} are linked in a vicinal disulfide bond at the C-terminus of the A2 domain (Figure 1.9)\textsuperscript{46}. Strikingly, all A2 interactions occur in this C-terminal portion, and this region is bounded by the β4-strand and the α6-helix, which contain the key recognition sites for ADAMTS13, which have been defined using peptide substrates\textsuperscript{60,69,70,71}. Deletion of Cys\textsuperscript{1669}-Cys\textsuperscript{1670} markedly enhances cleavage of VWF multimers by ADAMTS13\textsuperscript{72}.
Figure 1.11. Crystal structure of the ADAMTS13-DTCS fragment. Adapted from Akiyama et al. Panel A shows a schematic representation of the domain structures of full-length ADAMTS13 and ADAMTS13-DTCS. Panels B and C show the ribbon structure of ADAMTS13-DTCS (form 1) and ADAMTS13-DTCS (form 2), respectively.
1.4 ADAMTS13 Pathophysiology

1.4.1 Deficiency of ADAMTS13 causes Thrombotic Thrombocytopenic Purpura

Thrombotic Thrombocytopenic Purpura (TTP), first described by Eli Moschcowitz in 1924, is a potentially fatal syndrome. The clinical manifestations of this disease are thrombocytopenia, microangiopathic hemolytic anemia, neurological symptoms and signs, and various degrees of renal functional abnormalities. TTP is caused by the inability to cleave VWF due to hereditary or acquired deficiency of plasma ADAMTS13 activity.

Approximately 5% of all TTP cases are caused by hereditary mutations of ADAMTS13. The mutations are present throughout the gene, causing changes in amino acid composition, truncation of protein and impaired secretion. Most cases of adult TTP are caused by autoantibodies against ADAMTS13. In the earliest reports, inhibitors of ADAMTS13 were found in 65% to 96% of patients with acquired (idiopathic) TTP with severe deficiency (less than 5~10% of normal activity). In later prospective studies, a lower prevalence of anti-ADAMTS13 autoantibodies (31-44%) was reported (Table 1.2). Assessment of binding activity by ELISA has indicated that autoantibodies can be found in almost all patients with acquired (idiopathic) TTP who exhibit a severe deficiency of plasma ADAMTS13 activity. Autoantibodies against ADAMTS13 were also detected in six of seven patients with ticlopidine-associated TTP, and in two patients with clopidogrel-induced TTP. What triggers autoantibody formation is still not known.
Table 1.2. Prevalence of autoantibodies against ADAMTS13 in patients with idiopathic TTP

<table>
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<tr>
<th>Authors, year</th>
<th>Number of positive/investigated</th>
<th>% positive</th>
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<tr>
<td>Furlan et. al., 1998</td>
<td>20/24</td>
<td>83%</td>
</tr>
<tr>
<td>Tsai et. al., 1998</td>
<td>26/39</td>
<td>67%</td>
</tr>
<tr>
<td>Tsai et. al., 2001</td>
<td>31/41</td>
<td>76%</td>
</tr>
<tr>
<td>Veyradier et. al., 2001</td>
<td>30/59</td>
<td>51%</td>
</tr>
<tr>
<td>Rich et. al., 2002</td>
<td>26/29</td>
<td>90%</td>
</tr>
<tr>
<td>Study et. al., 2003</td>
<td>31/50</td>
<td>62%</td>
</tr>
<tr>
<td>Coppo et. al., 2004</td>
<td>17/31</td>
<td>55%</td>
</tr>
<tr>
<td>Zheng et. al., 2004</td>
<td>7/20</td>
<td>44%</td>
</tr>
</tbody>
</table>

1.4.2 Autoantibodies in the pathogenesis of Thrombotic Thrombocytopenic Purpura

Despite of the importance of autoantibodies in pathogenesis of TTP, and its presence correlates with more relapsing disease and poorer prognosis than its absence, the binding epitopes and functional consequences of many autoantibodies against various domains of ADAMTS13 remain poorly understood. A limited number of published studies demonstrated that the major binding epitopes of autoantibodies are localized to Cys and Spa. Using recombinant ADAMTS13 and variants expressed in mammalian cells, our lab also showed that approximately 30-45% of TTP patients harbored autoantibodies recognizing the middle TSP1 2-8 repeats and distal CUB domains. Cys and Spa have been shown to play a critical role in the recognition and cleavage of a peptidyl substrate (VWF73), denatured VWF, and VWF under shear stress. These domains have also been shown to be important for the \textit{in vivo} function of ADAMTS13. However, the
roles of the middle and distal C-terminal domains remain unclear. Understanding the biological and pathological significance of these autoantibodies against the middle and distal C-terminal domains will provide novel insight into the pathogenesis of TTP.

1.6 Specific Aims of the Thesis Research

The long-term goal is to understand of the biology of VWF processing by ADAMTS13, and thereby the pathophysiology of thrombotic complications. ADAMTS13 cleaves native VWF relatively poorly, and its preference for cleavage of the larger multimers is considered to lie in their greater susceptibility to deformation under shear stress or under flow. The specific hypothesis behind this thesis research is that physiological cofactors form ternary complexes with VWF and ADAMTS13 and, in addition to shear stress, cause the required conformational change of VWF to accelerate proteolytic processing by ADAMTS13 and that the ability of ADAMTS13 to recognize and cleave VWF is governed by exosite interactions with VWF.

Chapter 2 demonstrates that both FVIII and platelets can serve as cofactors for enhancing VWF proteolysis by ADAMTS13 by synergistically altering VWF conformation under shear stress. 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.

Chapter 3 demonstrates that a naturally-occurring type 2N VWF variant that exhibits significantly reduced FVIII binding is compromised in proteolytic cleavage by ADAMTS13 in the presence of FVIII, proportional to the defects of their FVIII binding. These results demonstrate the role the VWF propeptide and FVIII-binding site (D’D3 domains) in
regulating VWF proteolysis by ADAMTS13 and suggest an evolutionary advantage by limiting VWF proteolysis in cases of VWD. Currently, the developed assays for detecting ADAMTS13 cleavage of VWF require simultaneous cleavage of scissile bonds in adjacent subunits, greatly underestimating the true catalytic efficiency of ADAMTS13. Thus, Chapter 3 also describes a novel ELISA-based assay detects the N-terminal peptide (i.e. EQAPNVY) resulting from cleavage after the Tyr residue occurring anywhere along a VWF multimer, which dramatically increases the sensitivity of the ADAMTS13-mediated VWF cleavage assay.

Chapter 4 examines the specific exosite interactions that govern binding to the Spa domain of ADAMTS13 and their effects on VWF cleavage and binding of inhibitory anti-ADAMTS13 antibodies. This work, as part of two published papers, was primarily done by others in the lab. Here, I describe my contributions to the project. Following is a description of the crystallization, screening, data collection, and structure determination for an autolysis product of ADAMTS13, the ADAMTS13-CS fragment.

Chapter 5 explores the in vivo function of ADAMTS13, specifically the composition of Admats13^{1/-} mice thrombi, as well as the differential composition at the head and tail of the thrombus. We determined that infusion of recombinant FL-ADAMTS13 or a truncated ADAMTS13 variant into Admats13^{1/-} mice restored the kinetics of platelet/red blood cell accumulation and fibrin formation to those observed in wild-type mice. Our findings, revealing the apparent difference in thrombus composition, provide novel insight into the mechanism of ADAMTS13 function in vivo.
Chapter 6 concludes with a discussion regarding the implications of the results described in the preceding chapters as well as a comprehensive analysis of future works in the field of cofactor- and exosite interaction-mediated VWF proteolysis by ADAMTS13.

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CHAPTER 2: PHYSIOLOGICAL COFACTORS FACTOR VIII AND PLATELETS SYNERGISTICALLY REGULATE ADAMTS13 PROTEOLYTIC ACTIVITY

(Manuscript Title: Factor VIII and platelets synergistically accelerate cleavage of von Willebrand factor by ADAMTS13 under fluid shear stress)

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2.1 Overview

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 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 concentrations of FVIII (1 nM), the formation of the VWF cleavage product dramatically increases as a function of increasing platelets with a 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 a FVIII derivative that lacks an acidic region (a3), an anti-platelet glycoprotein 1bα (GP1bα) IgG, and a purified recombinant VWF-A1 domain or GP1bα-stripped 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.

2.2 Introduction

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\(^1,^2\) and the other may occur in solution (or
blood). The cleavage of the cell membrane-anchored UL-VWF by ADAMTS13 occurs very rapidly and requires low or almost no shear stress. 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. The soluble VWF cleaved from the endothelial cell membrane by ADAMTS13 remains ultra large in size and the multimer distribution is not different from that released from Weibel-Palade bodies upon histamine stimulation. 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 as compared with healthy individuals, suggesting that increased VWF proteolysis is correlated with increased fluid shear stress in vivo. However, under in vitro assay conditions, fluid shear stress alone 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 and we 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. In this study, we show that the combination of the two (FVIII and platelets) at physiological concentrations synergistically accelerate 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 GP1bα 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.

2.3 Materials and Methods

2.3.1 Preparation of plasma 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 15,000g. The supernatant was decanted and 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 re-dissolved with 10 ml PBS. Only 5 ml of clear supernatant (~1% of the column volume) was loaded onto a Sephacryl-300 gel filtration column (2.5 cm x100 cm, ~490 ml) (GE Healthcare,
Piscataway, NJ). The purity of VWF was determined by 8% SDS-polyacrylamide gel and Coomassie blue staining. The multimer distribution was determined by 1% mini-agarose gel electrophoresis and Western blotting as described previously\textsuperscript{10,13}. The amount of purified VWF was determined by absorbance at 280 nm corrected by absorbance at 320 nm (1 corrected absorbance at 280 nm equals to \(\sim1\) mg/ml). The purified plasma VWF is free of detectable FVIII, assessed by an enzyme-linked immunoassay established in the laboratory. Recombinant ADAMTS13 was expressed in stably transfected HEK293 cells and purified according to the protocol described previously\textsuperscript{11,14}. The contaminated proteins were removed by gel filtration on a Superose-6 column (GE Healthcare, Piscataway, NJ) with 20 mM HEPES, pH 7.5, 150 mM NaCl, and 5 mM CaCl\(_2\) (Figure 2.1). The amount of the purified proteins was determined by absorbance at 280 nm (corrected with light scattering at 320 nm) (1 corrected absorbance at 280 nm equals to \(\sim0.68\) mg/ml) as described previously\textsuperscript{11,14}. Recombinant human FVIII and FVIII derivative (FVIII-2RKR) were prepared as previously described\textsuperscript{10}. The final products were exchanged into 20 mM HEPES, 0.15 M NaCl, and 5 mM CaCl\(_2\), pH 7.5, and stored at -80 °C.
Figure 2.1. Initial purification of ADAMTS13-V5His. Recombinant ADAMTS13 was expressed in stably transfected HEK293 cells and purified. The contaminated proteins were removed by gel filtration on a Superose-6 column (GE Healthcare, Piscataway, NJ) with 20 mM HEpes, pH 7.5, 150 mM NaCl, and 5 mM CaCl$_2$ (Panel A). The amount of the purified proteins was determined by absorbance at 280 nm (corrected with light scattering at 320 nm) (1 corrected absorbance at 280 nm equals to ~0.68 mg/ml). Coomassie blue gel showing the fractions from the “B” and “C” peaks (Panel B). The fractions in the “B” peaks comprised the collected full-length ADAMTS13 (arrowhead).

2.3.2 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 (PRP) was prepared by centrifugation of the anti-coagulated whole blood at 1,000 rpm for 10 minutes. The PRP was then loaded onto a Sepharose-2B column (GE Healthcare, Piscataway, NJ) pre-equilibrated with Modified Tyrode’s buffer (20 mM HEpes pH 7.4, 137 mM NaCl, 5.6 mM Glucose, 1 g/l BSA, 1 mM MgCl$_2$, 2.7 mM KCl, 3.3 mM NaH$_2$PO$_4$). 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 minutes. The pellets were re-suspended in the same buffer to a platelet concentration of $20 \times 10^6$ µl, determined by counting in the hemacytometer.

2.3.3 Removal of GP1ba receptor from fresh platelets by O-sialoglycoprotein endopeptidase

Freshly isolated human platelets ($3 \times 10^9$/µl) were treated without or with various concentrations (0-400 µg/ml) of O-sialoglycoprotein endopeptidase (OSE) isolated from Pasteurella haemolytica$^{15,16,17}$ (Cedarlane Laboratories, Burlington, NC) at 25°C for 4 hours in Modified Tyrode’s buffer. OSE specifically cleaves proteins containing O-linked glycans, but spares N-linked glycoproteins or unglycosylated proteins$^{18,19,20}$. This metallo-endoprotease has been reported to specifically cleave the 25-kDa N-terminal fragment of platelet GPIbα, which is heavily O-glycosylated, while not affecting other receptors such as GPIIb-IIIa$^{21}$. The enzyme-digested fresh platelets were washed with Modified Tyrode’s buffer to remove the excessive protease, soluble GP1b and VWF prior to addition into the reactions.

2.3.4 Ristocetin-induced platelet agglutination (RIPA) 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 hours were mixed with purified plasma VWF (15 µg/ml, final concentration), and ristocetin (1 mg/ml) (Helena Laboratories, Beaumont, TX) in a glass tube with stirring at 1,100 rpm. The light transmission was recorded simultaneously in all the
channels for 4 minutes 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%.

2.3.5 Cleavage of multimeric VWF by ADAMTS13 under fluid shear stress

Purified plasma VWF (37.5 µg/ml or 150 nM of VWF subunits) was incubated with recombinant ADAMTS13 (25 nM) in the presence or absence of various other components (such as FVIII, FVIII-2RK R and platelets) in 20 mM HEPES, pH 7.5 containing 1 mg/ml BSA, 150 mM NaCl and 5 mM CaCl₂ 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, Pittsburg, PA) at rotation rate of 2,500 rpm for 10 min. The reaction mixtures were then denatured without reducing agents at 100 ºC for 5 min with a sample buffer [62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate (SDS), and 0.01% bromophenol blue] for the SDS-polyacrylamide gel or at 60 ºC 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 ºC, 120V for 150 min or in a 1% agarose gel at 4 ºC, 15 mA for 100 min in a Mini-protein III gel cassette (Bio-Rad, Hercules, CA). The proteins were then transferred onto a nitrocellulose membrane (Millipore, Billerica, MA) at 100 mA for 60 min from the SDS polyacrylamide gel and at 15V 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, Nebraska) in TBS containing 1% casein and 0.05% Tween 20. The fluorescent signals were collected by an Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, Nebraska) (22). The cleavage products were quantified by NIH ImageJ software after the fluorescent intensities were converted into the gray image signals.

2.4 Results

2.4.1 Factor VIII and platelets synergistically increase cleavage of VWF by ADAMTS13 under fluid shear stress

Previous studies have shown that FVIII\textsuperscript{10} or platelets\textsuperscript{12} 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. D'D3 domain and A1 domain, respectively\textsuperscript{22}. 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 (150x10\textsuperscript{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\textsubscript{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 (Figure 2.2). The proteolytic cleavage of VWF by ADAMTS13 in the presence of 1 nM of FVIII or 150-600×10³/µl of lyophilized platelets alone increased by 2-3 folds after 10 minutes of incubation on the PCR tube mixer (2,500 rpm) (Figure 2.2). However, when both FVIII (1 nM) and lyophilized platelets (150×10³/µl) were added into the reaction, more substantial increase in the formation of cleavage product was detected than in the presence of either FVIII or platelets alone (Figure 2.2). The amount of cleavage product formed when both FVIII and platelets were present was ~8 fold (Figure 2.2), greater than the sum of the product generated in the presence of either FVIII or platelets alone, suggesting a synergistic effect of FVIII and platelets in enhancing VWF proteolysis by ADAMTS13 under fluid shear stress.
Figure 2.2. Factor VIII and platelets synergistically accelerate cleavage of VWF by ADAMTS13 under shear stress. Purified human plasma VWF (~150 nM) was incubated with recombinant ADAMTS13 (25 nM) in the absence (-) or the presence (+) of recombinant FVIII (1 nM) and/or lyophilized platelets (150×10^3/µL) for 10 min under constant mixing on the MixMate vortexer at 2,500 rpm, which generates fluid shear stress (~32 dyn/cm²). The proteolytic cleavage product (350 kDa) (arrowhead) was determined by Western blotting with rabbit anti-VWF IgG, followed by IRdye 800-labeled goat anti-rabbit IgG. The image was obtained by an Odyssey imaging detection system. Negative controls including the omission of ADAMTS13, FVIII or platelet or addition of EDTA (10 mM) showed no detectable cleavage product.

Consistent with previously reported results, in the presence of increasing concentrations of platelets, VWF cleavage was increased (Figure 2.3). Addition of 1 nM FVIII to the reaction shifted the curve to lower concentrations of platelets (Figure 2.3), indicating that FVIII and platelets can synergistically increase VWF proteolysis by ADAMTS13. Conversely, in the absence of platelets FVIII alone increased the formation of cleavage product in a concentration dependent manner (Figure 2.4). However, in the presence of physiological concentration of platelets (~150x10^3/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) (Figure 2.4), which falls into the physiological range of human plasma FVIII concentrations.
Figure 2.3. Cleavage of VWF by ADAMTS13 in the presence of fixed concentration of FVIII and various concentrations of platelets under shear stress. Purified human plasma VWF (~150 nM) was incubated with recombinant ADAMTS13 (25 nM) in the absence (-FVIII) or the presence (+FVIII), but with increasing concentrations of lyophilized platelets (0-600x10^3/µl) at 25 ºC for 10 min under constant mixing at 2,500 rpm on the MixMate PCR mixer. The proteolytic cleavage product of VWF (350 kDa) (arrowhead) was determined by Western blotting. The 350 kDa band (Panel A, arrowhead) was quantified by densitometry using Image J software and the fold of increase compared with that in the absence of platelet and FVIII in Lane 1 was plotted against platelet concentrations (Panel B). The results are the mean ± standard error from three independent experiments.
Figure 2.4. Cleavage of VWF by ADAMTS13 in the presence of fixed concentration of platelets and various concentrations of FVIII under shear stress. Purified human plasma VWF (~150 nM) was incubated with recombinant ADAMTS13 (25 nM) in the absence (-) and the presence (+) of lyophilized platelets (150x10^3/µL) and various concentrations of recombinant FVIII (0, 0.5, 1, 2.5, 5 and 10 nM) for 10 min under constant mixing at 2,500 rpm on the MixMate PCR mixer. The proteolytic cleavage product (350 kDa) (Panel A, arrowhead) was detected by Western blotting as described. The 350 kDa-band of proteolytic cleavage product in the absence (-Plt) and in the presence of platelet (+Plt) was plotted against the concentrations of FVIII (Panel B). The data (fold of increase) represent the mean ± standard error from three independent experiments.
2.4.2 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 (KD, 0.3~0.5 nM) through its interaction with the D'D3 domain of VWF\textsuperscript{22}. This high affinity binding appears to be necessary for the enhancing effect of FVIII on VWF proteolysis by ADAMTS13 under shear stress\textsuperscript{10}. 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 of a B-domain less FVIII lacking the acidic (a3) region (i.e. FVIII-2RKR) that is responsible for high affinity binding to VWF\textsuperscript{22} and various concentrations of lyophilized platelets (0-600x10\textsuperscript{3}/\mu l) under the same conditions. We showed that in the presence of FVIII-2RKR (instead of wild type FVIII), platelets (up to 600x10\textsuperscript{3}/\mu l) increased the formation of proteolytic cleavage of VWF by ADAMTS13 by ~2-3 fold (Figure 2.5), 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.
Figure 2.5. Cleavage of VWF by ADAMTS13 in the presence of fixed concentration of FVIII-2RKR and various concentrations of platelets. Purified human plasma VWF (~150 nM) was incubated with ADAMTS13 (25 nM) in the presence of 1 nM of recombinant wild-type FVIII or a B-domain-deleted variant lacking the acidic region (a3) (FVIII-2RKR) and increasing concentrations of lyophilized platelets (0-600x10^3/µl) for 10 min at 25 °C under constant mixing at 2,500 rpm on the MixMate PCR mixer. The proteolytic cleavage product (350 kDa) (Panel A, arrowhead) was determined by Western blotting (Panel B). The quantification of 350 kDa band (fold Increase) was determined by densitometry. Results represent the mean ± standard error from three independent experiments.
2.4.3 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 GP1bα with the A1 domain of VWF\(^2\). 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 anti-GP1bα IgG (6D1) (Figure 2.6A and C), provided by Dr. Barry Coller at Rockefeller University, New York, NY\(^2\) or purified A1 domain (Figure 2.5B 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. 6D1 at a concentration of 0.8 µM (Figure 2.6A and C) or VWF-A1 domain at a concentration of 0.75 µM (Figure 2.6B) almost completely blocked proteolytic cleavage of VWF by ADAMTS13 despite of the presence of 1 nM FVIII, suggesting that the interaction between VWF-A1 and GP1bα may be more important in regulating cleavage of the VWF-A2 domain by ADAMTS13.

Furthermore, freshly isolated platelets pre-treated with 200 µg/ml of O-sialoglycoprotein endopeptidase (OSE) for 4 hours, which selectively removed a 25-kDa N-terminal fragment of the platelet surface receptor GP1bα, but spared receptor GPIIb-IIIa, exhibited a dramatically reduced rate-enhancing effect on the VWF proteolysis by ADAMTS13 in the presence of FVIII (1 nM) (Figure 2.6A and B). These results suggest that the synergistic rate-enhancing activity of platelets with FVIII depends on the specific VWF-
A1 and GP1bα interaction. 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 (Figure 2.6A and B), consistent with that previously reported\textsuperscript{12}. 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 (Figure 2.6A). Such a result was not observed using a 5% SDS-polyacrylamide gel by Shim et al\textsuperscript{12}, 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 (Figure 2.6A).

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 Figure 2.5C, OSE treatment dramatically reduced ristocetin-induced platelet agglutination in a concentration- dependent manner. In the presence of 50 µg/ml of OSE, the ristocetin-induced platelet agglutination was almost completely inhibited (Figure 2.6C), whereas the control platelets treated for 4 hours at 37 °C with Modified Tyrode’s buffer aggregated normally (Figure 2.6C). Together, our data support a hypothesis that the specific interactions between GP1bα receptor and VWF-A1 domain, but not the membrane scaffold interactions, may be necessary for the synergistic rate enhancing effect of platelets with FVIII on proteolytic cleavage of VWF by ADAMTS13 under physiologically relevant fluid shear stresses.
Figure 2.6. Disruption of VWF-platelet interactions inhibits cleavage of VWF by ADAMTS13 under shear stress. Purified plasma VWF (150 nM) was incubated with ADAMTS13 (25 nM), lyophilized platelets (150x10^3/µl), and FVIII (1 nM) in the absence or presence of increasing concentrations of 6D1 anti-GP1b IgG (panel A) or purified A1 domain (Panel B). In addition, purified VWF (150 nM) was incubated with ADAMTS13 (25 nM), and FVIII (1 nM) in the absence (Panel C & D, Lane 1) or the presence of various concentrations of fresh platelets (150x10^3/µl) that were pre-treated without (Panel C) or with 200 µg/ml of O-sialoglycoprotein endopeptidase (Panel D) for 4 hours and washed. The removal of GP1b receptor from the platelet surface was assessed by ristocetin-induced light scattering platelet agglutination assay (Panel E).

2.4.4 Assessing the amount of fluid shear stress generated in the 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 (Figure 2.7A) and a reaction system (Figure 2.7B) containing purified plasma VWF (~150 nM), recombinant ADAMTS13 (25 nM), recombinant FVIII (2 nM) and lyophilized platelets (150×10³/µl) exhibited a Newtonian behavior. This indicates that the observed increase in cleavage product can be directly correlated to the increase in fluid shear stress generated in the system, but not to 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 physiological concentrations of FVIII and platelets, but modestly increased in the absence of added FVIII and platelets (Figure 2.7C). Curve fitting of the data obtained from 3 independent experiments revealed a linear relationship between the shear stress and the fold enhancement in the VWF cleavage (Figure 2.7D). Using this standard function, the fluid shear stress generated in the MixMate PCR tube mixer and mini-vortexer was estimated to be ~32 dynes/cm² and ~75 dynes/cm², respectively (Figure 2.7C 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 amount of proteolytic cleavage product in different assays.
Figure 2.7. Assessing the fluid shear stress generated by various vortexing assays. Panels **A** and **B** demonstrate the 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 (150x10^3/µl) was placed on the cone-plate viscometer, respectively. Panel **C** shows a 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 (150x10^3/µL) for 10 min under increasing shear rates on a cone-plate viscometer (HAAKE RotoVisco1) (Lanes 1-8) or Mini-vortexer (Fisher Scientific) (Lanes 9-10) or MixMate PCR mixer (Eppendorf) (Lanes 11-12) for 10 min at rotation rate of 2,500 rpm. The proteolytic cleavage product (350 kDa) (arrowhead) was determined by Western blotting. Panel **D** demonstrates the ratio of the cleavage product (mean ± SD) (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/cm^2 and 32 dynes/cm^2 of shear stress, respectively.
2.5 Chapter 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$^{1,2,5}$ or cofactors$^6$, the proteolytic cleavage of soluble VWF by ADAMTS13 requires drastic manipulations, such as denaturation of VWF substrate with 1.5 M urea/guanidine$^{14,24}$ or exposure of soluble VWF to high fluid shear stress$^{10,11}$.

High fluid shear stress results in conformational changes of VWF multimers and opens up the binding sites and cleavage bond in the central A2 domain$^{25,26,27}$ 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 (Figures 2.2-2.4). Addition of FVIII or platelets to VWF alone can increase the formation of cleavage product (350 kDa) (Figures 2.2-2.4), suggesting that binding of FVIII or platelets to VWF may facilitate 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$^{10,12}$. These differences may result from the amount of shear stress applied and
the concentrations of VWF and ADAMTS13 used in the different assays. As shown in Figure 2.7, vortexing at the rotation rate of 2,500 rpm on a mini-vortexer generates \( \sim 75 \) dynes/cm\(^2\) of fluid shear stress, whereas vortexing at the same rotation rate on a MixMate PCR mixer generates only \( \sim 32 \) dynes/cm\(^2\) of fluid shear stress.

Further analyses demonstrate that FVIII and platelets act synergistically to accelerate VWF proteolysis by ADAMTS13 under shear stress (Figures 2.2-2.4). 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 GP1b\( \alpha \) 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 (Figure 2.5). Furthermore, the synergistic effect of platelets with FVIII is dramatically inhibited by addition of an increasing concentration of 6D1 (Figure 2.6A and C) or purified VWF-A1 domain (Figure 2.6B and D), which disrupts the specific platelet-VWF interactions. The requirement of such specific VWF-A1/GP1b\( \alpha \) 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 GP1b\( \alpha \) receptor has been removed by treatment with a metalloprotease (OSE) isolated from Pasteurella haemolytica, although the platelet-bound VWF may partially contribute to the increase in formation of the cleavage products (Figure 2.6).

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. 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\textsuperscript{18}.

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 dynes/cm\textsuperscript{2} shear force, the predicted peak force soluble VWF multimers experience ranges from 0.2 to 0.8 pN\textsuperscript{19} in the absence of platelets. The peak force increases to ~390 pN when two or more platelets are bound at either side of the scissile bond\textsuperscript{12,19}. This is the amount of shear stress if applied directly to a VWF fragment such as the A1A2A3 or A2 domain in which both ends are attached to a solid surface\textsuperscript{27,28}. However, this does not appear to be the case in our assay system as only a modest increase of VWF cleavage is observed with addition of physiological concentrations of lyophilized platelets alone (Figures 2.2-2.4). 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 \textit{in vitro} assay may underestimate the degree of VWF proteolysis since 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.
2.6 Chapter References


2.7 Supplemental Information

2.7.1 Towards a calculation of the average shear stress generated by the vortex method

*In vivo*, because of the tendency of erythrocytes to deform and aggregate, blood is a fluid with non-Newtonian behavior. This indicates that blood viscosity varies with the force applied to it. Blood viscosity is dependent on shear rate, thus viscosity decreases when the shear rate increases. Shear rate is approximated by the velocity gradient and is primarily determined by the diameter of vessels. A high shear rate is present when flow is fast and the vessel diameter is small; a low shear rate is present when flow is slow and the vessel has a large diameter. The non-Newtonian behavior of blood is, thus, a result of the tendency of erythrocytes to aggregate at low shear rates.

As described in the Results section, our *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 and could, thus, be considered a Newtonian system (Figure 2.7A and B).

Under this assumption, we may treat the 0.2 ml thin-walled PCR tube in a vortexer as a coaxial cylinder system with a small, but finite cylinder radius:
Given this setup, we can calculate that the average shear rate in the system (\( \gamma \)) is dependent on the angular velocity and the ratio of rotor radius to the container radius (along the boundary of the vessel):

\[
\gamma = \left( \frac{2\delta^2}{\delta^2 - 1} \right) \cdot \Omega = M \Omega = \frac{\pi}{30} \left( \frac{2\delta^2}{\delta^2 - 1} \right) \cdot n
\]  

(Eq. 2.1)
Looking at this setup, the “rotor edges” are indicative of the liquid-air interface. Using the reaction volume (20 µl) and the tube capacity (200 µl), and given Equation 2.2, we can make a rough estimate of the average shear rate ($\gamma$):

$$\Omega = \frac{2\pi n}{60} \sec^{-1} = 262 \sec^{-1} \quad (Eq. 2.2)$$

$$R_a - R_i \approx 200 \mu m \quad (Eq. 2.3)$$

$$\delta = \frac{R_a}{R_i} = \frac{0.2 \text{ mm}}{3.5 \text{ mm}} + 1 = 1.06 \quad (Eq. 2.4)$$

$$M = \left( \frac{2\delta^2}{\delta^2 - 1} \right) = 19.1 \quad (Eq. 2.5)$$

$$\gamma = 4.99 \times 10^3 \text{ sec}^{-1} \quad (Eq. 2.6)$$

$n = 2500 \text{ rpm}; \ R_i = 3.5 \text{ mm};$

$\rho(\text{protein}) \sim 1.35 \text{ kg/m}^3; \ \rho(\text{solution}) \sim 1.00 \text{ kg/m}^3$

From this estimate of the average shear rate ($\gamma$, Equation 2.6), we can calculate the average shear stress ($\tau$) using dynamic viscosity ($\eta$) acquired by a cone-plate viscometer in previous experiments:

$$\eta = 0.6 \text{ cP} = 6 \times 10^{-4} \text{ Pa} \cdot \text{s} \quad (Eq. 2.7)$$

$$\tau = \eta \gamma = 52.6 \text{ Pa} \quad (Eq. 2.8)$$
Figure S2.2. Representative reading from the reaction system on the cone-plate viscometer.

The geometry factor of the tube, which is found by simply using the approximate rotor geometry (Figure S2.1) is factored into the calculation from Equation 2.8 to give:

\[
A = \frac{3R_a^3}{\pi R_i^3} = \frac{3\delta^3}{\pi} = 0.96 \quad (Eq. 2.9)
\]

\[
\tau = \eta \gamma A = 2.88 \text{ Pa} \quad (Eq. 2.10)
\]

\[
= 28.8 \text{ dyne/cm}^2
\]

Thus the calculated shear stress of ~30 dynes/cm² is very close to the empirically-determined shear stress of ~32 dynes/cm². This provides further evidence that our
assumption of a Newtonian system is reasonable, however it must be noted that all experiments done by the vortex method are confirmed by repeating the experiments on the cone-plate viscometer (HAAKE RotoVisco1, Thermo Fisher Scientific, Waltham, MA), a well-defined and well-characterized tool for generating fluid shear stress.
CHAPTER 3: THE VWF D’D3 AND PROPEPTIDE DOMAINS PLAY A ROLE IN FACTOR VIII-MEDIATED ENHANCEMENT OF VWF PROTEOLYSIS BY ADAMTS13

(Manuscript Title: Compromised Proteolytic Cleavage of Type 2N von Willebrand Factor Variants by ADAMTS13 in the Presence of Factor VIII)

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3.1 Overview

Proteolytic cleavage of von Willebrand factor (VWF) by ADAMTS13 is enhanced by clotting factor VIII (FVIII). In this study, we test the hypothesis that naturally occurring type 2N VWF variants that are defective in FVIII binding may be compromised in proteolytic cleavage by ADAMTS13 in the presence of FVIII. Recombinant type 2N VWF variants (H817Q, R854Q, R782W, R782W/H817Q, and HPP-VWF) were expressed in human embryonic kidney cells. Binding of FVIII and proteolytic cleavage of the 2N VWF variants were determined. We showed that most 2N VWF variants exhibited reduced proteolytic cleavage by ADAMTS13 in the presence of FVIII under fluid shear stress. The impairment of FVIII in accelerating the cleavage of the 2N VWF variants was proportional to the defects in their FVIII binding. In contrast, these variants, except for HPP-VWF, appeared to be cleaved normally in the presence of ristocetin and platelets, which modulate VWF proteolysis via the A1 domain. These results further support the critical role of the VWF D’D3 domain and the VWF propeptide in regulating VWF proteolysis by ADAMTS13 under physiologically relevant shear stress. Our findings suggest that the compromised cofactor-dependent proteolysis of the 2N VWF variants may be advantageous for mitigating bleeding phenotype in cases of von Willebrand disease.

3.2 Introduction

Type 2N VWD includes cases with defective binding of FVIII. Genetic analyses have shown that most 2N mutations are located in the exons 18-27, which affect the propeptide cleavage site and the D’-D3 domains that contain the FVIII binding sites.
Patients with 2N VWD resemble those with mild hemophilia A as the result of variably reduced plasma FVIII activity, usually between 0.05 IU/ml and 0.40 IU/ml\textsuperscript{10,16}. Symptoms in cases of type 2N VWD are known to be correlated with low plasma FVIII activity\textsuperscript{17} and the structure and functionality of plasma VWF multimers. For instance, plasma VWF multimers in type 2N patients may be normal, increased, smeared, or significantly reduced, depending on the type of VWF mutations\textsuperscript{16}. A subset of 2N mutations (i.e. C788R/Y or C804F) in the D’ domain may affect intra-molecular disulfide bonds, thereby affecting the secondary structure, secretion, and multimerization of VWF\textsuperscript{18,19}. However, most other 2N VWF mutations do not involve a cysteine residue (i.e. H817Q, R854Q, R782W, R782W/H817Q, and HPP-VWF identified through the Blood Center of Wisconsin). All patients with type 2N mutations exhibit a reduced FVIII binding affinity to various extents.

We hypothesize that a type 2N VWF variant that exhibits significantly reduced FVIII binding may be compromised in proteolytic cleavage by ADAMTS13 in the presence of FVIII. This hypothesis stems from our previous observations, demonstrating the cofactor role of FVIII alone\textsuperscript{20} or with platelets\textsuperscript{21} in accelerating proteolytic cleavage of VWF by ADAMTS13 under mechanically induced fluid shear stress. In addition, a FVIII variant lacking the major VWF binding site (a3) in the light chain (i.e. FVIII-2RKR) has no effect in accelerating VWF proteolysis by ADAMTS13 under the same conditions\textsuperscript{20}.

The present study demonstrates for the first time that proteolytic cleavage of type 2N VWF variants by ADAMTS13 in the presence of FVIII is variably compromised. The impairment in FVIII-dependent proteolysis is proportional to the defects of their FVIII
binding. These results support the role of the VWF D’D3 domain and the VWF propeptide in regulating VWF proteolysis and suggest an evolutionary advantage by limiting VWF proteolysis in cases of VWD.

3.3 Materials and Methods

3.3.1 Constructs

Plasmids encoding a human full-length wild-type (WT) VWF (pvW198.1) or various type 2N VWF variants including pvW198.8 (H817Q)\textsuperscript{22,23}, pvW198.4 (R854Q)\textsuperscript{22,24,25}, pvW198.7 (R782W)\textsuperscript{23}, pvW198.9 (R782W/H817Q)\textsuperscript{23}, and HPP-VWF\textsuperscript{23} were prepared as described previously. These constructs were chosen because they represent a spectrum of FVIII binding affinity from nearly normal (i.e. H817Q) to severely defective (i.e. HPP-VWF). The HPP (hereditary persistent propeptide)-VWF has two mutations: one from the gene (\textDelta nt2269-2270) and the other by virtue of alternative splicing (\textDelta nt2282-2288)\textsuperscript{26}, resulting in the removal of furin cleavage site and persistence of the propeptide D1-D2 domains.

3.3.2 Preparation of plasma and recombinant VWF variants

Plasma VWF was purified from normal human plasma according to the method described previously\textsuperscript{21}. Serum-free conditioned media containing recombinant WT VWF and type 2N VWF variants were prepared from transiently transfected HEK293T cells (kindly provided by Dr. David Ginsburg at University of Michigan, Ann Arbor, MI). The conditioned media were concentrated 50-100 times with a 150 kDa MWCO Pierce
concentrator (Thermo-Scientific, Rockford, IL) according to the manufacturer’s recommendation. The amount of purified plasma VWF was determined by absorbance at 280 nm, corrected by absorbance at 320 nm (1 corrected absorbance at 280 nm equals to ~1 mg/ml), while recombinant WT and 2N variants in the conditioned media were determined by ELISA using purified plasma VWF for a calibration standard.

3.3.3 Preparation of recombinant FVIII and ADAMTS13

Recombinant full-length human ADAMTS13 and human FVIII were prepared as described previously. The final purification products were buffer exchanged with 20 mM HEPES, 0.15 M NaCl, and 5 mM CaCl$_2$, pH 7.5, and stored at -80 °C until use.

3.3.4 Quantitation of VWF concentrations by ELISA

The concentrations of WT and type 2N VWF variants in the concentrated conditioned media were determined by an enzyme-linked immunosorbent assay (ELISA). Briefly, a high binding microtiter plate (Nagle NUNC International, Rochester, NY) was coated with 100 µl of rabbit polyclonal anti-VWF IgG (1:2,000) (Dako, Carpinteria, CA) overnight. The remaining binding sites were blocked for 30 min with 150 µl/well of 2.5% BSA in PBS. Conditioned media containing recombinant WT and type 2N VWF variants diluted with PBS were added and incubated for 2 hours. After being washed with PBS, monoclonal horseradish peroxidase (HRP)-conjugated rabbit anti-VWF IgG (1:5,000) (Dako) was added for detection. A chromogenic substrate, 3,3′, 5,5′-tetramethylbenzidine (TMB) (Invitrogen, Frederick, MD), was used to develop color and stopped with 1.5 M H$_2$SO$_4$. The absorbance at 450 nm was determined by a SpectraMax190
spectrophotometer (Molecular Devices, Sunnyvale, CA). Purified plasma VWF was used for a calibration standard. All assays were repeated at least three times for consistency.

3.3.5 VWF multimer analysis

VWF multimers of recombinant WT VWF and type 2N VWF variants in the conditioned medium were determined by fractionation by 1% or 2% agarose gel electrophoresis, followed by Western blotting as described previously\textsuperscript{20,28}.

3.3.6 Binding of FVIII or ADAMTS13 to type 2N VWF variants

A high binding microtiter plate (Nagle Nunc International) was coated with 100 µl of rabbit polyclonal anti-VWF IgG (1:2,000) (Dako) overnight. The remaining binding sites were blocked for 30 min with 150 µl/well of 2.5% BSA in PBS. Recombinant WT and type 2N VWF variants diluted with PBS were added and incubated for 2 hours. After being washed with PBS, recombinant FVIII or ADAMTS13 at various concentrations was added and incubated for 1 hour. The bound FVIII or ADAMTS13 was detected by a monoclonal anti-FVIII HRP-conjugated antibody (1:3,000) (ESH-8HR) that recognizes the C2 domain in the light chain of FVIII (American Diagnostica Inc, Stamford, CT) or a monoclonal antibody against the disintegrin domain (1:5,000) (Green Mountain Antibodies, Burlington, VT), followed by a goat anti-mouse peroxidase-conjugated secondary antibody (1:5,000). A TMB (3,3′, 5,5′-tetramethylbenzidine) substrate (Invitrogen, Frederick, MD) was used for color reaction and stopped by addition of 1.5 M
H$_2$SO$_4$. The absorbance at 450 nm was determined on a SpectraMax190 microtiter plate reader (Mol. Devices).

### 3.3.7 Binding of type 2N VWF variants to FVIII

A high binding microtiter plate (Nagle Nunc International) was coated with 100 µl of sheep anti-FVIII IgG (1:2,000) (American Diagnostica Inc, Stamford, CT) overnight. The bound VWF was detected by rabbit anti-VWF (1:5,000) (A0082, Dako), followed by a goat anti-rabbit biotin-conjugated secondary antibody (1:5,000) and streptavidin-conjugated peroxidase (1:1,000). A TMB (3,3′, 5,5′-tetramethylbenzidine) substrate (Invitrogen, Frederick, MD) was used for color reaction and stopped by addition of 1.5 M H$_2$SO$_4$. The absorbance at 450 nm was determined on a SpectraMax190 microtiter plate reader (Mol. Devices).

### 3.3.8 Western blotting

The proteolytic cleavage of VWF by ADAMTS13 under denaturing conditions or fluid shear stress was determined by Western blotting as described previously$^{20,21,29}$. For assays under denaturing conditions, purified WT VWF and variants were pre-denatured at 37 °C for 2 hours with 1.5 M urea. The denatured VWF and variants (150 nM) were incubated with ADAMTS13 (0.2 nM) on a dialysis membrane floating on 5 mM Tris-HCl, pH 8.0, 1.5 M urea at 37 °C for 4 hours. The digested materials were fractionated with 1% agarose gel electrophoresis. For assays under fluid shear stress, purified plasma VWF (pVWF) or recombinant WT and type 2N VWF variants in the conditioned medium (37.5
µg/ml, ~150 nM subunits) were incubated with recombinant ADAMTS13 (5 nM) in the absence or in the presence of either a fixed concentration (5 nM) or various concentrations of FVIII (0-20 nM) in 20 mM HEPES, pH 7.5 containing 1 mg/ml BSA, 150 mM NaCl and 5 mM CaCl₂ in a 0.2 ml thin-walled PCR tube with dome caps (total volume, 20 µl) for 30 min at 37 °C. The reaction mixtures were then subjected to mixing in a 96-well Eppendorf MixMate vortexer (Fisher-Thermo Scientific, Rockford, IL) at 2,500 rpm for 2.5 min. The reaction mixtures were then denatured at 100 °C for 5 min with sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate, and 0.01% bromophenol blue). The denatured VWF was fractionated in a 5% Tris-glycine polyacrylamide gel in a Mini-protein III gel cassette (Bio-Rad, Hercules, CA) at 120V for 150 min. The proteins were then transferred onto a nitrocellulose membrane (Millipore, Billerica, MA) at 100 mA for 60 min. The proteolytic cleavage product of VWF (350 kDa, the dimer of C-terminal fragments) was detected by rabbit anti-VWF IgG (1:5,000) (Dako), followed by IRDye 800CW-labeled goat anti-rabbit IgG (1:20,000) (LI-COR Bioscience, Lincoln, NB) in TBS containing 1% casein and 0.05% Tween 20. The fluorescent signals were collected by an Odyssey infrared imaging system (LI-COR Bioscience)²⁰. The cleavage products were quantified by NIH ImageJ software after the fluorescent intensities were converted into the gray image signals.

3.3.9 ELISA for quantification of the cleaved VWF-A2

Recombinant WT and 2N VWF variants in the conditioned media (150 nM) were incubated with various concentrations of recombinant FVIII (0-20 nM) for 30 min at 37
°C. Recombinant ADAMTS13 (5 nM) was added to the VWF-FVIII mixture and subjected to vortexing at 2,500 rpm for 2.5 min. The reaction was then terminated by addition of 50 mM EDTA. The reaction mixture was diluted 1:200-400 with 0.5% BSA in PBS and added to a high binding microtiter plate (Nagle NUNC), which was coated with 100 µl of monoclonal anti-VWF cleaved A2 IgG (MAB27642) (R&D Systems, Minneapolis, MN) (1 µg/ml) that specifically recognizes the N-terminus of cleaved VWF after amino acid residue Tyr\textsuperscript{1605}, but not intact VWF\textsuperscript{30}. Incubation was carried out for 2 hours at 37 °C. After being washed with PBS, horseradish peroxidase (HRP)-conjugated rabbit anti-VWF IgG (1:5,000) (Dako) was added and incubated for 1 hour. A TMB substrate (Invitrogen, Frederick, MD) was used for color development. After being stopped with 1.5 M H\textsubscript{2}SO\textsubscript{4}, the absorbance at 450 nm was obtained from a SpectraMax190 spectrophotometer (Mol. Devices). Non-specific background absorbance resulted from binding of WT or a type 2N VWF variant to the wells without monoclonal anti-VWF A2 IgG was subtracted. Each sample was done three times for consistency. Statistical analysis was performed using one-way analysis of variance (ANOVA) or Student t-test. P values less than 0.05 was considered to be statistically significant.

3.3.10 Ristocetin-induced platelet agglutination

To assess the function of the expressed type 2N VWF variants to agglutinate platelets, lyophilized human platelets (Helena Laboratories, Beaumont, TX) were reconstituted with 20 mM Tris-HCl, pH 7.4, and 100 mM NaCl (20x10\textsuperscript{6}/µl). Lyophilized platelets (200 µl) were incubated at 37°C with WT, type 2N VWF variants (15 µg/ml), or purified VWF
dimer (15 and 30 µg/ml) in the presence of 10 µl of normal human plasma (to provide fibrinogen) in a glass tube stirring at 1,100 rpm. The light transmission was recorded simultaneously in all channels for 6 minutes after addition of 1.0 mg/ml ristocetin with a PAP4 aggregation module (Bio/Data Corporation, Horsham, PA).

3.3.11 Ristocetin and platelet-induced cleavage of 2N VWF variants by ADAMTS13 under shear stress

WT or type 2N VWF variants in conditioned medium (37.5 µg/ml) were incubated without or with lyophilized platelets (final concentration, 150 x 10^3/µl) and ristocetin (1.0 mg/ml) in 20 mM HEPES, pH 7.5 containing 1 mg/ml BSA, 150 mM NaCl and 5 mM CaCl₂ in a 0.2 ml thin-walled PCR tube with dome caps (total volume, 20 µl) for 30 min at 37 °C. Recombinant ADAMTS13 (5 nM) was added to the VWF/platelet/ristocetin mixture and subjected to vortexing at 2,500 rpm for 2.5 min as described previously²¹. The reaction mixtures were then denatured by heating at 100 °C for 5 min with a sample buffer (62.5 mM Tris-HCl, pH 6.8 containing 25% glycerol, 2% sodium dodecyl sulfate, and 0.01% bromophenol blue). The proteolytic cleavage of VWF variants was determined by Western blotting as described in the previous section.

3.4 Results

3.4.1 Multimer distribution of the expressed recombinant type 2N VWF variants

Previous studies have demonstrated that plasma VWF multimers in cases of type 2N VWD may be normal²⁴,²⁵,³¹, ultra large²⁵,³¹,³², or reduced³¹,³³, depending on the type of
mutations and zygosity. To determine the multimer distribution of recombinant type 2N variants expressed, serum-free media containing recombinant wild type VWF (WT) and type 2N variants (i.e. H817Q, R854Q, R782W, R782W/H817Q, and HPP-VWF) (Figure 3.1A) were collected from transiently transfected HEK293T cells. VWF multimer analysis was performed using 1% (Figure 3.1B) or 2% (Figure 3.1C) agarose gel electrophoresis, followed by Western blotting as described in the Methods. As shown in Figure 3.1, on a low-resolution agarose gel (1%) the multimer distribution of WT and type 2N VWF variants was normal (Figure 3.1B). On a high-resolution agarose gel (2%), the HPP-VWF variant that contains an uncleaved propeptide was larger for each ladder as expected (Figure 3.1C). The multimers in other 2N variants--including H817Q, R854Q, R782W, R782W/H817Q--were also normal (Figure 3.1B and C). These results demonstrate that the type 2N VWF variants selected for the study do not demonstrate any defects in multimerization.
Figure 3.1. Schematic representation of type 2N mutations, and multimer distribution of variants. Panel A. Human pro-VWF comprises a signal peptide, propeptide (D1-D2), furin cleavage site, D'-D3 domains, and A1-A2-A3 domains. In the distal C-terminus, there are D4-C1-C2 and CK domains. The sites for FVIII binding, potential disulfide bond formation (-S-S-), and furin cleavage (blue arrow) are indicated. Mutations that result in type 2N VWD are indicated below the domain structure. They affect the furin cleavage site (i.e. HPP-VWF) or the D'-D3 domain (i.e., H817Q, R854Q, and R782W). Panel B and Panel C show the VWF multimer distribution in the conditioned medium after being fractionated on 1% and 2% gel, respectively. Western blotting with rabbit anti-human VWF IgG (1:5,000) was used to visualize the VWF multimers. Purified plasma VWF (pVWF) was used as a normal control (Panels B and C, lane 7).
3.4.2 Binding of FVIII or ADAMTS13 to type 2N VWF variants

Type 2N mutations primarily affect the D'-D3 domains of VWF that contain the FVIII binding sites\(^9,11,16\). Patients with the mutations exhibit variably reduced levels of plasma FVIII activity (0.02 to 0.40 IU/ml) and reduced FVIII binding\(^10,16,17\). To determine the total binding of FVIII to recombinant type 2N VWF variants, full-length FVIII at various concentrations (0-40 nM) was incubated with recombinant WT or type 2N variants captured by polyclonal anti-VWF IgG on a microtiter plate. The bound FVIII was detected by peroxidase-conjugated monoclonal anti-FVIII IgG as described in the Methods. As shown, FVIII bound type 2N VWF variants in a concentration-dependent manner, but with variably reduced affinities as compared with WT VWF. At any given concentration, the total amount of FVIII that bound to VWF was progressively reduced in the following order: WT>H817Q>R854Q> R782W>R782W/H817Q>HPP-VWF (Figure 3.2A). When FVIII was immobilized on the microtiter plate and binding of WT and type 2N VWF variants to FVIII was determined, the results were highly concordant (Figure 3.2B).

As three-body interactions between VWF, ADAMTS13, and FVIII are critical for proteolytic cleavage of VWF under fluid shear stress\(^20\), we determined the binding of ADAMTS13 to type 2N variants. The results showed that recombinant ADAMTS13 bound WT and type 2N VWF variants with similar affinities under these conditions (Figure 3.2C). These results suggest that type 2N mutations do not affect the VWF-ADAMTS13 interactions.
Figure 3.2. Binding of FVIII or ADAMTS13 to recombinant type 2N VWF variants. 100 µl of FVIII (0-40 nM) (Panel A) or ADAMTS13 (0-25 nM) (Panel C) was incubated with WT and type 2N variants captured by rabbit anti-VWF IgG (1 µg/well). Panel B shows the binding of VWF to immobilized FVIII captured by rabbit anti-FVIII IgG (1 µg/well). Bound FVIII, VWF or ADAMTS13 was determined by peroxidase-conjugated monoclonal anti-FVIII IgG (1:2,000) (Panel A), anti-VWF IgG (1:5,000) (Panel B) or anti-V5 IgG (1:1,000) (Panel B), followed by incubation with TMB substrate. The specific binding was determined by the total absorbance at 450 nm in the presence of VWF minus that in the absence of immobilized VWF. The results are the mean from three independent experiments (n=3).
3.4.3 Cleavage of type 2N variants by ADAMTS13 under denaturing conditions is normal

To determine whether mutations on the D’D3 domain or at the cleavage site of VWF affect overall susceptibility to proteolysis, WT and type 2N or HPP-VWF variants were pre-denatured with 1.5% urea and then incubated with recombinant ADAMTS13 (0.4 nM) for 4 hours. The cleavage of pre-denatured VWF was determined by 1% agarose gel and Western blotting as described in the Methods. The cleavage of type 2N (H817Q, R854Q, R782W, and compound heterozygous R782W/H817Q) or HPP-VWF variants to ADAMTS13 proteolysis under static and denaturing conditions was quite similar to the cleavage of WT VWF (Figure 3.3A). No statistically significant difference in the cleavage product formation was observed among all the VWF variants and between the variants and WT VWF (p>0.05) (Figure 3.3B). These results suggest that there is no intrinsic defect in the susceptibility of type 2N or HPP-VWF to proteolysis by ADAMTS13 under static and denaturing conditions.
Figure 3.3. Western blotting analysis of the proteolytic cleavage of type 2N VWF variants in the presence of FVIII under denaturing conditions. WT and type 2N VWF variants (150 nM) pre-denatured with 1.5 M urea were incubated with ADATMS13 (0.4 nM) in the absence (-) or in the presence (+) of EDTA (20 mM) at 37 °C for 4 hours on a dialysis membrane floating over 5 mM Tris-HCl, pH 8 and 1.5 M urea. The proteolytic cleavage of VWF was determined by 1% agarose gel electrophoresis and Western blot (Panel A). Relative cleavage efficiency was quantified from the ratio of the cleavage product (arrowhead) to the high molecular weight VWF (HMW), which was normalized to 1 for WT. One-way ANOVA analysis of variance was used to determine the significance of differences among the group with a $p$ value of greater than 0.05. The means and standard errors of the means from three independent experiments are shown (Panel B).
3.4.4 Compromised proteolytic cleavage of type 2N VWF variants by ADAMTS13 in the presence of FVIII under fluid shear stress

Our previous studies have shown that binding of FVIII alone\textsuperscript{20} or in combination with platelets\textsuperscript{21} to VWF dramatically enhances proteolytic cleavage of VWF by ADAMTS13 under fluid shear stress. We tested the hypothesis that type 2N mutations that result in reduced FVIII binding may affect FVIII-dependent proteolysis of VWF under shear stress. As shown, in the absence of FVIII, the VWF cleavage product of 350 kDa was barely detectable. In the presence of 5 nM FVIII, the cleavage product increased dramatically for WT VWF (~ 9 fold).

However, the fold increase in the cleavage product for the type 2N VWF variants was reduced in the following order: H817Q>R854Q> R782W>R782W/H817Q>HPP-VWF (Figure 3.4). There was nearly normal cleavage for H817Q and severely defective cleavage for HPP-VWF under these conditions.
Figure 3.4. Western blotting analysis of the proteolytic cleavage of type 2N VWF variants in the presence of FVIII under fluid shear stress. WT and type 2N VWF variants in conditioned medium (~150 nM) were incubated with ADAMTS13 (~5 nM) in the absence (-) or in the presence (+) of a fixed concentration of FVIII (5 nM) at 25 °C for 2.5 min under vortexing-induced fluid shear stress (rotation rate, 2,500 rpm). EDTA (20 mM) (+) was included in lane 3 as a negative control. Western blotting with rabbit anti-VWF IgG (1:5,000) was used to determine the cleavage product (~350 kDa) indicated by the arrowhead (Panel A). Quantitation of the 350 kDa bands was performed using ImageJ software (Panel B). The results (mean ± standard error, n=3) were expressed as the fold increase in the cleavage product from WT and type 2N variants in the presence of FVIII relative to that in the absence of FVIII.

Additional evidence from the kinetic study further supports this hypothesis. In the presence of increasing concentrations of FVIII (0-20 nM), the proteolytic cleavage of type 2N VWF variants remained variably reduced as compared with that of WT VWF, assessed either by our novel quantitative ELISA (Figure 3.5) or Western blotting (Figure 3.6). There was severely impaired proteolytic cleavage of R782W/H817Q and HPP-VWF
by ADAMTS13 even in the presence of 20 nM FVIII (Figure 3.4 and Figure 3.5). Together, these results demonstrate that naturally occurring type 2N VWF variants that are defective in FVIII binding are also compromised in proteolytic cleavage by ADAMTS13 in the presence of FVIII under physiologically relevant fluid shear stresses.
Figure 3.5. Kinetic analysis of proteolytic cleavage of type 2N VWF variants in the presence of various concentrations of FVIII under fluid shear stress. WT or type 2N VWF variants (150 nM) were incubated with ADATMS13 (5 nM) at 25 °C for 2.5 min in the presence of various concentrations of FVIII (0-20 nM) under vortex induced fluid shear stress (rotation rate, 2,500 rpm). The proteolytic cleavage of VWF at the specific Tyr-Met bond was determined by an ELISA described in detail in the Methods. The percentage of cleaved VWF as a function of increasing concentrations of FVIII observed in WT and type 2N VWF variants (Panel A) or the relative fold reduction of the cleavage product formation in type 2N VWF variants compared with that in WT (Panel B) in the presence of 20 nM of FVIII was presented (mean ± standard error) from three independent experiments (n=3). One (*) and two stars (**) indicated that the difference in the cleavage product formation between a type 2N variant and WT VWF was statistically significant (p<0.05) and highly significant (p<001), respectively.
Figure 3.6. Western blotting analysis of the proteolytic cleavage of type 2N VWF variants in the presence of various concentrations of FVIII under fluid shear stress. Panel A. Purified plasma VWF (pVWF) (as a reference), WT or type 2N VWF variants in the conditioned medium (~150 nM) were incubated at 25°C for 2.5 min under fluid shear stress with ADAMTS13 (~5 nM) in the presence of and various concentrations of FVIII (0-20 nM). EDTA (20 mM) was included in lane 1 for a negative control in each blot. The cleavage product (~350 kDa) was determined by SDS-polyacrylamide gel (5% Tris-glycine) electrophoresis and Western blotting as described in the Methods. Panel B. The quantitation of the specific cleavage product (~350 kDa) was performed using ImageJ software. The fold increase (mean ± standard error) was expressed as a function of FVIII concentrations from three independent experiments (n=3).
3.4.5 Type 2N VWF variants are normal or nearly normal in ristocetin-induced platelet aggregation

Ristocetin, an antibiotic that specifically binds the A1 domain of VWF, modifies interaction between VWF-A1 and the platelet receptor glycoprotein 1ba (GP1ba). Type 2N mutations located in the D’D3 domain of VWF are not expected to affect VWF-platelet interaction. To assess the function of type 2N VWF variants, commercially available and lyophilized platelets from healthy donors were incubated with type 2N VWF variants in the presence of 1.0 mg/ml ristocetin and a small amount (5%) of normal human plasma (to provide fibrinogen). The percentage of platelet aggregation was determined by a light transmission approach. The results showed that the total final aggregation of lyophilized platelets in the presence of the same final concentration (15 µg/ml) of WT, H817Q, R854Q, R782W, R782W/H817Q, and HPP-VWF was 86%, 65%, 73%, 65%, 73%, and 50%, respectively (Figure 3.7A).

To rule out the possibility that adding a small amount of normal human plasma into the reaction may obscure the results in assessing platelet aggregation, a dimeric VWF (500 kDa) (Figure 3.7B, inset) (15 or 30 µg/ml), isolated by size exclusion chromatography, was used in the same assay. No agglutination or aggregation of lyophilized platelets was observed, while plasma-derived multimeric VWF aggregated platelets normally (Figure 3.7B), suggesting that a small amount of normal human plasma added into the assay is not sufficient to cause normal platelet aggregation without the supplement of functional
multimeric VWF. Together, these results demonstrate that the type 2N VWF variants are normal or nearly normal for agglutinating platelets enhanced by ristocetin.

Figure 3.7. Effect of type 2N mutations on ristocetin-induced platelet agglutination and VWF proteolysis by ADAMTS13. WT and type 2N VWF variants (15 µg/ml) were incubated with lyophilized human platelets (20x10⁶/µl) in the presence of 5% normal human plasma and 1.0 mg/ml ristocetin (Panel A). The light transmission was recorded simultaneously in all channels for 6 minutes at 37°C. Multimeric VWF (WT) (15 µg/ml) or dimeric VWF (Dimer) (15 and 30 µg/ml) purified from plasma (Inset) was incubated with lyophilized human platelets (20x10⁶/µl), 5% normal human plasma, and 1.0 mg/ml ristocetin (Panel B). The light transmission was recorded simultaneously in all three channels for 6 minutes at 37°C. An increase in light transmission is indicative of platelet agglutination and aggregation. The inset shows the multimer distribution of WT and dimeric VWF by 1% agarose gel electrophoresis and Western blotting.
3.4.6 Proteolytic cleavage of type 2N VWF/platelet complexes by ADAMTS13 in the presence of ristocetin

Binding of platelets in the presence of ristocetin appears to alter VWF conformation by a different mechanism from FVIII, thereby accelerating proteolytic cleavage of VWF by ADAMTS13\textsuperscript{21}. To assess the effect of ristocetin/platelets on proteolysis of type 2N variants, WT or variants were incubated with ADAMTS13 in the presence of lyophilized platelets and ristocetin for 5 min under constant shear stress. Proteolytic cleavage was determined by 5\% SDS-polyacrylamide gel electrophoresis and Western blotting as described previously\textsuperscript{21}. We showed that the cleavage of all type 2N variants, except for HPP-VWF, was quite similar to that of WT-VWF (Figure 3.8). However, there was a statistically significant reduction (42\%) in the cleavage product of HPP-VWF, compared with WT (p<0.01) (Figure 3.8B). These results suggest that HPP-VWF binds platelets nearly normally under static conditions, but appears to be moderately compromised in ristocetin/platelet enhanced proteolysis under fluid shear stresses.
Figure 3.8. Effect of type 2N mutations on ristocetin-induced VWF proteolysis by ADAMTS13. Panel A. WT or type 2N VWF variants in the conditioned medium (~150 nM) were incubated with lyophilized platelets (150x10^3/µl) in the presence of ristocetin (1.0 mg/ml) at 25 °C for 2.5 min under fluid shear stress. The proteolytic cleavage of VWF was determined by 5% SDS-polyacrylamide gel and Western blotting as described in the Methods. Panel B. ImageJ densitometry was used to determine the relative fold increase in the presence of ristocetin and platelets and compared with that in the absence of both. The data presented (mean ± standard error) is from three independent experiments (n=3). Statistical analysis was performed using Student’s t-test. Double stars indicate that the difference in the VWF cleavage product formation between WT and HPP-VWF was statistically significant (p<0.01).

3.5 Chapter Discussion

The present study demonstrates for the first time that naturally occurring VWF mutations (i.e. H817Q, R854Q, R782W, R782/H817Q, and HPP-VWF) found in cases of type 2N VWD result in compromised proteolysis by ADAMTS13 in the presence of FVIII under fluid shear stress (Figures 3.4, 3.5, and 3.6), but not under denaturing conditions (Figure
3.3). The reduced proteolytic cleavage of the 2N variants appears to be proportional to the impairment of their FVIII binding (Figure 3.2). For instance, H817Q, which results in mild defects for FVIII binding, exhibits nearly normal cleavage by ADAMTS13 in the presence of various concentrations of FVIII, while HPP-VWF, which results in the most severe defects for FVIII binding, is dramatically impaired in proteolysis by ADAMTS13 under the same conditions (Figures 3.4, 3.5, and 3.6). Even in the presence of high concentrations of FVIII (20 nM), no enhancement of the cleavage product formation, assessed either by ELISA (Figure 3.5) or by Western blotting (Figures 3.4 and 3.6), was detected. These results are reminiscent of those we have reported previously, in which a FVIII variant with a major VWF binding site (a3) removed (i.e. FVIII-2RKR) did not enhance the proteolytic cleavage of WT VWF by ADAMTS13 under fluid shear stress. Together, these findings, both from the investigation of the mutant cofactor (i.e. FVIII) and the mutant substrate (i.e. VWF), underscore the critical role of FVIII-VWF interactions in regulating proteolysis of VWF under physiologically relevant conditions.

To date, quantitation of the specific cleavage of multimeric VWF at the Tyr\textsuperscript{1605}-Met\textsuperscript{1606} bond by ADAMTS13 has been cumbersome and problematic. The detection of the 350 kDa product (the dimer of two C-terminal fragments) requires simultaneous cleavage of two adjacent Tyr-Met bonds, which occurs rarely under physiological conditions. Our novel ELISA-based assay detects the N-terminal peptide (i.e. EQAPNVY) resulting from cleavage after the Tyr residue occurring anywhere along a VWF multimer, which dramatically increases the assay sensitivity. Furthermore, the assay is highly specific for detecting the ADAMTS13 cleavage product, but not from the cleavage by other
leukocyte proteases such as cathepsin G, proteinase 3, and elastase (Figures 3.9 and 3.10), which are known to cleave VWF at or near the Tyr$^{1605}$-Met$^{1606}$ bond$^{33}$. This sensitive and reproducible ELISA assay will fundamentally change how we assess the biological activity of ADAMTS13.

Figure 3.9. Validation of the novel ELISA method for assessing proteolytic cleavage of VWF by ADAMTS13. Panel A. Schematic representation of the A1-A2-A3 domains of a mature VWF subunit, the cleavage site for ADAMTS13, cathepsin G, elastase, and proteinase 3, as well as the binding site for the monoclonal anti-VWF A2 IgG. Panels B to E show the signals (absorbance at 450 nm) detected by ELISA after 10 min of incubation of pre-denatured WT VWF (150 nM) with ADAMTS13 (0-40 nM) (Panel B), cathepsin G (0-36 nM) (Panel C), proteinase 3 (0-400 nM) (Panel D), and elastase (0-400 nM) (Panel E).
It remains somewhat puzzling why the ELISA does not detect the cleavage of VWF by cathepsin G, which also cleaves the Tyr-Met bond. This may be related to the additional cleavages occurring elsewhere on VWF as demonstrated previously, which may destroy the binding epitopes for anti-cleaved A2 IgG (capturing antibody) or anti-VWF IgG (detecting antibody). Moreover, the N-terminal cleavage products resulting from digestion with proteinase 3 (Figure 3.9D and 3.10C) and elastase (Figure 3.9E and 3.10A) contains two extra amino acids (methionine and valine), which may block the binding of the anti-cleaved A2 antibody. In addition, similar non-specific cleavage of VWF elsewhere by proteinase 3 and elastase was reported, resulting in the loss of antibody binding epitopes necessary for detection. A linear increase (by ELISA) as opposed to a plateau (by Western blotting) of the cleavage product of WT VWF as a function of increasing concentrations of FVIII (Figure 3.6) supports the assertion that our novel ELISA is a much better assay for assessing ADAMTS13 activity.
Figure 3.10. Proteolytic cleavage of VWF by leukocyte proteases. VWF (~150 nM) was incubated with elastase (200 nM, Panel A), cathepsin G (20 nM, Panel B), or proteinase 3 (200 nM, Panel C) at 25 °C for 2.5 min under fluid shear stress. The proteolytic cleavage of VWF was determined by 5% SDS-polyacrylamide gel and Western blotting as described in the Methods.

These results, although interesting, present a significant question about the physiological relevance of having compromised proteolysis of VWF in cases of type 2N VWD. The clinical phenotype in patients with type 2N VWD is usually mild, with bleeding episodes only after trauma or surgery. It has been postulated that plasma FVIII clotting activity is the major determinant of the clinical phenotype. We hypothesize that plasma VWF may play a role in modulating bleeding severity. For instance, patients with HPP-VWF or R763G, which disrupts the furin cleavage site and results in a persistence of the propeptide, exhibit dramatically reduced plasma FVIII activity (as low as 0.05-0.1 U/ml)\textsuperscript{23,25}. However, plasma VWF multimers in these patients are smeary or ultra large in
size with no visible satellite bands\textsuperscript{25}. This evidence is consistent with the notion of impaired proteolysis of VWF by ADAMTS13 \textit{in vivo}. In addition, the cleavage of HPP-VWF by ADAMTS13 in the presence of ristocetin and platelets is also modestly reduced (Figure 3.8), despite its nearly normal activity in agglutinating lyophilized platelets induced by ristocetin (Figure 3.7). The discrepancy between the nearly normal platelet agglutination and reduced ristocetin/platelet-accelerated cleavage may lie in the assay conditions. The cleavage assay is done under fluid shear stress (\textasciitilde 32 dynes/cm\textsuperscript{2}), while the platelet agglutination assay is performed in the (relative) absence of shear. The reduced proteolysis of VWF in cases of type 2N VWD may have a protective role in mitigating bleeding tendency.

In summary, we demonstrate that recombinant type 2N VWF variants exhibit normal or expected patterns of multimerization, normal binding to ADAMTS13, and normal to subnormal platelet agglutination in the presence of ristocetin, but variably reduced FVIII binding. More importantly, the proteolytic cleavage of type 2N VWF variants by ADAMTS13 is variably reduced in the presence of FVIII under fluid shear stress. The impairment of ADAMTS13-mediated proteolysis of these variants appears to be correlated primarily with the defects in their FVIII binding or partially with platelet binding under fluid shear stress as in the case of HPP-VWF. Our findings, by examining the susceptibility of naturally occurring type 2N VWF mutants to ADAMTS13, further underscore the important role of FVIII and propeptide in regulating VWF proteolysis under physiologically relevant shear stresses. We speculate that this may have an
evolutionary advantage by limiting proteolytic cleavage of a hemostatic protein like VWF in the case of a bleeding disorder such as VWD.

### 3.6 Chapter References


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CHAPTER 4: VWF PROTEOLYSIS BY ADAMTS13 IS EXQUISITELY CONTROLLED AT AN ADDITIONAL LEVEL BY EXOSITE BINDING

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4.1 Overview

Previous studies have shown that ADAMTS13 spacer domain is required for recognition and cleavage of von Willebrand factor (VWF) under static/denaturing conditions. Sequence alignment of the spacer domains from human, murine and zebrafish ADAMTS13 with those from human and murine ADAMTS1, a closely related member of the ADAMTS family, has allowed us to identify a potential motif Arg$^{659}$-Arg-Tyr-Gly-Glu$^{664}$ (or RRYGEE) within the spacer domain of human ADAMTS13 that may participate in substrate recognition. A deletion of all these six amino acid residues (RRYGEE) from the ADAMTS13 spacer domain (construct del6aa) resulted in dramatically reduced proteolytic activity toward VWF73 peptide, denatured VWF, native VWF under fluid shear stress, and ultra large VWF on endothelial cells. Site-directed mutagenesis, kinetic analyses, and peptide inhibition assays have further identified the important role of the amino acid residues Arg$^{659}$, Arg$^{660}$ and Tyr$^{661}$ (or RRY) in proteolytic cleavage of various substrates under both non-physiological and physiological conditions. In addition, modification of an exosite in the spacer domain generated ADAMTS13 variants with reduced inhibition by anti-ADAMTS13 IgGs while preserving or enhancing specific activity. Molecular modeling using the existing crystal structure of ADAMTS13-DTCS fragment and VWF-A2 domain indicated a shift in hydrophobicity and subsequent opening of the VWF-A2 binding pocket on the ADAMTS13 spacer domain, potentially resulting in increased hydrophobic interactions in the pocket and hydrogen bonding on the exterior of the pocket. Together, these results shed light on the importance of exosite binding in the spacer domain in the substrate recognition and
pathogenesis of acquired TTP. Furthermore, the crystallization, screening, data collection, and structure determination for an autolysis product of ADAMTS13, the ADAMTS13-CS fragment, provides the basis for beginning crystallization studies of complexes to probe amino acid specificity of ADAMTS13 exosite binding with VWF and autoantibody recognition.

4.2 Introduction

ADAMTS13-Spa appears to directly interact with the distal portion (amino acid residues Glu^{1660} - Arg^{1668}) of the VWF73 peptide^{11}. The deletion of these C-terminal 9 amino acid residues from the VWF73 results in dramatically reduced proteolytic cleavage of the shorter peptide by ADAMTS13 under static conditions. The importance of ADATMS13-Spa is also highlighted by the fact that this region is frequently targeted by anti-ADAMTS13 autoantibodies in patients with acquired TTP^{12,13,14}. Epitope mapping studies have suggested that the anti-ADAMTS13 immunoglobulin G (IgG) in some TTP patients specifically binds the amino acid residues between Tyr^{572} and Asn^{579} or between Val^{657} and Gly^{666} on the ADAMTS13 spacer domain^{14,15,16,17,18}. Both antibody mapping results and sequence alignment findings provide hints to investigate the role of the amino acid residues between Arg^{659} and Glu^{664} in the ADAMTS13 spacer domain in substrate recognition. Our lab has published data that demonstrate the critical role of the amino acid residues Arg^{659}, Arg^{660} and Tyr^{661} (RRY) within the ADAMTS13 spacer domain in proteolytic cleavage of various substrates including VWF73, denatured VWF, native VWF under fluid shear stress, and cell bound
UL-VWF under static and fluid shear stress conditions\textsuperscript{11}. In this chapter, I will present my work done in this project, which included some work on peptide inhibition assays and molecular modeling of the spacer domain with our designed peptide.

In another recent study\textsuperscript{19}, our lab hypothesized that a modification of the exosite site 3 in the spacer domain of ADAMTS13 might generate ADAMTS13 variants with reduced inhibition by anti-ADAMTS13 IgGs in patients with acquired TTP, while their proteolytic activity was preserved or enhanced\textsuperscript{19}. To test this hypothesis, we engineered a series of novel recombinant ADAMTS13 variants by replacing several surface charged or hydrophobic residues in the exosite 3 of the spacer domain of ADAMTS13 with those that have similar chemical structure. Proteolytic activity and sensitivity of these novel ADAMTS13 variants to inhibition by patients’ autoantibodies were assessed. We showed that two of twenty-four novel ADAMTS13 variants exhibit enhanced specific proteolytic activity, but are more resistant to inhibition by anti-ADAMTS13 autoantibodies in at least 10/12 patients with acquired TTP\textsuperscript{19}. These novel gain-of-function and autoantibody-resistant variants may be applicable for therapeutics of acquired TTP patients with inhibitors, as well as hereditary TTP. In this chapter, I will present my work done in this project, which included molecular modeling of the WT and mutants spacer domains with the VWF-A2 domain.

Finally, in the search to determine the ADAMTS13 exosite binding with VWF and autoantibody recognition sites on ADAMTS13, we sought to crystallize the full-length ADAMTS13 protein. The structures of the ADAMTS13-DTCS fragment, obtained at 2.6-Å and 2.8-Å resolution, revealed linearly distributed exosites that recognize distinct substrate regions\textsuperscript{22}. With intimate knowledge of the full-length structure of ADAMTS13,
which includes the Metalloprotease and C-terminal domains, we can begin to probe amino acid specificity of ADAMTS13 exosite binding with VWF and autoantibody recognition. This chapter describes the crystallization, screening, data collection, and structure determination for an autolysis product of ADAMTS13, the ADAMTS13-CS fragment.

4.3 Materials and Methods

4.3.1 Preparation of recombinant ADAMTS13

For stable expression, Chinese hamster ovary (CHO-s) cells were used. Cells were cultured in 6-well plate and transfected with plasmid DNA mixed with lipofactmine-2000 (Invitrogen) (1:3 weight to volume) in serum-free medium. The cells were selected under G418 (1 mg/ml) for 10-15 days. The clones expressing ADAMTS13 were identified by Western blotting or immunofluorescent staining. The expression of ADAMTS13 was carried out in 3-layer tissue culture flasks with serum-free DMEM/F12 medium without phenol supplemented with insulin, transferrin, and selenium (Roche). Conditioned medium was collected every day for 10 days. The recombinant ADAMTS13 (His-V5 tagged) was purified with ion exchange and Ni-chelating affinity chromatographies as previously described\textsuperscript{23}. The amount of the purified proteins was determined by absorbance at 280 nm (corrected with light scattering at 320 nm) (1 corrected absorbance at 280 nm equals to ~0.68 mg/ml for full-length and mutant ADAMTS13) as described previously\textsuperscript{24}. 24
The FL-ADAMTS13 for crystallization screening was a kind gift from Baxter Pharmaceuticals (F. Scheiflinger).

4.3.2 Preparation of recombinant fluorescein-labeled VWF73

A plasmid encoding VWF73 with substitutions by 2 cysteine residues at the amino residues Gln\(^{1599}\) and Pro\(^{1611}\) flanking the Tyr-Met bond was a kind gift from Dr. John Owen (Department of Medicine, Wake Forest University School of Medicine). Recombinant VWF73 was expressed, purified, and labeled with fluorescein-5-maleimide (Pierce). The amount of purified fluorescein-labeled VWF73 designated rF-VWF73 was determined by the absorbance at 280 nm (1 corrected absorbance at 280 nm equals to ~0.94 mg/ml). Because of the homo-quenching effect resulting from close proximity of 2 labeled-fluorescent dyes, no fluorescence emits. When the Tyr-Met bond is cleaved by ADAMTS13, 2 fluorescent dyes are separated, and fluorescence emits at excitation wavelength of 485 nm, which can be detected at the emission wavelength of approximately 540 nm.

4.3.3 Preparation of synthetic peptides

Several short amino acid peptides corresponding to the amino acid residues (Arg\(^{659}\)-Glu\(^{664}\) or RRYGEE) of ADAMTS13 spacer domain were chemically synthesized at Peptide 2.0 (Chantilly, VA). Three perfectly matched peptides (RRYGEE, RRY and GEE) and 2 scrambled peptides with identical amino acid residues (EGRYR and RYR) were synthesized. All peptides were purified by HPLC to greater than 92% in purity. The Mass spectrometry analysis showed that the masses [M +2H+]\(^2+\) for RRYGEE, EGRYR,
RRY, RYR, and GEE were 406.2, 406.2, 248.5, 248.5, and 335.0, respectively. The peptides were lyophilized and supplied in 0.1% trifluoroacetate salt. For reconstitution, distilled water was added to concentration of 10 mg/ml. The stock peptides were stored in aliquots at -80 °C.

4.3.4 Proteolytic cleavage of fluorescein-labeled VWF73 peptide

Maleimide-fluorescein-labeled VWF73 derived from the central A2 domain of VWF (i.e. rF-VWF73) (2 µM) was incubated with ADAMTS13 (0.2 nM) in 5mM Bis-Tris, pH 6.0 containing 25 mM CaCl₂ and 0.005% Tween 20 in a total volume of 200 µl in a 96-well white plate (Corning, NY). The rate of fluorescence generation was monitored at 37 °C with a fluorescent microtiter plate reader (Molecular Devices, Sunnyvale, CA) (Ex/Em 485/535nm) every minute for 30 min. Pooled NHP was used as a reference. Each sample was assayed three times. The data were expressed as means and standard errors of the means (Mean ± SEM). The relative proteolytic activity compared with that in wild type ADAMTS13 was plotted against ADAMTS13 variants.

For the peptide inhibition assay, rF-VWF73 (1 µM) was incubated with various concentrations (0-200 µM) of synthetic peptides as indicated for 10 min. Then recombinant ADAMTS13 (2 nM) was added and proteolytic cleavage of rF-VWF73 was monitored at 37 °C on a fluorescent microtiter plate reader (Gemini XPS) (Ext. 485 nm and Emission 540 nm) every minute for 20 min. The maximal rate of fluorescent generation per second (units/sec) was determined by SoftMax Pro® software version 7.0 and plotted against the log concentrations of synthetic peptides used. The experiments were repeated under the same conditions for three times. The concentration achieving
half maximal inhibition (IC_{50}) was determined by fitting the data into a non-linear equation using GraphPad Prism software (La Jolla, CA).

4.3.5 Proteolytic cleavage of multimeric VWF

Purified plasma VWF (150 nM) was incubated with wild type ADAMTS13 and variants in the conditioned medium at the final concentrations of 0.2 nM and 0.04 nM or 2 nM and 10 nM in the purified forms (total volume 150 µl) at 37 °C for 4 hours on a dialysis membrane (0.25 µm, pore size) floating over 50 ml dialysis buffer (10 mM Tris-HCl, pH 8.0 containing 1.5 M urea in a conical tube. The digested material (50 µl) was withdrawn and denatured with 50 µl sample buffer (70 mM Tris-HCl, pH6.5, 2.4% SDS, 0.67 M urea, and 4 mM EDTA) at 60 °C for 20 minutes. The denatured VWF was fractionated with 1% (wt/vol) SeaKem HGT agarose (Cambrex, East Rutherford, NJ) gel in a Mini-protein III gel cassette (Bio-Rad, Hercules, CA), 15 mA for 90 min. The protein was then transferred onto a nitrocellulose membrane (Millipore, Billerica, MA), 100 mA for 20 min. The proteolytic cleavage of VWF multimers were detected by Western blotting with rabbit anti-VWF IgG (1:5,000) and IRDye 800CW-labeled goat anti-rabbit IgG (1:10,000) (LI-COR Bioscience, Lincoln, Nebraska) in TBST containing 1% casein. The fluorescent signal was determined by an Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, Nebraska). The relative proteolytic activity was determined by densitometry of the cleavage product and uncleaved high molecular weight VWF multimers.
4.3.6 Crystallization screening and optimization

Initial screening for crystallization conditions for FL-ADAMTS13 was carried out by the hanging-drop vapor-diffusion method using Index Screen, Crystal Screen, SaltRx Screen, PEG/Ion Screen, Grid Screen MPD and Grid Screen Ammonium Sulfate kits (Hampton Research, Aliso Viejo, California, USA). A volume of 1 µl protein solution was manually mixed with an equal amount of reservoir solution and the droplets were allowed to equilibrate against 0.5 ml reservoir solution at 4°C and 25°C.

4.3.7 Data collection and structure refinement

X-ray data were collected to their diffraction limit and were processed using DENZO. The structure was determined by molecular replacement using AmoRe. The initial phases were improved by non-crystallographic symmetry (NCS) averaging and solvent flattening using DPHASE (S. Stayrook, personal communication). The resulting electron density maps were fit using Coot. The structure was refined using CNS. Refinement included simulated annealing, restrained isotropic temperature factor refinement, a bulk solvent correction, and monitoring of the free $R$-factor with 5% of the data omitted. NCS restraints were imposed. Figures were prepared using MOLSCRIPT, Coot, and PyMol.

4.3.8 Molecular modeling

The interaction between the spacer domain of ADAMTS13 and RRYGEE or VWF73 peptide was modeled using the HHpred server plugin using an energy minimization algorithm (GROMACS4) in PyMol software (http://www.pymol.org/).
4.4 Results

4.4.1 Synthetic peptides derived from the ADAMTS13 spacer domain or an isolated spacer domain of ADAMTS13 inhibits proteolytic cleavage of VWF by ADAMTS13

Deletion of several amino acid residues or substitution of a single amino acid residue within the spacer domain may result in protein structural change or misfolding. To rule out such a possibility, we synthesized short peptides based on amino acid sequence of the RRYGEE motif in ADAMTS13 spacer domain and used these synthetic peptides for the inhibition assays. The peptides RRYGEE and RRY, which perfectly match the sequence of spacer domain, dramatically inhibited proteolytic cleavage of rF-VWF73 (Figure 4.1A) and multimeric VWF (Figure 4.1B-E) by wild-type ADAMTS13 in a concentration-dependent manner. At 200 μM, RRYGEE and RRY inhibited proteolytic cleavage of rF-VWF73 by ADAMTS13 activity by approximately 85% and 90%, respectively.

The synthetic peptide GEE, and scrambled peptides RYR and EYREGR, however, exhibited much less inhibitory activity on proteolytic cleavage of rF-VWF73 even at the highest concentration (200 μM) tested (Figure 4.1A). The inhibitory effect of RRYGEE (Figure 4.1B) and RRY (Figure 4.1C) was also observed in proteolytic cleavage of multimeric VWF under fluid shear stress.

The IC_{50}s were estimated to be less than 50 μM for both peptides (Figure 4.1F). The peptides GEE (Figure 4.1D) and RYR (scrambled) (Figure 4.1E) at concentration of 200 μM did not exhibit significant inhibitory effects in this assay. Together, these data suggest that the amino acid residues Arg^{659}, Arg^{660} and Tyr^{661} in the ADAMTS13 spacer domain...
domain may involve in direct binding interaction with the central A2 domain of soluble VWF that is exposed by truncation or under high fluid shear stresses.
Figure 4.1. Synthetic peptides derived from ADAMTS13 spacer domain inhibit cleavage of VWF73 and multimeric VWF by ADAMTS13. Panel A. rF-VWF73 peptide (1 µM) was incubated with various concentrations (0-200 µM) of synthetic peptides as indicated for 10 min. Then recombinant ADAMTS13 (2 nM) was added and proteolytic cleavage of rF-VWF73 was monitored at 37 ºC on a fluorescent microtiter plate reader every minute for 20 min. The maximal rates of fluorescent generation per second were plotted against the log concentrations of synthetic peptides used. The data presented are means ± SD of three independent measurements. Panel B to E. Purified plasma-derived VWF (37.5 µg/ml) was incubated at 37 ºC with various concentrations (0-200 µM) of synthetic peptides RRYGEE (Panel B) or RRY (Panel C) or GEE (Panel D) or RYR (Panel E) in the absence or presence of 20 mM EDTA for 60 min. Then, ADAMTS13 (50 nM), recombinant FVIII (10 nM), and lyophilized platelets (210x10^6 /ml) were added into the reaction. The reaction mixture was subjected to vortexing (2,500 rpm) for 5 min. The proteolysis of VWF was determined by multimer analysis on 1% agarose gel and Western blot. High molecular weight multimer is indicated as HMW. The proteolytic cleavage product (350 kDa) is indicated with the arrowhead. Panel F shows the quantification of the 350-kDa cleavage product by densitometry in relation to the concentration of synthetic peptides (Mean ± SD, n=3, except for RYR).
4.4.3 Molecular modeling of VWF73 and RRYGEE complex

Molecular modeling of VWF73 based on the crystal structure of the VWF-A2 domain\textsuperscript{28} docked by the peptide RRYGEE using energy minimization algorithm (GROMACS4) revealed that the amino acid residues RRY fit into the pocket formed at the carboxyl terminal end of the VWF73 and Tyr-Met peptidyl bond, whereas the amino acid resides GEE reside outside of this pocket (Figure 4.2). These data suggest that direct engagement of the amino acid residues Arg\textsuperscript{659}, Arg\textsuperscript{660} and Tyr\textsuperscript{661} with the central A2 domain of VWF may play a critical role for the efficient cleavage of VWF under both non-physiological and physiological conditions.

![Figure 4.2. Proposed model of VWF73 and spacer domain peptide complex.](image)

**Figure 4.2. Proposed model of VWF73 and spacer domain peptide complex.** The $\alpha$-helix and $\beta$-sheet are derived from the crystal structure of VWF-A2 domain. The C-terminal 9 amino acid residues of VWF73 peptide are indicated in blue. The sticks represent the amino acid residues (RRYGEE) found in the ADAMTS13 spacer domain. The complex between VWF73 and RRYGEE was modeled with an energy minimization algorithm with GROMACS4 software. The general model was carried out using PyMol software. Panel B is viewed at 325° rotation of the Panel A. The amino acid residues RRY make direct contact with the C-terminal 9 amino acid residues EAPDLVLQR in blue and Tyr-Met bond, whereas the residues GEE serve as a linker.
4.4.7 Molecular modeling of ADAMTS13-VWF specific interactions in the Cys-rich and Spacer domains

To gain insight into the mechanisms underlying the enhanced activity of ADAMTS13 variants, we performed molecular modeling using the existing crystal structure of ADAMTS13-DTCS fragment\(^{23}\) and VWF-A2 domain\(^{28}\). As shown, the spacer domain comprises ten β-sheets (i.e. β1-10), a pocket formed by various β sheets containing a cluster of hydrophobic residues (L591, F592, L637, F638, L668, and T669), and a ring formed by Y661 and Y665 lined by basic residues R568, R589, R660, and R636 (Figure 4.3). This pocket appears to directly interact with the α6-helix (residues between D1653 and R1668) in the central A2 domain of VWF. The hydrophobic residues in the A2 domain presumably face the exosite 3 to make strong hydrophobic contacts in conjunction with some hydrogen bonds outside the pocket (Figure 4.3D). A substitution of R with K residue or Y with F residue or vice versa appears to alter the hydrophobicity in the exosite 3 (Figure 4.3C). The corresponding changes of hydropathy index were as follows: \(R \rightarrow K\): -4.5 \rightarrow -3.9 and \(Y \rightarrow F\): -1.3 \rightarrow +2.8, thereby enhancing the interaction between VWF and ADAMTS13. Furthermore, a substitution of F592 with Y may open up the pocket even more, thereby better engaging substrate. There was also a corresponding backbone shift which appears to take place in the β2, β5, β6, and β9 sheets to compensate for the increased hydrophobicity. These changes allow greater engagement of the exosite 3 with A2 domain, particularly the amino acid residues between residues D1653 and R1668.
Figure 4.3. Modeling of ADAMTS13-spacer and VWF-A2 interaction. A. Surface representation of ADAMTS13-DTCS fragment; B. Close-up view of the hydrophobic cluster in the exosite of ADAMTS13 spacer domain. This pocket contains a cluster of hydrophobic residues (L591, F592, L637, P638, L668, T669, and ring of Y661 and Y665), lined by basic residues (R568, R589, R636, and R660) supported by 8 β sheets (i.e. β1, 2, 3, 6, 7, 8, 9, and 10). C. A substitution of these surface residues with those in yellow appears to increase hydrophobicity of this pocket. D. VWF-A2 (1653-1668) forms an amphipathic helix (α6). Here shown are the hydrophobic residues facing to the top and charged residues to the bottom. This amphipathic helix may govern specificity to the ADAMTS13-spacer exosite by inserting its hydrophobic side into the pocket.
4.4.5 Crystallization of ADAMTS13

We conducted initial crystal screens for ADAMTS13 carried out by the hanging-drop vapor-diffusion method using Index Screen, Crystal Screen, SaltRx Screen, PEG/Ion Screen, Grid Screen MPD and Grid Screen Ammonium Sulfate kits (Hampton Research, Aliso Viejo, California, USA) at 4°C and 25°C. Crystals were monitored for precipitate and microcrystal growth under an inverted microscope. From this initial set of screens, we were able to obtain 6 potential hits from solutions 4 (A), 12 (B), 20 (C) of the Crystal Screen 1 kit and solutions 10 (D), 14 (E), and 23 (F) of the PEG/Ion Screen kit. We performed grid optimization screens using the initial conditions and we were able to consistently grow crystals at 25°C for 6 conditions (Figure 4.4). Preliminary diffraction data for these crystals was negative, except for one condition (Condition D, 0.17 M Ammonium Sulfate, 0.23 M HEPES pH 7.0, 33% PEG 8000) which yielded a low-resolution, 6.5Å broad diffraction pattern (Figure 4.5). This pattern could likely be attributed to the very thin, needle-like morphology of the crystals.
Figure 4.4. Crystals of human FL-ADAMTS13 grown by the hanging-drop method. (a) Microcrystals were initially obtained from solutions 4 (A), 12 (B), 20 (C) of the Crystal Screen 1 kit and solutions 10 (D), 14 (E), and 23 (F) of the PEG/Ion Screen kit. The crystals were obtained using the optimized conditions.
Figure 4.5. Diffraction pattern obtained for human FL-ADAMTS13 crystals grown by the hanging-drop method (following optimization from Condition D). The diffraction pattern was obtained to a resolution of 6.54Å for needle-shaped crystals under the conditions 0.17 M Ammonium Sulfate, 0.23 M HEPES pH 7.0, 33% PEG 8000.

Overall, we screened over 900 conditions, out of which we got ~26 putative hits. The specific conditions that yielded diffractable crystals (Condition F, Figure 4.6) were: 0.18M Sodium citrate tribasic dihydrate pH~6, 17% (v/v) (+/-)-2-Methyl-2,4-pentanediol (MPD), 22% tert-Butanol pH 6.0. These crystals formed after ~4 weeks at 298K. This condition was the result of initial optimization from a hit containing 0.1M Sodium citrate tribasic dihydrate pH 5.6, 35% tert-Butanol, which yielded no crystals in the initial screens, but did yield a productive precipitate. We used PEG 4000, PEG 8000, and MPD as additives in subsequent screens. This set of crystals was the result of 4 optimization
screens after using MPD as an additive (higher concentration of salt, lower concentrations of precipitant).

**Figure 4.6. Crystals of human FL-ADAMTS13 grown by the hanging-drop method (Condition F).** The conditions were 0.18M Sodium citrate tribasic dihydrate pH~6, 17% (v/v) (+/-)-2-Methyl-2,4-pentanediol (MPD), 22% tert-Butanol pH 6.0. These crystals formed after ~4 weeks at 298K. This condition was the result of initial optimization from a hit containing 0.1M Sodium citrate tribasic dihydrate pH 5.6, 35% tert-Butanol, which yielded no crystals in the initial screens, but did yield a productive precipitate.

4.4.6 Structure determination

Data were collected (Figure 4.7) to the diffraction limit (2.2 Å) and the structure solved as described in Methods. An unbiased view of the electron density was obtained by solvent flattening and non-crystallographic symmetry averaging. The phases obtained by molecular replacement were improved and bias removed by symmetry averaging and solvent flattening. The structure agrees with that previously reported\(^{23}\). A summary of the crystallographic data and refinement statistics are presented in Table 4.1.
Figure 4.7. Diffraction patterns and Wilson plots obtained for human FL-ADAMTS13 crystals grown by the hanging-drop method (following optimization from Condition F). The diffraction pattern (Panels A and C) was obtained to a resolution of 2.2Å for single crystals under the conditions 0.18M Sodium citrate tribasic dihydrate pH~6, 17% (v/v) (+/-)-2-Methyl-2,4-pentanediol (MPD), 22% tert-Butanol pH 6.0. The Wilson plots (Panels B and D) shown are for these diffraction patterns obtained from two different crystals grown under the same conditions.
Table 4.1. Data Collection and Refinement Statistics

<table>
<thead>
<tr>
<th>Data Collection</th>
<th></th>
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<tr>
<td>Space group</td>
<td>P212121</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>39.11, 55.88, 69.84</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90, 90, 90</td>
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<tr>
<td>Wavelength (Å)</td>
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<tr>
<td>Resolution range (Å)</td>
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<td>R merge</td>
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<tr>
<td>l/σl</td>
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<tr>
<td>Completeness (%)</td>
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<td>Redundancy</td>
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</table>

<table>
<thead>
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<th>Refinement</th>
<th></th>
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<tbody>
<tr>
<td>Maximum resolution (Å)</td>
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</tr>
<tr>
<td>No. reflections</td>
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<td>R work/R free (%)</td>
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</tr>
<tr>
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<td>0.009</td>
</tr>
<tr>
<td>rmsd bond angle (°)</td>
<td>0.962</td>
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Oddly, we were able to reproducibly acquire high resolution diffraction patterns (2-2.5Å) that encompassed the P212121 space group. This space group cannot accommodate a protein as large as FL-ADAMTS13. In addition, molecular replacement (MR) with ADAMTS13-DTCS did not yield a favorable solution. The PHASER output indicated that, rather than being 145 kDa, the product was more likely around 20-30 kDa. Using multiple iterations of MR, we were finally able to find a suitable solution using the structure of human ADAMTS1 catalytic domain and cysteine-rich/spacer domains (apo-form, PDB code 2V4B). We later confirmed this by truncating the ADAMTS13-DTCS fragment to CS and performing MR. The determined boundaries of the solved structure
were from M$^{432}$ (in TSP-1) to R$^{670}$ (in Spa). The structure (Figure 4.8A) encompasses 8 residues in the 1st TSP-1 repeat, in addition to the entire cysteine-rich domain (C$_A$ and C$_B$) and the majority of the spacer domain.

Our structure agrees very well (Table 4.2) with the analogous portion of the ADAMTS13-DTCS structure previously reported (RMSD = 0.02Å, 1817 atoms, 232 residues). A region within the C$_A$ domain (Q$^{478}$ – R$^{528}$) does vary slightly from ADAMTS13-DTCS (RMSD = 0.08Å, 379 atoms, 51 residues), however there are no significant differences in structure other than a shift in the $\alpha$4 helix from G$^{479}$–C$^{483}$ (Figure 4.8B, Table 4.2). As with the ADAMTS13-DTCS structure, our structure shows 5 intermolecular disulfide bonds in Cys, in addition to 2 free cysteine residues.

Figure 4.8. Structure of ADAMTS13-CS and structural alignment with ADAMTS13-DTCS. Panel A. Ribbon structure of ADAMTS13-CS in stereo. Blue indicates the ADAMTS13-Cys domain, while red indicates the ADAMTS13-Spa domain. Disulfide bonds (and free cysteines are indicated in yellow. Panel B. Structural alignment of ADAMTS13-CS (red) with the analogous portion of the published ADAMTS13-DTCS$^{23}$ structure (teal). The G$^{479}$–C$^{483}$ alpha helix is slightly shifted in ADAMTS13-CS.
**Table 4.2. Structural alignment of ADAMTS13-CS and ADAMTS13-DTCS**

<table>
<thead>
<tr>
<th>Region</th>
<th>RMSD (Å)</th>
<th>No. of atoms</th>
<th>No. of residues</th>
</tr>
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<tbody>
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<td>M&lt;sup&gt;432&lt;/sup&gt;-R&lt;sup&gt;670&lt;/sup&gt;</td>
<td>0.02</td>
<td>1817</td>
<td>232</td>
</tr>
<tr>
<td>Q&lt;sup&gt;478&lt;/sup&gt; - R&lt;sup&gt;528&lt;/sup&gt;</td>
<td>0.08</td>
<td>379</td>
<td>51</td>
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<tr>
<td>G&lt;sup&gt;479&lt;/sup&gt;-C&lt;sup&gt;483&lt;/sup&gt;</td>
<td>0.15</td>
<td>31</td>
<td>6</td>
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</table>

### 4.5 Chapter Discussion

The crystal structure of the ADAMTS13-DTCS fragment demonstrates that amino acid residues Arg<sup>659</sup>, Arg<sup>660</sup>, Tyr<sup>661</sup>, and Tyr<sup>665</sup> form a surface loop facing the solvent (Figure 4.2). This loop may directly interact with the central A2 domain, presumably the C-terminal end of VWF73 peptide (Gly<sup>1660</sup>-Arg<sup>1668</sup>) as the deletion of this C-terminal end from the VWF73 peptide resulted in an approximately 20-fold reduction of the cleavage rates by wild-type ADAMTS13.13 Such a rate reduction appears to be similar to that after the deletion of ADAMTS13 spacer domain<sup>8,10</sup>. It remains to be elucidated, however, how ADAMTS13-Spa gains access to the central A2 domain of the UL-VWF polymers anchored on endothelial cells. The newly released UL-VWF polymers from human endothelial cells after histamine stimulation appeared to be highly susceptible to ADAMTS13 whether in the presence<sup>25,26</sup> or in the absence<sup>27</sup> of fluid shear stress. Spa, particularly the residues Arg<sup>659</sup>, Arg<sup>660</sup>, and Tyr<sup>661</sup>, play a role in recognition of cell-bound UL-VWF polymers<sup>11</sup>. The importance of Spa, particularly the amino acid residues Arg<sup>659</sup>, Arg<sup>660</sup>, and Tyr<sup>661</sup> in substrate recognition *in vivo*, is highlighted by the results of antibody mapping. Luken et.
al.\textsuperscript{28,29} have shown that anti-ADAMTS13 IgG isolated from several patients with acquired idiopathic TTP directly targets against the amino acid residues between Val\textsuperscript{657} and Gly\textsuperscript{666}, which contains the triple peptide (Arg-Arg-Tyr). However, the role of this triple peptide in substrate recognition does not undermine the importance of the other C-terminal noncatalytic domains of ADAMTS13.

For instance, the precise engagement of amino acid residues Arg\textsuperscript{349}, Leu\textsuperscript{350}, and Val\textsuperscript{352} in the disintegrin domain with VWF-A2 domain\textsuperscript{30} and interactions between the VWF-D4-CK domain with the more distal C-terminal domains of ADAMTS13\textsuperscript{30} also may play a role for productive cleavage of multimeric VWF in solution under physiologically relevant conditions. The results from genetic studies in mice and humans may further support the role of more distal C-terminal domains for ADAMTS13 function.

We conclude that the amino acid residues Arg\textsuperscript{659}, Arg\textsuperscript{660}, and Tyr\textsuperscript{661} in the ADAMTS13 spacer domain play a critical role in recognition and proteolytic cleavage of peptidyl substrates, soluble VWF, and cell-bound VWF polymers under both static and fluid shear conditions. The reduced proteolytic activity of the spacer mutants in many cases is associated with the impaired binding interactions with the central A2 domain of VWF. Our findings may provide novel insight into the structure-function relationship of ADAMTS13 and shed some light on the pathogenesis of TTP and perhaps other arterial thrombotic disorders related to compromised VWF proteolysis by ADAMTS13.

In the effort to crystallize full-length ADAMTS13, we were able to obtain a high resolution structure of the ADMTS13-CS fragment, presumably a product of autolysis under the crystallization conditions (0.18M Sodium citrate tribasic dihydrate, 17% (v/v) (+/-)-2-Methyl-2,4-pentanediol (MPD), 22% tert-Butanol pH 6.0). The nature of this
autolysis, which is reproducible under these conditions, is unknown. There is evidence\(^{31}\) that pH can affect the activity of ADAMTS13, however there are no substantial reports on autolysis by the Metalloprotease domain. We will probe the exact conditions under which autolysis occurs to remedy this issue.

4.6 Chapter References


5. Tsai HM. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood* 1996; 87: 4235-44.


CHAPTER 5: ADAMTS13 PLAYS A ROLE IN REGULATING THROMBUS COMPOSITION AND THROMBUS FORMATION DYNAMICS \textit{IN VIVO}

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5.1 Overview

Proteolytic cleavage of ultra large von Willebrand factor (ULVWF) released from endothelial cells by ADAMTS13 metalloprotease is critical for maintaining normal hemostasis. However, the effect of infusing ADAMTS13 on thrombus composition remains poorly understood. In this study, we determined the morphology and composition of thrombi formed in carotid arteries after topical application of FeCl₃ in *Adams13*⁻/⁻ mice receiving PBS, recombinant human full-length ADAMTS13 (FL) and carboxyl-terminal truncated variant after spacer domain (S), using scanning electron microscopy and quantitative image analysis. We showed that in *Adams13*⁻/⁻ mice 5 min after FeCl₃ injury, formed arterial thrombi were comprised ~39% platelets, ~26% red blood cells, and ~35% fibrin. The arterial thrombi in these mice were structurally deformed. An infusion of recombinant FL (final concentration of 10 nM) significantly reduced the accumulation of platelets (~18%) but increased the fibrin network (57%) without affecting the composition of red blood cells (~25%) in the arterial thrombi formed at the same time point after FeCl₃ injury. Similar effects on the morphology and composition of FeCl₃-induced arterial thrombi were observed after infusion of recombinant S (10 nM) into *Adams13*⁻/⁻ mice.

Kinetic analysis showed that there was a decrease in platelet accumulation over the time of 30 min during thrombus formation with a slight increase in accumulation of red blood cells and formation of fibrin in *Adams13*⁻/⁻ mice. But, the infusion of recombinant FL and S into *Adams13*⁻/⁻ mice restored the kinetics of platelet/red blood cell accumulation and fibrin formation to those observed in wild-type mice. Our findings, revealing the
apparent difference in thrombus composition, provide novel insight into the mechanism of ADAMTS13 function *in vivo*, which may shed more light on the pathogenesis of thrombotic thrombocytopenic purpura and other arterial thrombotic disorders associated with deficiency of plasma ADAMTS13 activity.

5.2 Introduction

Studies have shown that the cleavage of endothelial ULVWF occurs very rapidly with or without fluid shear stress, suggesting that newly-released ULVWF on endothelial cell surface may be in its “open” conformation. However, the VWF cleaved from endothelial cells by ADAMTS13 remains ultra-large by multimer analysis, suggesting that further proteolytic processing of these released VWF multimers, likely to occur in microvasculature, may be necessary to reduce the size.

Exposure of the soluble VWF to high shear stress or denaturants may open up the binding and cleavage sites, which are normally buried under the β-sheet in the central A2 domain. Moreover, binding of factor VIII (FVIII), platelets or both to soluble VWF also increases its cleavage by ADAMTS13 under shear stress. These results suggest a cofactor-dependent mechanism regulating VWF proteolysis by ADAMTS13 under physiologically relevant conditions.

In addition, as mentioned previously, the role of the C-terminal domains of ADAMTS13 *in vivo* has long been controversial. For instance, a study from our lab reported that a C-terminally truncated ADAMTS13 variant after the spacer domain expressed by an in utero injection of lentiviral vector eliminated plasma ULVWF and inhibited ferric chloride (FeCl₃)–induced arterial occlusion in the carotid artery of *Adams13* mice.
while another study\textsuperscript{18} showed that a naturally occurring murine ADAMTS13 variant truncated after the sixth TSP1 repeat (ADAMTS13S/S) was less efficacious than full-length ADAMTS13 in inhibiting FeCl\textsubscript{3}-induced thrombosis in the mesenteric arteriole\textsuperscript{18}.

Finally, it has been reported\textsuperscript{19} that a recombinant murine ADAMTS13 variant truncated after the eighth TSP1 repeat, infused into \textit{Adams13}\textsuperscript{+/−} mice, was not able to cleave newly released ULVWF/VWF strings on the endothelial cells in the mesenteric arterioles/venules\textsuperscript{19}.

These discrepant results prompted our lab to systematically investigate the structure-function relationship of ADAMTS13 \textit{in vivo} using a recombinant protein strategy and a murine thrombosis model\textsuperscript{20}. 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 \textit{Adams13}\textsuperscript{+/−} mice with efficacy similar to that of full-length ADAMTS13, suggesting that the amino terminus of ADAMTS13, specifically the variable region of the spacer domain, is crucial for modulation of arterial thromboses under physiological conditions\textsuperscript{20}.

Even with these results, the effect of infusing ADAMTS13 or ADAMTS13 variants on thrombus formation and overall thrombus composition is poorly understood. Understanding of this process can elucidate possible unknown functions of ADAMTS13 in thrombus formation and inform researchers about the usefulness of soluble ADAMTS13 as a potential therapy for TTP and other arterial thrombotic disorders.

In this study, we examine thrombi after FeCl\textsubscript{3} injury in \textit{Adams13}\textsuperscript{+/−} mice before and after injection of FL-ADAMTS13 and variants, to determine: 1) the time-dependent
composition of thrombi (time after injury); 2) the differential composition at the head and tail of the thrombus; and 3) the change in composition with reconstitution of normal thrombosis. We show that infusion of recombinant FL-ADAMTS13 and an ADAMTS13 variant truncated after either the eighth thrombospondin type 1 repeat into Adamts13−/− mice restored the kinetics of platelet/red blood cell accumulation and fibrin formation to those observed in wild-type mice. Our findings reveal the effect of ADAMTS13 deficiency on thrombus composition and thrombus formation dynamics over time, providing novel insight into the mechanism of ADAMTS13 function in vivo. These results may further elucidate the pathogenesis of thrombotic thrombocytopenic purpura and other arterial thrombotic disorders.

5.3 Materials and Methods

5.3.1 Preparation of carotid artery samples and fixation for microscopy

C57BL/6 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 retro-orbital 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 (~10 nmol/L). The ADAMTS13, variant, or PBS were infused via a jugular vein into Adams13−/− mice (~1 ml blood for ~14 g body weight). This resulted in final concentrations of ~10 nmol/L ADAMTS13 in mouse circulation. In the indicated number of minutes after protein infusion, injury was induced
by exposing the carotid artery to 10% ferric chloride (FeCl$_3$) (anhydrous) soaked on a piece of filter paper (1x2 mm) for 2 minutes. The filter paper was then removed, and the artery sections were flushed with PBS and fixed in 2.5% glutaraldehyde in 0.1M Sodium Cacodylate buffer pH 7.4.

Figure 5.1. Schematic showing preparation of carotid artery samples in C57BL/6 mice.

5.3.2 Immunofluorescent microscopy of carotid artery samples

After being washed with phosphate-buffered saline (PBS), arteries were fixed with 1% paraformaldehyde in PBS (non-permeabilized) for 10 min. The arteries were then flash-frozen in OCT medium (Tissue-Tek) and sectioned in a cryostat (5-µm sections) at -25°C. Sections were placed onto microscope slides and allowed to adhere at room temperature. The sections were blocked with 2.5% bovine serum albumin (BSA) in PBS for 30 min and incubated with a primary antibody (monoclonal anti-VWF, polyclonal anti fibrinogen, monoclonal anti-GP1b, or polyclonal anti-CD34) (1:200) for 2 h at 22°C,
followed by a fluorescein-conjugated secondary antibody diluted in 0.5% BSA in PBS for 1 h. The sections were then incubated with Alexa Fluor® 647 conjugated transferrin (Invitrogen) (1:500) for 10 min, and mounted with medium containing 4’,6 diamidino-2-phenylindole (DAPI) (1:5) (Vector Lab, Burlingame, CA, USA) after three washes with PBS. The fluorescent signals were determined under a multi-color fluorescent microscope. The images were obtained using SPORTadvanced software (Sterling Heights, MI) at magnifications of 100X and 600X.

5.3.3 Scanning electron microscopy of carotid artery samples

Sample fixation, dehydration, and preparation of samples were performed according to the method of Weisel and Nagaswami. High definition photographs (3000X magnification) were obtained using a Philips/FEI XL20 scanning electron microscope (4nm resolution) (FEI, Hillsboro, OR, USA) at 10 KV accelerating voltage. To control for composition heterogeneity in the analysis of the thrombus, we examined several areas (at least 12 according to a grid and the size of the thrombus) for each thrombus, in an unbiased manner at low magnification and at least a dozen digital images were taken at the highest resolution.

5.3.4 Quantitative analysis of micrographs by FOAMS (Fast Object Acquisition and Measurement System)

Image processing analysis was carried out by a modified version of FOAMS (Fast Object Acquisition and Measurement System), which is a Matlab®-based program initially designed to help geologists wishing to initiate in vesicle texture studies. We have
modified the FOAMS program to perform size analysis on 1 µm$^2$ grid sections of SEM micrographs, based on the established method by Silvain et. al.$^{24}$ Analysis of the size characteristics of each of these sections, based on grayscale representation of analyzable objects, is cumulatively expressed as a total quantitative measure.

For *Adams13*−/− mice thrombi, images were processed by Adobe Photoshop® for preparation for size analysis. Briefly, the required input for FOAMS is a set of images with distinct shades of gray corresponding to various analyzable objects (bubbles, crystals, etc.). Shades of gray have values between 0 and 255 (called Digital Numbers, or DN). For instance, all “bubble” objects might have a DN value of 0 (black) and the other objects intermediate values until white (DN = 255)$^{22}$. Once images were processed, they were input into the FOAMS program, which superimposed a grid with 1 µm$^2$ squares and identified and analyzed the size of objects (platelets, red blood cells, fibrin, and macrophages) in each grid section based on the DN.

5.3.5 *Focused ion beam milling and scanning-transmission electron microscopy of fixed artery sections*

SEM/SED/STEM was performed using a Strata DB235 Focused Ion Beam (FEI) during FIB milling. An electron beam at 20 kV was used to do the initial "trench" digging, and 2 kV at a slight tilt for the final thinning. After excising ~100 nm thick FIB sections with a needle and mounting them on carbon grids, they were visualized by a JEOL 2010 TEM at 60 kV.
5.4 Results

5.4.1 Scanning electron microscopy indicates differences in the morphology and composition of WT and Adamts13\(^{-/-}\) mice arterial thrombi

In order to determine the necessary components of arterial thrombi to track, we used immunofluorescent microscopy to observe the predominant species of molecules (platelets, fibrin, red blood cells, macrophages, and VWF) in extracted arterial thrombi. After using antibodies to probe all of the aforementioned components, we found that we were able to detect fibrin and platelets reproducibly enough to serve as a standard for measuring the thrombus composition (Figure 5.2). In subsequent scanning electron microscopy images we were also able to reproducibly identify a large number of red blood cells. Thus, we determined that the optimal components for quantifying arterial thrombus composition would be fibrin, platelet, and red blood cell content.
Figure 5.2. Immunofluorescent staining of carotid artery sections of WT mice after FeCl$_3$-induced thrombosis. Hematoxylin-Eosin staining of a frozen carotid artery section showing vessel walls and thrombi of wild-type C57BL/6 mice 30 minutes after FeCl$_3$ induced thrombosis (Panel A). Immunofluorescent staining of a different section of the same frozen carotid artery of wild-type mice 30 minutes after FeCl$_3$ induced thrombosis (Panel B). Blue dots indicate nuclei in vessels stained by DAPI. Also shown are Fibrin (red), platelet-specific CD41 (green), and merged images.

In this study, we also determined the morphology and composition of thrombi formed using scanning electron microscopy and quantitative image analysis in carotid arteries after topical application of FeCl$_3$ in $Adams13^{+/}$ mice receiving PBS, recombinant human full-length ADAMTS13 (FL) and carboxyl-terminal truncated variant after spacer domain (S). We showed that in $Adams13^{+/}$ mice 5 minutes after FeCl$_3$ injury, formed
arterial thrombi that were very platelet- and red blood cell-rich (Figure 5.3B and D), compared to the thrombi of wild-type mice 5 minutes after FeCl$_3$ injury (Figure 5.3A and C). The arterial thrombi in these mice were deformed ultrastructurally (Figure 5.3D) compared to the thrombi of wild-type mice (Figure 5.3C).

Figure 5.3. Scanning electron microscopy analysis of thrombi from the carotid arteries of *Adams13/-* mice after FeCl$_3$-induced arterial thrombosis. Carotid artery sections showing vessel walls and thrombi of wild-type C57BL/6 mice 0 and 30 minutes after FeCl$_3$ induced thrombosis (Panels A and C). Carotid artery sections showing vessel walls and thrombi of *Adams13/-* C57BL/6 mice 0 and 30 minutes after FeCl$_3$ induced thrombosis (Panels B and D). The thrombi were fixed, processed and examined on a FEI Quanta FEG SEM.
5.4.2 Kinetic analysis indicates that infusion of FL-ADAMTS13 or C-terminal truncated ADAMTS13 variant restores the kinetics of platelet/red blood cell accumulation and fibrin formation

Infusion of recombinant FL (final concentration of 10 nM) significantly reduced the accumulation of platelets (~18%) but increased the formation of fibrin network (57%) without affecting the composition of red blood cells (~25%) in the arterial thrombi at the same time point after FeCl₃ injury (Figure 5.4).

A similar effect on the morphology and composition of FeCl₃-induced arterial thrombi was observed after infusion of recombinant S (10 nM) into Adamts13⁻/⁻ mice. Kinetic analysis showed that there was a decrease in platelet accumulation over a time of 30 min following FeCl₃ injury, with a slight increase in accumulation of red blood cells and fibrin formation in Adamts13⁻/⁻ mice. Infusion of recombinant FL and S into Adamts13⁻/⁻ mice restored the kinetics of platelet/red blood cell accumulation and fibrin formation to those observed in wild-type mice (Figure 5.4).
Figure 5.4. Time-dependent thrombus formation by scanning electron microscopy. Carotid artery sections from C57BL/6 mice were removed the indicated number of minutes after topical application of 10% FeCl₃ for 5 min under a fluorescent microscope. Fully-formed thrombi were exposed in the carotid artery of wild type mice, Adamts13⁻/⁻ mice receiving PBS, full-length ADAMTS-13 (FL-A13), and MDTCS, 10 nM. The thrombi were fixed, processed and examined on a FEI Quanta FEG SEM. The thrombus composition from wild-type, Adamts13⁻/⁻ mice receiving PBS, FL, and S (10 nM) was quantified (n=15) by the Fast Object Acquisition and Measurement System, which is a Matlab®-based program to perform size analysis on 1 µm² grid sections of SEM micrographs. Analysis of the size characteristics of each of these sections, based on grayscale representation of analyzable objects, was cumulatively expressed as a total quantitative measurement. The kinetic data of fibrin, platelets and red blood cells are shown.
5.4.3 *An apparent increase in platelet density at the tail of thrombi in* *Adamts13<sup>−/−</sup> *mice is corrected by infusion of FL-ADAMTS13 or C-terminal truncated ADAMTS13 variant*

Using Focused Ion Beam milling, we were able to generate sections of the thrombi (Figure 5.5A) which spanned from the head to the tail, allowing analysis of differential composition in the primary areas. Preliminary analysis indicates an apparent increase in platelet density at the tail of thrombi in *Adamts13<sup>−/−</sup>* mice at 10 min after FeCl<sub>3</sub> injury, while there was no apparent difference in composition at the head (Figure 5.5B).
Figure 5.5. Focused Ion Beam milling with subsequent EM visualization of arterial thrombus sections. Carotid artery sections from mice were removed 30 min after topical application of 10% FeCl₃ under a fluorescent microscope. Panel A shows a representative SEM/SED/STEM image of mouse artery surface during FIB milling. An electron beam at 20 kV was used to do the initial "trench" digging, and 2 kV at a slight tilt for the final thinning. Trenches were dug every 100 microns from the point that was marked as the site of injury. Panel B shows the images of excising FIB sections with a needle and mounting them on carbon grids, visualized by a JEOL 2010 TEM at 60 kV. Sections at 0, 100, and 300 microns from the marked site of injury are shown. Mostly platelets are seen, with occasional red blood cells and macrophages.
5.5 Chapter Discussion

We have seen that ADAMTS-13-MDTCS appears to be sufficient for proteolytic cleavage of ULVWF in vivo; thereby offering systemic protection of mice against FeCl₃ induced arterial thrombosis in the carotid artery. Imaging analysis indicates that normal thrombi appear to be well-formed and fibrin-rich, whereas Adamts13⁻/⁻ C57BL/6 mice exhibit thrombi with multiple malformations and areas enriched with platelets and erythrocytes (less apparent fibrin). Imaging analysis indicates that normal thrombi appear to be well-formed and fibrin-rich, whereas Adamts13⁺/⁻ C57BL/6 mice exhibit thrombi with multiple malformations and areas enriched with platelets and erythrocytes (less apparent fibrin). Preliminary study of the formation dynamics as a function of time and with infusion of FL-ADAMTS13 and MDTCS indicates an apparent reconstitution of the normal thrombus composition upon full formation of the thrombus, however it appears that the early stages of formation differ from that of wild-type mice. Using focused ion beam (FIB) milling, we study the differential composition of the thrombi at the head and tail, informing us about the potential for embolism or other complications.

The FIB milling technique, although potentially very helpful, is wrought with technical difficulties. Quantitation of the acquired TEM images is very difficult because the morphology of the platelets, fibrin, and red blood cells is distorted in the images. This is likely due to a well-known phenomenon where a thin “film” is left by the laser trench digging on the excised section. This film could potentially interfere with subsequent visualization. We endeavor to use live confocal microscopic imaging to determine the extent of malformation of the arterial thrombi in Adamts13⁺/⁻ mice, and to determine
whether infusion of ADAMTS13 or variants has a differential effect on the thrombus composition at different areas of the thrombus.

Our findings, revealing the apparent difference in thrombus composition, provide novel insight into the mechanism of ADAMTS13 function in vivo, which may shed more light on the pathogenesis of thrombotic thrombocytopenic purpura and other arterial thrombotic disorders associated with deficiency of plasma ADAMTS13 activity.

5.6 Chapter References


CHAPTER 6: SUMMARY, GENERAL DISCUSSION, AND FUTURE PLANS

6.1 Summary

The regulation of proteolytic cleavage of multimeric VWF by ADAMTS13 under physiological conditions remains an unanswered question in the field. It is currently unknown how ADAMTS13 preferentially cleaves UL-VWF under flow shear stress to generate sizes of VWF multimers that normally circulate in plasma, ranging from 500 kDa to 2,000 kDa. Several hypotheses have been proposed to explain this phenomenon: 1) modulation of cleavage by high fluid shear stress; 2) binding of platelets or protein cofactors to VWF; 3) the exosite interactions between VWF and ADAMTS13. Multimeric VWF is highly sensitive to shear stresses. At a critical shear stress of 35 ± 3.5 dynes/cm², VWF changes its conformation from globular to elongated in shape, which has been shown by atomic force microscopy. Therefore, it is conceivable that the larger the VWF multimers, the more sensitive they are to deformation by high shear stress. The work of this thesis has demonstrated that FVIII and platelets cooperatively accelerate proteolytic cleavage of soluble VWF by ADAMTS13 through an alteration of VWF substrate conformation under physiologically relevant fluid shear stresses. We have established the critical role of the VWF propeptide and FVIII- and platelet-binding domains of VWF in regulating proteolysis of VWF by ADAMTS13 under physiologically relevant shear stress using type 2N VWF mutants. Through site-directed mutagenesis, kinetic analyses, and peptide inhibition assays we have identified a
substrate-binding exosite encompassing ADAMTS13 residues Arg$^{659}$, Arg$^{660}$ and Tyr$^{661}$ that exhibits an important role in proteolytic cleavage of VWF under both non-physiological and physiological conditions. In addition, modification of an exosite binding region of ADAMTS13 yielded ADAMTS13 variants with reduced inhibition by autoantibodies and enhanced specific activity. Finally, we have demonstrated that infusion of an ADAMTS13 variant, truncated after the Spacer domain, can restore thrombus composition and the kinetics of platelet and fibrin accumulation in $Adams^{13-/-}$ mice. Together, these results suggest an important physiological role of cofactor binding to VWF and VWF interactions with ADAMTS13 exosites in regulating ADAMTS13 function in hemostasis, in addition to shear-induced unfolding of VWF.

6.2 General Discussion

6.2.1 VWF binding to cofactors, and subsequent increased susceptibility to ADAMTS13 cleavage, is an autoregulatory mechanism

The results presented in Chapter 2 and Chapter 3 suggest that binding of FVIII or platelets to VWF may facilitate 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. FVIII and platelets, thus, serve as physiological cofactors for VWF proteolysis by ADAMTS13, whose purpose is to lower the free energy required for conversion of substrate (multimeric VWF) to product (cleaved VWF) (Figure 6.1). Together, these findings, both from the investigation of the mutant cofactor (i.e. FVIII) and the mutant substrate (i.e.
VWF), underscore the critical role of FVIII-VWF interactions in regulating proteolysis of VWF under physiologically relevant conditions.

Figure 6.1. Schematic showing the cofactor effect of FVIII and platelets in VWF proteolysis by ADAMTS13. FVIII and platelets serve as physiological cofactors for VWF proteolysis by ADAMTS13, whose purpose is to lower the free energy required for conversion of substrate (multimeric VWF) to product (cleaved VWF). The reaction coordinate to the right indicates that the large free energy barrier shown can be overcome by using cofactors. FVIII and platelets overcome this free energy barrier by mechanical means, altering VWF conformation, making the Tyr$^{1665}$-Met$^{1606}$ cleavage bond more easily accessible by ADAMTS13.

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 GP1b$\alpha$ receptor and the VWF-A1 domain. However, 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, approximately 0.5-1 nM\textsuperscript{11,12}, whereas plasma VWF (subunit) concentration is approximately 50 nM\textsuperscript{12}, suggesting that approximately one FVIII is bound to every 50 VWF subunits (equal to ½ to one UL-VWF multimer). Similarly, less than 3 platelets may be bound to every 1,000 VWF subunits\textsuperscript{12}. 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\textsuperscript{13}.

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 dynes/cm\textsuperscript{2} shear force, the predicted peak force soluble VWF multimers experience ranges from 0.2 to 0.8 pN\textsuperscript{14} in the absence of platelets. The peak force increases to ~390 pN when two or more platelets are bound at either side of the scissile bond\textsuperscript{14}. This is the amount of shear stress if applied directly to a VWF fragment such as the A1A2A3 or A2 domain in which both ends are attached to a solid surface\textsuperscript{10,14}.

To investigate how the FVIII level in plasma modulates the size of VWF multimers, our lab has performed some studies to reconstitute plasma FVIII levels in fVIII\textsuperscript{-/-} mice\textsuperscript{15}. The results showed that the reconstitution of plasma FVIII by overexpressing human FVIII abrogates a massive accumulation of high molecular weight VWF multimers released from endothelial cells triggered by hydrodynamic injection. Accordingly, increased VWF antigen level in hydrodynamic injection groups drop to the basal level similar to that in
$fVIII^{+/−}$ mice without hydrodynamic injection\textsuperscript{15}. These findings further suggest that binding of FVIII to VWF might alter the rate of processing of VWF by ADAMTS13. However, it is also of importance to note that there is a minor discrepancy between the biochemical observations presented in this thesis and the aforementioned animal studies. In the \textit{in vitro} studies, the presence of FVIII leads to a selective removal of the larger multimers of VWF that coincides with the increase of the smaller ones. The diminished high molecular weight VWF multimers are due to ADAMTS13-mediated proteolysis. However, the animal study shows the disappearance of the accumulated high molecular weight VWF multimers in plasma by hydrodynamic injection of FVIII do not correspondingly increase the smaller multimers\textsuperscript{15}. It is speculated that the removal of high molecular weight VWF multimers is caused either by an enhanced proteolytic cleavage by ADAMTS13 and/or by an increase the clearance of the high molecular weight VWF multimers. This peculiar occurrence underscores the dichotomy that exists between FVIII and VWF when they are bound together: on one hand, FVIII stability in plasma is contingent upon binding to VWF, while on the other hand, VWF is protected from clearance, and even conformationally altered, in the presence of FVIII. Given that one FVIII molecule binds per every ~50 VWF subunits, there remains a perplexing issue regarding how FVIII can cause such a drastic conformational change to the A2 domains of multiple VWF monomers in a multimer.
6.2.2 The collection of data about ADAMTS13 exosites informs the role of shear stress in the dynamic interactions taking place between ADAMTS13 and VWF

The results of Chapter 4 indicate an identified substrate exosite in Spa. These exosites work by effectively shifting the equilibrium of a reaction towards product conversion by utilizing long-range interactions with the substrate (Figure 6.2). This work adds to the wealth of knowledge about exosites in Spa, Cys, and Dis that have been previously identified. *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. Such a globular VWF is neither active for ligand binding (such as interacting with platelet GP1bα) nor sensitive to ADAMTS13 proteolysis, 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. 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. High fluid shear stress results in conformational changes of VWF multimers and opens up the binding sites and cleavage bond in the central A2 domain normally buried in the hydrophobic core of the native VWF-A2 domain (Figure 6.3).
Figure 6.2. Schematic showing the substrate exosite effect on enzymatic activity, as seen with ADAMTS13 and VWF. Interactions between VWF and several ADAMTS13 exosites—substrate binding sites distant from the active site—enhance protease activity. This exosite binding, indicated in the schematic as P1-S1, P2-S2, and P3-S3, supports the main substrate interaction with the enzyme active site. This serves to shift the equilibrium to product formation because the substrate can engage the active site more intimately. Several ADAMTS13 domains interact with distinct sequences on an extended segment of the VWF-A2 domain. Each of these interactions is relatively weak, but together they cooperate to enhance ADAMTS13 substrate specificity, which is critical for hemostasis.
Figure 6.3. Proposed model for *in vivo* cleavage of VWF by ADAMTS13. Studies have shown that 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. High fluid shear stress results in conformational changes of VWF multimers and opens up the binding sites and cleavage bond in the central A2 domain normally buried in the hydrophobic core of the native VWF-A2 domain. More distinct exosite interactions occur with the Cys-rich, Spa, and Dis domains that ultimately govern the specificity of ADAMTS13 for VWF and orient the Met active site proximal to the Tyr1605-Met1606 cleavage bond. This results in efficient cleavage of VWF by ADAMTS13 only at sites where VWF has an optimal conformation (likely at the vessel walls).

6.2.3 The co-existence of ADAMTS13 substrate exosites and inhibitory autoantibody binding sites suggests a flexibility in ADAMTS13 structure that has been utilized by nature

In Chapter 4, it was shown through peptide inhibition studies and molecular modeling that amino acid residues Arg$^{659}$, Arg$^{660}$, and Tyr$^{661}$ (or RRY) play an important role in
proteolytic cleavage of various substrates under both non-physiological and physiological conditions. This site, which is commonly thought to be a major antigenic epitope for the majority of TTP patient autoantibodies targeting the Cys-rich and Spa domains, is very surface-exposed. It follows that this will likely be a site of binding to VWF because these residues, which reside on a loop, appear to readily interact with the α6 helix of the VWF A2 domain, as shown by modeling studies (Figure 6.4). It still remains perplexing, however, how this region can be a major antigenic epitope, given the strong interaction between ADAMTS13 and VWF. Inhibition studies have suggested that the inhibiting activity of these autoantibodies may not be entirely competitive. It becomes much more imperative that, instead of defining epitope regions for autoantibody binding, that we discover, at an amino acid level, the critical interactions that take place between the antibodies and ADAMTS13 to gain insight into whether there is a unique type of allostery taking place when there is a putative ADAMTS13-VWF-antibody complex that forms.
Figure 6.4. Model of ADAMTS13 MDTCS showing surface-exposed exosites. Adapted from Akiyama et al. MDTC was built from the existing DTCS crystal structure docked onto a homology-modeled ADAMTS13 metalloprotease domain, based on the structures of ADAMTS1 (PDB: 2V4B), ADAMTS4 (PDB: 3B2Z), and ADAMTS5 (PDB: 3B8Z). Sequence alignment (Panel A) indicates sequence identities of 22.8% (ADAMTS1), 23.5% (ADAMTS4) and 22.0% (ADAMTS5). MODELLER v 9.11, run by the UCSF Chimera GUI (Graphical User Interface) was used to generate the initial model. Dead-end elimination was used to optimize orientation of all side chains and polar hydrogens, and optimized bond geometry by energy minimization with GROMACS v 4.5.5, using the GROMOS96 43a1 force field. The model of MDTCS (Panel B) indicates the ADAMTS13 exosites identified to date, showing that they are all surface-exposed. The exosite containing Arg$_{659}$, Arg$_{660}$ and Tyr$_{661}$ is shown in purple.
In addition, **Chapter 4** showed that modification of an exosite in Spa generated ADAMTS13 variants with reduced inhibition by anti-ADAMTS13 IgGs while preserving or enhancing specific activity. Molecular modeling using the existing crystal structure of ADAMTS13-DTCS fragment and VWF-A2 domain indicated a shift in hydrophobicity and subsequent opening of the VWF-A2 binding pocket on the ADAMTS13 spacer domain, potentially resulting in increased hydrophobic interactions in the pocket and hydrogen bonding on the exterior of the pocket. Despite the polyclonal nature of anti-ADAMTS13 IgGs, the inhibitory activity appears to be largely mediated through binding to Spa, as the mutations in this region dramatically reduced binding and inhibition by patients’ autoantibodies. Although it was not previously noted in the Chapter, a level of concern has been raised about the exact nature of the gain-of-function mutants’ ability to accelerate VWF proteolysis. Preliminary binding studies have shown that the binding of these ADAMTS13 variants (particularly M4 and M5) is almost unchanged compared to wild-type ADAMTS13, which directly contradicts the modeling analysis mentioned above. I reason that modification of exosites in Spa can have effects on other exosites in the region to alter the specificity of binding altogether. This has potential usefulness when trying to intelligently design gain-of-function enzymes using the same approach with other potential exosites. Indeed, if we can decipher the exact relationship between changes in exosites proximal to each other, we could embark upon a process of *de novo* design, which would allow for us to evade a specific panel of patient antibodies.

Furthermore, the crystallization, screening, data collection, and structure determination presented in **Chapter 4** for an autolysis product of ADAMTS13, the ADAMTS13-CS fragment, provides the basis for beginning crystallization studies of complexes to probe
amino acid specificity of ADAMTS13 exosite binding with VWF and autoantibody recognition. The present structure suggests a linear correspondence between the ADAMTS13 domains and their interaction sites in the A2 domain of VWF. We suggest that Spa contains an exosite that primarily determines catalytic efficiency by interacting with α6 of the VWF A2 domain. Other studies have identified 3 other VWF segments that interact with the MD, T1, and C domains of ADAMTS13, suggesting that the catalytic cleft, plus exosite binding sites, make cooperative, modular contacts along the length of VWF. Furthermore, the M domains of ADAMTS4 and ADAMTS5 do not retain specific catalytic activity. The inclusion of the proximal C-terminal domains enhances their aggrecanase activity, suggesting that these ADAMTSs function through multiple exosites, as observed in the ADAMTS13-VWF system. It is, thus, reasonable to conclude that, much like VWF structure in the presence of varying environments of shear stress, ADAMTS13 can adopt multiple configurations to accommodate a multitude of potential substrate conformations.

6.2.4 The observed autolysis of ADAMTS13 may point to other autoregulatory functions of ADAMTS13

Our efforts to crystallize full-length ADAMTS13 in Chapter 4 yielded a high resolution structure of the ADAMTS13-CS fragment, presumably a product of autolysis under the crystallization conditions. The nature of this autolysis, which is reproducible under these conditions, is unknown. The lack of substantial reports on autolysis by Met presents the possibility for an intriguing and potentially very informative set of experiments. The autolysis appears to occur under conditions of low pH and high ionic strength.
It is well known that intense exercise can result in acidosis, or low pH. The propensity of ADAMTS13 to undergo autolysis at low pH may result in a transient decrease in active enzyme immediately following exercise. It has been shown that habitual physical exercise is associated with an overall decreased risk of acute heart disease. However, intense exercise may trigger acute myocardial infarction\(^\text{20}\). A number of studies have shown that strenuous physical exercise resulted in an activation of the hemostatic system\(^\text{20}\). One study, using human volunteers, investigated the effect of moderate and strenuous exercise on experimental arterial thrombus formation in men\(^\text{21}\). Thrombogenesis was measured in 15 sedentary healthy male volunteers at rest or immediately after exercise tests. The results showed that moderate exercise did not affect arterial thrombus formation. However, exercise of heavy intensity (conditions under which acidosis is likely to occur) was shown to increase the risk for arterial thrombosis\(^\text{21}\). These findings, combined with the potential of ADAMTS13 to engage in autolysis at low pH and high ionic strength conditions, may be able to shed light on explaining the potential susceptibility to arterial thrombosis with heavy exercise.

6.2.5 The in vivo correction of platelet and fibrin accumulation with infused ADAMTS13 suggests the importance of ADAMTS13 localization

The data presented in Chapter 5, along with published data from Xiao et. al.\(^\text{22}\), demonstrate the direct correlation between anti-arterial thrombotic activity of ADAMTS13 (or variants) and its VWF-cleavage activity under fluid shear stress, suggesting the critical role of ADAMTS13-mediated VWF proteolysis in modulation of arterial thrombosis. With growing interest in the therapeutic potential of recombinant
ADAMTS13\textsuperscript{23} or gene transfer\textsuperscript{24} in TTP and many other diseases, the findings showing the change in thrombus morphology and composition may provide the molecular basis for rational design of therapeutic targets.

The most rational thought when considering these results is whether determining the kinetics of platelet and fibrin accumulation from the head to the tail of the thrombus is a fruitful exercise. I contend that this knowledge is essential to the hypothesis, given the nature of the carotid artery occlusion model. Presumably, the FeCl\textsubscript{3} model derives its usefulness from the ability to cause a targeted mechanical injury to a specific site in the vessel. This allows for the spread of a thrombus from this site of injury, effectively creating a gradient of VWF, fibrin, and platelet concentrations. By virtue of the fact that ADAMTS13 is expressed in regions specifically where it is needed, it is reasonable that the gradient of ADAMTS13 across a thrombus moves more slowly than the gradient of VWF, effectively allowing for the accumulation of platelets without allowing uncontrolled aggregation. It is, thus, important to study the differential kinetics of platelet and fibrin accumulation to ensure that exogenously-expressed ADAMTS13 follows a similar gradient to the endogenous ADAMTS13. Whereas our current experiments have indicated that this may be an issue\textsuperscript{22}, we must endeavor to create ways to specifically target the delivered ADAMTS13, whether through ectopic expression in platelets, or by addition of chemical moieties to target the enzyme to platelet surface glycoproteins.
6.3 Future Plans

6.3.1 Improvement of methods to determine kinetics of VWF proteolysis

A major limitation of the Western blot assay to quantify VWF cleavage by ADAMTS13 is that detection of the 350 kDa dimer requires simultaneous cleavage of scissile bonds in adjacent subunits. However, only one cleavage event is required to decrease multimer size. The fractional saturation of VWF with FVIII or platelets in our reactions is likely small, thus a “double cleavage” event is unlikely, and we are likely underestimating the true magnitude of the rate of proteolysis by ADAMTS13. Consumption of multimers to lower molecular weight species requires a single cut, while accumulation of monomer largely requires two or more depolymerization events. It would be prudent to develop an assay that can detect single cleavage events to establish the magnitude of the rate enhancing effect of cofactors on VWF proteolysis and to provide a more meaningful measurement of ADAMTS13 function.

Our novel ELISA-based assay presented in Chapter 3 detects the N-terminal peptide (i.e. EQAPNVY) resulting from cleavage after the Tyr residue occurring anywhere along a VWF multimer, which dramatically increases the assay sensitivity. Furthermore, the assay is highly specific for detecting the ADAMTS13 cleavage product, but not from the cleavage by other leukocyte proteases such as cathepsin G, proteinase 3, and elastase. Although this assay presents a potentially useful assay, it is limited in its applicability because of the potential interference of the assay by platelet cofactors. We, thus, have endeavored to design an even better assay to probe VWF cleavage by ADAMTS13.
Multimeric VWF contains free thiols at both amino terminal (D’D3 domain) and carboxyl-terminal (C domain) ends. The perceived experiment is that one end of the VWF may be attached to a magnetic amine-derivatized beads and the other end of VWF may be attached to maleimide-biotin. This is possible because the estimated free thiols are much less than the number of protomers within a multimer. By separating the magnetic bead-labeled VWF and detecting the cleaved biotin-labeled VWF, this strategy could simplify detection and provide more sensitive quantitative estimation of single VWF cleavage events by ADAMTS13. The thiols would be labeled with magnetic amine-derivatized beads using the compound N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), an amine reactive N-hydroxysuccinimide (NHS) ester that reacts with amine groups to form an amide bond. The spacer arm of SPDP contains a pyridyl disulfide group that will react with sulfhydryls to form a reversible disulfide bond with the protein of interest. The remaining sulfhydryl group of VWF would then be labeled with biotin via its maleimide (NHS) group. The cleavage products, which would consist of various lengths of VWF can be detected by an ELISA format using a streptavidin-coated microtiter plate to capture the cleaved VWF fragments.

The same approach to find potential exosites in Spa, described in Chapter 4, can be used to find potential exosites in other areas of ADAMTS13, particularly in the distal C-terminal domains. These are particularly interesting because 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. It is reasonable to hypothesize that this initial contact serves as the “initial engagement” of VWF by ADAMTS13, and regulates VWF susceptibility to ADAMTS13 binding under the
application of further shear stress. Sequence alignments of the human ADAMTS13 TSP-1 repeats (Figure 6.5) or the CUB domains (Figure 6.6) of various species indicate a similar pattern of sequence conservation that was observed with the identified Spa exosites. Identification of exosites in these distal C-terminal domains could provide information about differential specificity of ADAMTS13 for VWF under different shear environments.

Figure 6.5. Sequence alignment of human ADAMTS13 TSP-1 repeats. An amino acid sequence alignment of human ADAMTS13 TSP-1 repeats indicates a consensus conserved region from residues 11 to 16 (CSVSCG) which may play an important role in initial substrate recognition.

Figure 6.6. Sequence alignment of ADAMTS13 CUB domains from various species. An amino acid sequence alignment of ADAMTS13 CUB domains from various species indicates a consensus conserved region from residues 1215 to 1220 (AIGRPL) which may play an important role in initial substrate recognition.
6.3.2 Deciphering the relevance of ADAMTS13 autolysis

In Chapter 4, the presumed autolytic activity of ADAMTS13 leaves room for many interesting questions. To probe the effects of various solution conditions on the proposed autolysis of ADAMTS13, we can set up conditions to screen the effect of pH, ionic strength, and hydrophobicity on ADAMTS13 autolysis. Although the determined boundaries of the solved structure were found to be from $M^{432}$ (in TSP-1) to $R^{670}$ (in Spa), we would also need to determine specific cleavage products resulting from ADAMTS13 autolysis. Perhaps the required fragment is the most abundant product, resulting in it consistently being the fragment that was able to crystallize. Preliminary identification of the cleavage products by western blotting, followed by detailed analysis of the exact cleavage products generated by mass spectrometry, can help us uncover this potentially exciting phenomenon.

6.3.3 Achieving amino acid resolution of ADAMTS13 interactions

In light of the importance of ADAMTS13 exosite binding presented in Chapter 4, along with the established importance of exosites in Spa for inhibitory autoantibody recognition, we can use our knowledge of crystallization of the ADAMTS13-CS fragment to undergo crystallization trials of ADAMTS13-CS bound to inhibitory antibodies. Our lab has isolated a repertoire of over 200 clones encoding single-chain fragments of variable region (scFV) by panning phage display libraries against full-length ADAMTS13 and a C-terminal fragment comprising of TSP1 5-8 and the CUB domains. These monoclonal scFV(s) would be expressed in E. coli and purified, and the binding
epitopes of these monoclonal antibodies can be determined by crystallization of the antibodies in complex with ADAMTS13-CS.

6.3.4 Rational design of ADAMTS13 for therapeutic purposes

Ultimately, the information from these studies can be used to inform an intelligent design of ADAMTS13 that fulfills a core set of qualifications: 1) minimal construction, to allow for optimal delivery as a therapeutic; 2) sufficient activity, to allow for small amounts of the therapeutic to be delivered; 3) targetability, to allow for the addition of chemical moieties to target the enzyme to platelet or endothelial surface receptors; and 4) immune evasion, to allow the enzyme to be active, even in the presence of inhibitory antibodies. Distinct structural information about the ADAMTS13-VWF interface and the ADAMTS13-autoantibody interface can different approaches to protein design. I propose that a directed evolution approach, using a library of exosite mutants, can be used to initially identify desirable candidates. This can be followed by a chimeric approach, which would allow us to combine, delete, and replace domains in the enzyme candidates to design a minimal, effective enzyme. Using this enzyme candidate as the model, we can then develop de novo design techniques based on patient autoantibody screening. Once we know the profile of the patient antibodies, we can design enzymes that have perturbed or entirely missing epitopes. This methodology presents a new and exciting direction for research on ADAMTS13, which combines our studies of the basic biochemistry and structural biology of ADAMTS13 with protein engineering to develop a potentially useful therapeutic.
6.4 Conclusions

The work presented in this thesis begins to tie together the complex nature of the association between VWF and ADAMTS13, which is controlled at multiple levels by mechanical, chemical, and allosteric factors. The ability of VWF to sense all of these contributions and allow itself to be optimally presented to ADAMTS13 is nothing short of fascinating. Shear stress is highest at the walls, where VWF can be optimally “stretched” to allow exposure of the ADAMTS13 cleavage bond. FVIII and platelet binding to VWF also occurs optimally where the lowest velocity occurs (at the vessel walls), further underscoring the importance of VWF to maintenance of hemostasis at the endothelium. Recruitment of FVIII and platelets by VWF not only allows for enhancement of ADAMTS13 activity, but it also allows for recruitment of clotting factors to the specific areas where they are needed. Thus, it becomes clearer why FVIII and platelets interact with VWF at such low densities—providing a restraint to the inherent feedback mechanisms of the coagulation cascade.

The data presented in this thesis, along with data from Gao et. al.⁴, seem to suggest that substrate exosite binding between ADAMTS13 exosites in Spa and VWF-A2 are, to some extent, modular and portable. Efficient substrate cleavage, which in a typical system would likely be tightly regulated, does not depend on a fixed spacing between the scissile bond and distal residues that bind the Cys-rich and Spa domains. This has important consequences that arise from the ability of VWF to undergo many different conformational changes in the presence of varying amounts of shear stress and in different vessels. The ability to accommodate a large change in substrate structure
suggests that ADAMTS13 is a particularly flexible enzyme, and can alter the relationships among the various non-catalytic domains that bind VWF. It is likely that fluid shear stress introduces a variety of conformational changes in VWF rather than one well-defined substrate structure. In that case, stretched VWF multimers may expose a varying number of binding sites for ADAMTS13. The presence of multiple exosites in multiple domains becomes particularly important because of the potential heterogeneity in VWF conformations.

This principle has particular usefulness when thinking about autoantibody binding of ADAMTS13. The large amount of VWF conformations available to ADAMTS13 may leave, at any particular time, some exosites that are unoccupied by VWF. These exosite regions, which are particularly surface-exposed and easily accessible by antibody variable fragment epitope recognition sites, likely have been targeted by these pathogenic antibodies. The very nature of VWF and blood vessel shear stress may be able to explain the effectiveness and somewhat odd inhibitory capacity of acquired TTP patient autoantibodies.

Overall, the study of the combination of mechanical, chemical, and allosteric effects on VWF conformation and, subsequently, ADAMTS13 binding provides a massive contribution to the field of thrombosis research. This work serves as the foundation upon which we can study how proteins, in general, are not merely a product of specific interactions, but are controlled by many outside forces. With this knowledge, we can begin to decipher how nature can take advantage of this ability to modulate protein environments to allow optimal localization of proteins.
6.4 Chapter References


