Published in final edited form as:

J Thromb Haemost. 2008 July ; 6(7): 1135–1143. doi:10.1111/j.1538-7836.2008.02991.x.

COVALENT REGULATION OF ULVWF STRING FORMATION AND ELONGATION ON ENDOTHELIAL CELLS UNDER FLOW CONDITIONS†

Ying Li¹, Hiuwan Choi¹, Zhou Zhou¹, Leticia Nolasco³, Henry J. Pownall², Jan Voorberg⁴, Joel L. Moake^{1,3}, and Jing-fei Dong^{1,*}

¹Section of Thrombosis Research, Department of Medicine, Baylor College of Medicine, Houston, Texas, USA ²Section of Atherosclerosis and vascular disease, Department of Medicine, Baylor College of Medicine, Houston, Texas, USA ³Department of Bioengineering, Rice University, Houston, Texas, USA ⁴Department of Plasma Proteins, Sanquin-AMC Landsteiner Laboratory, Amsterdam, the Netherlands

Abstract

Background and Objectives: The adhesion ligand von Willebrand factor (VWF) is a multimeric glycoprotein that mediates platelet adhesion to exposed subendothelium. On endothelial cells, freshly released ultra-large (UL)VWF multimers form long string-like structures to which platelets adhere.

Methods: The formation and elongation of ULVWF strings were studied in the presence of the thiol-blocking N-ethylmaleimide (NEM). The presence of thiols in ULVWF and plasma VWF multimers was determined by maleimide-PEO₂-Biotin labeling and thiol-chromatography. Finally, covalent re-multimerization of (UL)VWF was examined in a cell- and enzyme-free system.

Results: We found that purified plasma VWF multimers (but significantly less efficient when thiols are blocked) adhere to and elongate ULVWF strings under flow conditions. The formation and propagation of ULVWF strings were dose-dependently reduced by blocking thiols on VWF with NEM, indicating that ULVWF strings are formed by the covalent association of perfused VWF to ULVWF anchored to endothelial cells. The association is made possible by the presence of free thiols in VWF multimers and by the ability of (UL)VWF to covalently re-multimerize.

Conclusion: The data provide a mechanism by which the thrombogenic ULVWF strings are formed and elongated on endothelial cells. This mechanism suggests that the thiol-disulfide state of ULVWF regulates the adhesion properties of strings on endothelial cells.

INTRODUCTION

The adhesion ligand von Willebrand factor (VWF) is synthesized in endothelial cells as monomers that first form disulfide-linked dimers through the C-terminal cysteine residues (1;2). Subsequently, an unknown number of dimers forms disulfide-linked multimers via the N-terminal cysteine residues (3-5). Once synthesized, VWF multimers are either constitutively secreted or targeted to the storage granules Weibel-Palade bodies (6). It has

†This work was supported by NIH grants HL71895 and HL30914 and the Mary R. Gibson Foundation. J.F.D is an Established Investigator of the American Heart Association

*Address correspondence to this author at: Thrombosis Research Section, Department of Medicine, BCM286, N1319, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Telephone: 713-798-1078; FAX: 713-798-1384; e-mail: jfdong@bcm.tmc.edu

been suggested that VWF multimers secretion through the constitutive pathway are relatively small and hemostatically less active. In contrast, VWF multimers in storage granules are rich in the ultra-large (UL) forms (7;8) that are very active, capable of spontaneously agglutinating platelets (9;10). Following vascular injury, ULVWF multimers may be released as a defensive measure to prevent excessive bleeding. However, their presence could also result in devastating thrombosis when ULVWF multimers are secreted from inflamed endothelium and/or when it is inadequately cleaved, such as in the case of thrombotic thrombocytopenic purpura (11).

Upon release, ULVWF multimers are rapidly, but partially cleaved by the metalloprotease ADAMTS-13 to smaller forms that circulate in blood. The cleavage converts the hyperactive and prothrombotic ULVWF, which can spontaneously bind platelets with a high affinity (9;10), to smaller multimers that remain hemostatically active, but are no longer prothrombotic. A key question is: What structure-function relationship underlies the difference between ULVWF multimers and their plasma counterparts? A simple explanation may be that a ULVWF multimer has more GP Ib-binding sites than plasma VWF. If this is the case, the primary difference should be in the avidity of GP Ib-VWF interaction. However, ULVWF forms high strength bonds with GP Ib spontaneously and at the single bond level (9), whereas plasma VWF binds GP Ib only in the presence of either high fluid shear stress or modulators such as ristocetin. Ristocetin-induced VWF-GP Ib interaction shares many characteristics with shear-induced binding (12). Together, these data suggest that ULVWF and plasma VWF adopt different conformations that make the former constitutively active and the latter inactive, but can be activated by modulator- or fluid shear stress-induced structural changes. Such a structural change has indeed been demonstrated as VWF multimers transit from a globular to an elongated rope-like structure upon exposure to shear stress (13), but the biochemical base for the structural change is not known. We have recently reported that VWF multimers purified from normal plasma contain surface exposed thiols in the D3 and C domains that form disulfide bonds upon exposure to pathological high shear stress (14). This shear-induced thiol-disulfide exchange enhances VWF binding to platelets, suggesting that the shear-induced thiol-disulfide exchange activates plasma VWF.

In contrast to plasma VWF, ULVWF multimers are released in active forms that are rapidly assembled to string-like structures on endothelial cells under flowing conditions (15;16). These ULVWF strings are extremely long, unlikely to have derived from a single Weibel-Palade body and are linked together by adherent platelets (15;17). Platelets rapidly tether and adhere to these strings, leading to eventual formation of platelet-VWF clumps. How the strings are formed is unknown, but the fact that they form progressively over time to eventually become visible by conventional light microscopy (16) suggests that it involves linear elongation as well as lateral association that “stacks” multimers into thick fibrillar structures. The unique morphology of ULVWF strings and the role of thiol-disulfide exchange in activating plasma VWF led us to hypothesize that ULVWF strings are formed by covalent lateral and linear association of (UL)VWF multimers. We tested this hypothesis by determining how the formation of ULVWF strings on endothelial cells is promoted and regulated by covalent means.

MATERIAL AND METHODS

Production and purification of plasma VWF and ULVWF

Plasma VWF was purified from human cryoprecipitate (Gulf coast regional blood bank, Houston, TX) as previously described (14;18). VWF antigen level and multimer pattern were determined by a sandwich ELISA (Ramco Laboratories, Houston TX) and 1% SDS-agarose gel electrophoresis followed by the Western blot with a polyclonal VWF-antibody (Bethyl Laboratories, Montgomery, Texas), respectively.

To obtain a VWF variant with the A2 domain replaced by the green fluorescence protein (GFP), cDNA for the GFP-VWF chimera (19) was transfected into HEK 293 cells (ATCC, Manassas, VA) using lipids as carriers. Cells stably expressing the chimera were selected by growing them in DMEM medium containing 10% fetal calf serum and the selection drug Geneticin (final concentration 500 µg/ml, Invitrogen, Carlsbad, CA). To harvest GFP-VWF, confluent cells were washed with phosphate buffered saline (PBS) and then incubated with the serum-free medium (Opti-Pro SFM, Invitrogen) for 48 hrs. Cell supernatant was collected and the chimera purified through a sepharose 4B gel filtration column (PD-10, Amersham Bioscience, Uppsala, Sweden). Fractions from the void volume (fractions 4-6, 1 ml/fraction) were collected and the yield was determined by ELISA.

ULVWF multimers were produced from culture of human umbilical cord vein endothelial cells (HUVECs) as described previously (9;18). The use of human umbilical cords was approved by the Institutional Review Board of Baylor College of Medicine. In order to induce ULVWF secretion, confluent cells were stimulated with 100 µM of histamine in serum-free M199 medium containing 10 µg/ml of insulin, 5 µg/ml of transferrin, 1% of L-glutamine and 3% PSN for 30 min at 37°C. The activated endothelial cells were washed repeatedly and forcefully to release membrane-anchored ULVWF. The conditioned medium was then collected and centrifuged at $950 \times g$ for 10 min to remove cell debris. The VWF level and multimeric composition of ULVWF in supernatants were determined by 1% SDS-agarose gel electrophoresis and immunoblots using a polyclonal VWF antibody (Bethyl Laboratories).

Preparation of antibody coated polystyrene beads

Two types of beads were prepared and used to mark ULVWF strings in real-time under flowing conditions. First, fluoresbrite plain YG microspheres (Polysciences Inc. Warrington, PA) were washed with 1 ml of borate buffer (0.1 M boric acid, pH 8.5) and then centrifuged for 5 min at $10,000 \times g$ at room temperature. The bead pellet was suspended in 0.1 ml of borate buffer and incubated with a goat-anti-GFP antibody (40 µg, Abcam, Cambridge, MA) overnight at room temperature under gentle rotation. At the end of incubation, the coated beads were centrifuged at $3000 \times g$ for 10 minutes and then incubated with 1% of bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO) for 30 min at room temperature to block non-specific binding sites. The beads were then washed with and suspended in PBS containing 10 mg/ml BSA, 0.1% NaN₃, and 5% glycerol to a final density of 5.68×10^8 /ml beads and stored at 4°C until used. Second, the same technique was also used to coat non-fluorescent beads (Polybead® Microspheres, Polysciences Inc.) with a polyclonal VWF antibody (DAKO Corp, Carpinteria, CA). The beads coated with BSA were identically prepared and used as controls.

Endothelial cell culture and parallel-plate flow chamber system

To induce ULVWF strings to form on activated endothelial cells, HUVECs were placed in 3.5 cm culture dishes coated with 1% gelatin and maintained in Medium 199 (Invitrogen) containing 10% heat-inactivated fetal calf serum and 0.2 mM of L-glutamine until confluent. Before experiments, cultured endothelial cells were washed with PBS and incubated with 20 µM of histamine (Sigma-Aldrich) for 3 min at room temperature. The dish with endothelial cells was assembled to form the bottom of the parallel-plate flow chamber and loaded onto a Nikon Eclipse TE300 inverted stage microscope (Garden City, NY) as previously described (15). The activated endothelial cells were perfused with washed platelets and various concentrations of VWF multimers purified from human plasma cryoprecipitate (or GFP-VWF) suspended in Ca⁺⁺, Mg⁺⁺-free Tyrode's buffer (138 mM sodium chloride, 5.5 mM glucose, 12 mM sodium bicarbonate, 2.9 mM potassium chloride, and 0.36 mM sodium phosphate dibasic, pH 7.4) for 3-12 min at a flow rate of 0.2 ml/min (calculated wall shear

stress of 2.5 dyn/cm² with approximately 1 cp of viscosity). The formation of ULVWF strings was recorded by a digital camera and quantified by counting the numbers of ULVWF strings in 20 continuous review fields ($\times 200$). In the case of branched ULVWF string networks, each network was counted as a single string for quantification purpose. In a subgroup of experiments, plasma VWF multimers were first treated with 100 μ M of maleimide-PEO₂-Biotin (MPB, Pierce Biotechnology, Rockford, IL), which blocks thiols, for 13 min at room temperature and then dialyzed against 1 L of Tyrode's buffer overnight to remove free MPB. The MPB treated plasma VWF multimers were then perfused.

To determine the effect of blocking thiols on the formation of ULVWF strings, histamine-stimulated HUVECs were perfused at a flow rate of 0.2 ml/min for 3 min with various concentrations of N-ethylmaleimide (NEM from Sigma and dissolved in complete Tyrode's buffer), which reacts with and irreversibly blocks thiols in VWF multimers. Unbound NEM was then removed by perfusing Ca⁺⁺ and Mg⁺⁺- free Tyrode's buffer for additional 3 min followed by perfusion of washed or lyophilized platelets (Biodata Corp, Horsham, PA) or polystyrene beads coated with VWF antibody for 3 min. The numbers of ULVWF strings formed were counted after 3 min perfusion. Acquired images were analyzed offline using MetaMorph software (Universal Images, West Chester, PA). Because NEM is membrane permeable, it could potentially affect the intracellular multimerization of VWF monomers. To address this concern, experiments were repeated using MPB (Pierce Biotechnology), which is not membrane permeable.

To directly visualize adhesion of plasma VWF (or GFP-VWF) multimers to ULVWF strings, 100 μ g/ml of the chimeric GFP-VWF, 50 μ l of fluorescent anti-GFP beads, and 50 μ l of regular beads coated with a polyclonal VWF antibody were suspended in the complete Tyrode's buffer (a final volume of 1 ml) and perfused over ULVWF strings secreted from histamine-stimulated HUVECs for 3 min. Adhesion of both types of beads was detected and recorded in real-time under bright field and with a FITC filter to co-localize VWF-GFP to ULVWF strings.

Cell viability test

To determine whether NEM is toxic to HUVECs during perfusion, we conducted two sets of experiments to measure the viability of HUVECs after NEM treatment. First, cultured HUVECs were treated with 0.5 - 20 mM of NEM for 3 min (the perfusion time). After washing to remove free NEM, cells were examined for morphological changes associated with cyto-toxicity. Second, HUVECs detached by 0.1% trypsin (5 min at 37°C) were treated with NEM for 3 min and then diluted 300-fold with the culture medium. Cell suspension was centrifuged at 1200 $\times$ g for 10 min to remove NEM-containing medium. The cells resuspended in PBS were incubated with the cell viability dye 7-Amino-Actinomycin D (7-AAD, Total Cytotoxicity and Apoptosis Detection kit, ImmunoChemistry technology, Bloomington, NM) for 10 min at room temperature and analyzed on a Beckman-Coulter EPICS-XL flow cytometer.

Reduction and re-assembly of (UL)VWF multimers

ULVWF and plasma VWF multimers were incubated with reduced glutathione (GSH, Sigma Aldrich) at a final concentration of 200 μ M for 15 min at 37°C. After treatment, samples were sequentially diluted by Veronal buffered saline (28 mM Sodium 5,5-diethylbarbiturate, 125 mM NaCl, pH 7.4) to remove GSH. Aliquots from undiluted and diluted fractions were incubated with SDS sample buffer at 56°C for 15 min and then separated by SDS-1% agarose gel electrophoresis. (UL)VWF multimeric patterns were visualized by immunoblotting as previously described (11). Control samples were not treated with GSH, but underwent the same dilutions.

Detection of free thiols in ULVWF and purified plasma VWF

As previously described (14), purified plasma VWF and ULVWF multimers produced from activated HUVECs were incubated with 100 μ M of MPB (Pierce Biotechnology) at room temperature in phosphate-buffered saline (PBS). Maleimide reacts with the thiol group on a cysteine residue to form a stable carbon-sulfur bond. After 5 min incubation, the reaction was quenched by 200 μ M of GSH. The labeled ULVWF or purified plasma VWF multimers were then separated on 5% SDS-PAGE under reducing conditions and detected by immunoblotting using HRP-conjugated streptavidin (Pierce Biotechnology). The blots were then stripped and reprobed with a polyclonal VWF antibody to correlate the MPB labeling with ULVWF and plasma VWF multimers.

We have recently demonstrated that free thiols exist on the surface of plasma VWF and form disulfide bonds upon exposure to fluid shear stress (14). The same technology was used to determine whether ULVWF also contain the surface exposed free thiols. Briefly, 2 mg of thiopropyl activated thiol sepharoseTM 6B beads (GE Healthcare, Piscataway, NJ), a mixed disulfide formed between 2,2'-dipyridyl disulfide and glutathione coupled to CNBr-activated Sepharose 6B, were washed extensively with distilled water and suspended in 1 ml of the binding buffer (0.2M NaCl, 0.1M Tris-HCl, pH7.5) (20). The pore sizes (10,000 to 4,000,000) are irrelevant for the application because activated thiols are present in the pores and on bead surface. Washed beads were incubated with 1 μ g of ULVWF or VWF multimers purified from human plasma cryoprecipitate for 15 min at room temperature with constant rotation. The bead-bound ULVWF or plasma VWF was washed with binding buffer 3 times and then released from the beads with 20 mM of DTT. VWF eluted from the beads was separated on 5% SDS-PAGE and detected by immunoblotting with a polyclonal VWF antibody (DAKO Corp). The same technique was also used to detect free thiols in the recombinant GFP-VWF chimera.

Statistical Analysis

All experimental data are presented as mean $\pm$ SEM. The Student's *t* test was used for pair or group comparison and a *p* value of less than 0.05 was considered to be statistically significant.

RESULTS

Plasma VWF adhered and elongated ULVWF strings under flowing conditions

As shown in Figure 1, perfusion of plasma VWF significantly increased the numbers of strings on endothelial cells. Furthermore, the strings that formed were longer, with the average length (from three view-fields) increasing from 318.2 ± 16.4 μ m in the absence to 999.8 ± 128.8 μ m in the presence of soluble VWF (Mann-Whitney Rank Sum Test, *n* = 5, *p* < 0.05). However, increase in ULVWF string numbers was not observed when plasma VWF multimers were pretreated with 2 mM of NEM (unbound NEM removed by dialysis) before perfusion.

ULVWF strings with plasma VWF perfusion also had more branches, often forming string networks on endothelial cells (Figure 2A-D). To further determine whether ULVWF string networks are also formed when ADAMTS-13 is deficient (because ULVWF strings are not rapidly cleaved, but rather allowed to propagate), we also examined data previously generated by perfusing plasma samples from five patients with congenital or acquired TTP and determined that such string networks were also very frequent, found in 48% of images (two examples are provided as Figure 2E and F).

To visually verify if VWF multimers had indeed adhered to ULVWF strings, we perfused the chimeric GFP-VWF together with fluorescent beads coated with anti-GFP antibody and regular beads coated with anti-VWF antibody. The recombinant GFP-VWF expressed in 293 cells was captured by the thiol-active beads (Figure 3A), indicating that it contains thiols as VWF multimers purified from human cryoprecipitate do. The GFP antibody beads (but not beads coated with BSA) tethered to ULVWF strings marked by regular VWF antibody beads (Figure 3B & C), suggesting that soluble VWF multimers had indeed adhered to ULVWF strings. We used the fluorescent beads because GFP intrinsic fluorescence was too weak to be directly detected in real-time.

NEM blockage of ULVWF strings under flow conditions

To further determine if the formation of ULVWF strings involves covalent association of VWF multimers, we perfused the thiol-reactive agent NEM over histamine-stimulated HUVECs. NEM dose-dependently reduced the numbers of ULVWF strings formed on endothelial cells (Figure 4A). ULVWF strings formed after NEM treatment were also more rigid with less adherent platelets (Figure 4B). A similar effect was also observed by perfusion of 200 μ M of membrane impermeable MPB (data not shown), indicating that the results were not due to intracellular actions of NEM. The observed effect was not caused by NEM on platelets because it was observed when ULVWF strings were marked by lyophilized platelets (Figure 4A) or polystyrene beads coated with anti-VWF antibody (Figure 4C).

To address the concern that NEM, which is membrane permeable (21), impairs ULVWF release at high concentrations, we activated HUVECs in the presence 10 mM of NEM for 3 min. The levels of ULVWF antigen detected in the conditioned medium by ELISA were similar at 51.6 ± 26.1 and 57.8 ± 12.9 ng/ml (after the conditioned medium was concentrated 10 fold) for the NEM treated and untreated samples, respectively.

For the study, NEM was tested in a dose range of 0.5 – 10 mM, higher than studies conducted under static conditions (up to 1 mM), because of consideration that NEM may act less efficiently under flow conditions than in a static system with a longer incubation period. We considered this possibility because NEM in previous studies was used in significantly higher concentrations in experiments conducted under flow conditions (22;23) as compared to that in static assays (24;25). To determine whether NEM at the high concentration is cytotoxic to HUVEC during the 3 min perfusion period, cultured HUVECs were treated with up to 20 mM of NEM for 3 min and then examined for morphological changes and viability. As shown in Figure 5, cells remained morphologically intact (Figure 5A-C) and viable when treated with NEM up to 10 mM (Figure 5E). However, they underwent significant cell shrinkage and detachment with 20 mM of NEM (Figure 5D). Taken together, these data suggest that ULVWF strings can be formed and elongated through covalent lateral and linear association between perfused plasma VWF and anchored ULVWF multimers.

ULVWF multimers did not contain surface exposed thiols

Data described in the previous section suggest that the thiol-disulfide state of (UL)VWF multimers play a critical role in the formation and elongation of ULVWF strings under flowing conditions. This is consistent with our recent observation that VWF multimers either purified from cryoprecipitate or in plasma contain surface exposed thiols (14). Here, we found that MPB also labeled ULVWF multimers (Figure 6A). However, the activated thiol beads, which form mixed disulfides with surface exposed, but not buried, thiols due to the large bead sizes (± 90 μ m in diameter), failed to capture ULVWF (Figure 6B), suggesting that the thiols are not exposed on the surface of ULVWF quaternary structure.

Reversible de-assembly and re-multimerization of (UL)VWF

The data from the active thiol chromatography suggest a structural difference between the two forms of VWF and also demonstrate the ability of VWF to undergo changes in thiol-disulfide state. To further examine the reactivity of thiols in VWF multimers, we examined the rate of VWF re-multimerization in a cell- and enzyme-free environment. ULVWF freshly secreted from cultured HUVECs and plasma VWF were incubated with 200 μ M of GSH, which is a thiol active agent that reduces disulfide bonds by acting as an electron donor. Both forms of VWF multimers were rapidly, but only partially reduced, resulting in the loss of large and ultra-large multimers (Figure 7). However, as GSH was gradually reduced by sequential dilutions, both forms of VWF multimers fully re-multimerized to their original sizes and patterns without significant cross-over (*e.g.* plasma VWF re-multimerizes to ULVWF or *vice versa*). Treatment of ULVWF with Veronal buffered saline alone resulted in neither reduction nor re-multimerization.

DISCUSSION

We show that formation and elongation of ULVWF strings is dose-dependently blocked by the thiol reactive agent N-ethylmaleimide (Figure 4) and promoted by perfusing plasma VWF multimers in a thiol-dependent manner (Figure 1). The latter is directly visualized by perfusing GFP-VWF chimera over histamine-activated endothelial cells in real time (Figure 3). The observed effect is not caused by NEM blockage of intracellular VWF multimerization because the effect is also observed with membrane impermeable maleimide-PEO₂-Biotin. It is not mediated by GP Iba, which expresses a functionally critical thiol upon platelet activation (26-28), because it is observed without platelets (Figure 4C). Finally, it is also not due to NEM-related cytotoxicity during the short perfusion period (Figure 5). Taken together, these data suggest that the formation of ULVWF strings involves covalent lateral association as well as linear elongation of (UL)VWF multimers. The lateral association, which has previously been demonstrated between soluble and immobilized VWF multimers (29), allows (UL)VWF multimers to form fibrillar structures that can be reviewed under a regular light microscope without adherent platelets (16). The extensive covalent lateral and linear associations may also result in the formation of ULVWF string networks on endothelial cells (Figure 2, the branched ULVWF strings are also formed without soluble VWF, but at a much lower rate).

The results also indicate a potential thiol-disulfide exchange among anchored ULVWF and perfused soluble VWF multimers (intermultimer disulfide bonds). However, unlike VWF multimers purified from plasma (14), ULVWF newly released from cultured endothelial cells (never in contact with plasma) contain thiols that are “buried” in the quaternary structures (Figure 6) and may become exposed when anchored ULVWF strings are stretched by fluid shear stress and the tensile/torque forces on adherent platelets. Shear stress has indeed been previously shown to induce significant conformational changes (13) and to promote thiol-disulfide exchange of VWF multimers (14). It remains unclear as to whether the thiol-disulfide exchange is spontaneous or promoted by redox molecules such as protein disulfide isomerase (PDI), the intrinsic PDI-like activity associated with the VWF propeptide (30), and small redox molecules (such as GSH and GSSG) associated with endothelial cells (31;32).

The finding that thiols are exposed on the surface of plasma VWF, but “buried” in ULVWF multimers (Figure 5) and that the shear-induced thiol-disulfide exchange significantly increases VWF adhesion activity (14) suggests that the state of these thiols may serve as a structural marker to distinguish between newly released ULVWF and plasma VWF multimers. The former is hyperactive, capable of spontaneously agglutinating platelets (9;10), whereas the latter is inactive, but can be activated by fluid shear stress (14;33;34).

Further support to covalent formation of ULVWF strings comes from the observation that GSH partially reduced ULVWF or plasma VWF multimers are able to rapidly re-multimerize to restore their original multimer sizes once GSH is removed. The process occurs, independent of intracellular machinery for inducing and maintaining protein disulfide bonds (Figure 7). The data are important for this study for three reasons. First, the results demonstrate that thiols in (UL)VWF multimers are very active, capable of forming new disulfide bonds or being rearranged (39-41). The thiol reactivity provides a key structural base for data presented in Figure 1,2, and 4. Second, the data demonstrate that the thiol-disulfide state of (UL)VWF multimers could change in response to changes in redox environment, for instance, oxidative stress, which is a well known risk factor for thrombosis (35). Finally, ULVWF and its plasma counterpart re-multimerize to their original size without apparent cross over, e.g. ULVWF remultimerizes partially to the size of plasma VWF or *vice versa*. This strict pattern suggests that the re-multimerization is not random, but follows a particular pattern of folding plasticity and provide additional support for structural differences between ULVWF and plasma VWF multimers.

In summary, we have shown that ULVWF multimers secreted by and anchored to endothelial cells can covalently associate with VWF multimers in solution to form long, thick, and networked string-like structures. This covalent association may involve thiols that are exposed on the surface of plasma VWF multimers, but “buried” in the quaternary structure of ULVWF multimers freshly released from endothelial cells. The difference in the thiol-disulfide states may provide a structural basis for the different adhesion activities of ULVWF and its plasma counterpart. The results provide new insights into a mechanism by which (UL)VWF multimers form platelet-decorated strings and promote platelet-rich thrombosis found in the high shear environment of microcirculation.

REFERENCES

1. Voorberg J, Fontijn R, Calafat J, Janssen H, van Mourik JA, Pannekoek H. Assembly and routing of von Willebrand factor variants: the requirements for disulfide-linked dimerization reside within the carboxy-terminal 151 amino acids. *J.Cell Biol.* 1991; 113:195–205. [PubMed: 2007623]
2. Isaacs NW. Cystine knots. *Curr.Opin.Struct.Biol.* 1995; 5:391–395. [PubMed: 7583638]
3. Wagner DD, Lawrence SO, Ohlsson-Wilhelm BM, Fay PJ, Marder VJ. Topology and order of formation of interchain disulfide bonds in von Willebrand factor. *Blood.* 1987; 69:27–32. [PubMed: 3539228]
4. Marti T, Rosselet SJ, Titani K, Walsh KA. Identification of disulfide-bridged substructures within human von Willebrand factor. *Biochemistry.* 1987; 26:8099–8109. [PubMed: 3502076]
5. Purvis AR, Gross J, Dang LT, Huang RH, Kapadia M, Townsend RR, Sadler JE. Two Cys residues essential for von Willebrand factor multimer assembly in the Golgi. *Proc.Natl.Acad.Sci.U.S.A.* 2007; 104:15647–15652. [PubMed: 17895385]
6. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu.Rev.Biochem.* 1998; 67:395–424. 395-424. [PubMed: 9759493]
7. Brouland JP, Egan T, Roussi J, Bonneau M, Pignaud G, Bal C, Vaiman M, Andre P, Herve P, Mazmanian GM, et al. In vivo regulation of von willebrand factor synthesis: von Willebrand factor production in endothelial cells after lung transplantation between normal pigs and von Willebrand factor-deficient pigs. *Arterioscler.Thromb.Vasc.Biol.* 1999; 19:3055–3062. [PubMed: 10591687]
8. Tsai HM, Nagel RL, Hatcher VB, Seaton AC, Sussman II. The high molecular weight form of endothelial cell von Willebrand factor is released by the regulated pathway. *Br.J.Haematol.* 1991; 79:239–245. [PubMed: 1958482]
9. Arya M, Anvari B, Romo GM, Cruz MA, Dong JF, McIntire LV, Moake JL, Lopez JA. Ultralarge multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX complex: studies using optical tweezers. *Blood.* 2002; 99:3971–3977. [PubMed: 12010796]

10. Moake JL, Chow TW. Increased von Willebrand factor (vWf) binding to platelets associated with impaired vWf breakdown in thrombotic thrombocytopenic purpura. *J.Clin.Apheresis*. 1998; 13:126–132. [PubMed: 9828023]
11. Moake JL, Rudy CK, Troll JH, Weinstein MJ, Colannino NM, Azocar J, Seder RH, Hong SL, Deykin D. Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N.Engl.J.Med*. 1982; 307:1432–1435. [PubMed: 6813740]
12. Dong JF, Berndt MC, Schade A, McIntire LV, Andrews RK, Lopez JA. Ristocetin-dependent, but not botrocetin-dependent, binding of von Willebrand factor to the platelet glycoprotein Ib-IX-V complex correlates with shear-dependent interactions. *Blood*. 2001; 97:162–168. [PubMed: 11133756]
13. Siedlecki CA, Lestini BJ, Kottke-Marchant KK, Eppell SJ, Wilson DL, Marchant RE. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. *Blood*. 1996; 88:2939–2950. [PubMed: 8874190]
14. Choi H, Aboulfatova K, Pownall HJ, Cook R, Dong JF. Shear-induced disulfide bond formation regulates adhesion activity of von willebrand factor. *J.Biol.Chem*. 2007; 282:35604–35611. [PubMed: 17925407]
15. Dong JF, Moake JL, Nolasco L, Bernardo A, Arceneaux W, Shrimpton CN, Schade AJ, McIntire LV, Fujikawa K, Lopez JA. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood*. 2002; 100:4033–4039. [PubMed: 12393397]
16. Padilla A, Moake JL, Bernardo A, Ball C, Wang Y, Arya M, Nolasco L, Turner N, Berndt MC, Anvari B, et al. P-selectin anchors newly released ultralarge von Willebrand factor multimers to the endothelial cell surface. *Blood*. 2004; 103:2150–2156. [PubMed: 14630802]
17. Lopez JA, Dong JF. Shear stress and the role of high molecular weight von Willebrand factor multimers in thrombus formation. *Blood Coagul.Fibrinolysis*. 2005; 16(Suppl 1):S11–6. S11–S16. [PubMed: 15849521]
18. Moake JL, Turner NA, Stathopoulos NA, Nolasco LH, Hellums JD. Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. *J.Clin.Invest*. 1986; 78:1456–1461. [PubMed: 3491092]
19. Romani, d.W.; Rondaij, MG.; Hordijk, PL.; Voorberg, J.; van Mourik, JA. Real-time imaging of the dynamics and secretory behavior of Weibel-Palade bodies. *Arterioscler.Thromb.Vasc.Biol*. 2003; 23:755–761. [PubMed: 12676800]
20. Wang S, Regnier FE. Proteomics based on selecting and quantifying cysteine containing peptides by covalent chromatography. *J.Chromatogr.A*. 2001; 924:345–357. [PubMed: 11521884]
21. Leichert LI, Jakob U. Global methods to monitor the thioldisulfide state of proteins in vivo. *Antioxid.Redox.Signal*. 2006; 8:763–772. [PubMed: 16771668]
22. Epstein DL, Patterson MM, Rivers SC, Anderson PJ. N-ethylmaleimide increases the facility of aqueous outflow of excised monkey and calf eyes. *Invest Ophthalmol.Vis.Sci*. 1982; 22:752–756. [PubMed: 7076421]
23. Pang Y, Sakagami M, Byron PR. The pharmacokinetics of pulmonary insulin in the in vitro isolated perfused rat lung: implications of metabolism and regional deposition. *Eur.J.Pharm.Sci*. 2005; 25:369–378. [PubMed: 15979534]
24. Jensen LH, Renodon-Corniere A, Wessel I, Langer SW, Sokilde B, Carstensen EV, Sehested M, Jensen PB. Maleimide is a potent inhibitor of topoisomerase II in vitro and in vivo: a new mode of catalytic inhibition. *Mol.Pharmacol*. 2002; 61:1235–1243. [PubMed: 11961142]
25. Bowes TJ, Gupta RS. Induction of mitochondrial fusion by cysteine-alkylators ethacrynic acid and N-ethylmaleimide. *J.Cell Physiol*. 2005; 202:796–804. [PubMed: 15389563]
26. Burgess JK, Hotchkiss KA, Suter C, Dudman NP, Szollosi J, Chesterman CN, Chong BH, Hogg PJ. Physical proximity and functional association of glycoprotein Ib α and protein-disulfide isomerase on the platelet plasma membrane. *J Biol Chem*. 2000; 275:9758–9766. [PubMed: 10734129]

27. Titani K, Takio K, Handa M, Ruggeri ZM. Amino acid sequence of the von Willebrand factor-binding domain of platelet membrane glycoprotein Ib. *Proc.Natl.Acad Sci U.S.A.* 1987; 84:5610–5614. [PubMed: 3497398]
28. Kenny D, Jonsson OG, Morateck PA, Montgomery RR. Naturally occurring mutations in glycoprotein Ibalpha that result in defective ligand binding and synthesis of a truncated protein. *Blood.* 1998; 92:175–183. [PubMed: 9639514]
29. Savage B, Sixma JJ, Ruggeri ZM. Functional self-association of von Willebrand factor during platelet adhesion under flow. *Proc.Natl.Acad.Sci.U.S.A.* 2002; 99:425–430. [PubMed: 11756664]
30. Mayadas TN, Wagner DD. Vicinal cysteines in the prosequence play a role in von Willebrand factor multimer assembly. *Proc.Natl.Acad.Sci.U.S.A.* 1992; 89:3531–3535. [PubMed: 1565649]
31. Ramachandran N, Root P, Jiang XM, Hogg PJ, Mutus B. Mechanism of transfer of NO from extracellular S-nitrosothiols into the cytosol by cell-surface protein disulfide isomerase. *Proc.Natl.Acad.Sci.U.S.A.* 2001; 98:9539–9544. [PubMed: 11493694]
32. Sullivan DC, Huminiecki L, Moore JW, Boyle JJ, Poulsom R, Creamer D, Barker J, Bicknell R. EndoPDI, a novel protein-disulfide isomerase-like protein that is preferentially expressed in endothelial cells acts as a stress survival factor. *J.Biol.Chem.* 2003; 278:47079–47088. [PubMed: 12963716]
33. Chow TW, Hellums JD, Moake JL, Kroll MH. Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. *Blood.* 1992; 80:113–120. [PubMed: 1611079]
34. Zhang JN, Bergeron AL, Yu Q, Sun C, McIntire LV, Lopez JA, Dong JF. Platelet Aggregation and Activation under Complex Patterns of Shear Stress. *Thromb.Haemost.* 2002; 88:817–821. [PubMed: 12428100]
35. Loscalzo J. Oxidative stress in endothelial cell dysfunction and thrombosis. *Pathophysiol.Haemost.Thromb.* 2002; 32:359–360. [PubMed: 13679676]
36. Stenina OI, Topol EJ, Plow EF. Thrombospondins, their polymorphisms, and cardiovascular disease. *Arterioscler.Thromb.Vasc.Biol.* 2007; 27:1886–1894. [PubMed: 17569883]
37. Xie L, Chesterman CN, Hogg PJ. Control of von Willebrand factor multimer size by thrombospondin-1. *J.Exp.Med.* 2001; 193:1341–1349. [PubMed: 11413189]
38. Pimanda JE, Annis DS, Raftery M, Mosher DF, Chesterman CN, Hogg PJ. The von Willebrand factor-reducing activity of thrombospondin-1 is located in the calcium-binding/C-terminal sequence and requires a free thiol at position 974. *Blood.* 2002; 100:2832–2838. [PubMed: 12351392]
39. Verweij CL, Hart M, Pannekoek H. Expression of variant von Willebrand factor (vWF) cDNA in heterologous cells: requirement of the propolypeptide in vWF multimer formation. *EMBO J.* 1987; 6:2885–2890. [PubMed: 3500851]
40. Wise RJ, Pittman DD, Handin RI, Kaufman RJ, Orkin SH. The propeptide of von Willebrand factor independently mediates the assembly of von Willebrand multimers. *Cell.* 1988; 52:229–236. [PubMed: 3124962]
41. Mayadas TN, Wagner DD. In vitro multimerization of von Willebrand factor is triggered by low pH. Importance of the propolypeptide and free sulfhydryls. *J.Biol.Chem.* 1989; 264:13497–13503. [PubMed: 2788160]

Figure 1. Perfusion of plasma VWF increase the number of ULVWF strings

Washed platelets suspended in the Tyrode's buffer were perfused over histamine-stimulated HUVECs for 3 min in the presence or absence of plasma VWF multimers. The formation of ULVWF strings was quantified by counting the numbers of strings in 20 continuous review fields (200x). For comparison, HUVECs were also perfused with plasma VWF pretreated with 2 mM of NEM (free NEM was removed by dialysis) for 15 min at room temperature (the Student's *t* test, *n* = 6).

Figure 2. The formation of ULVWF string networks on endothelial cells under flowing conditions

When washed platelets were perfused over histamine-stimulated HUVECs, individual ULVWF strings were formed on endothelial cells (A). The number of ULVWF strings increased significantly when plasma VWF multimers were perfused together with washed platelets (B). Perfusion of plasma VWF multimers also promoted cross-linking of ULVWF strings to form networks on endothelial cells to attract more platelets (C & D). A similar network structure of ULVWF strings was also commonly observed when plasma from patients with TTP was perfused (E & F). The figure is representative of 4-10 individual experiments (bar = 100 μ m for A - E, bar = 250 μ m for F).

Figure 3. Plasma VWF adhered to ULVWF strings under flowing conditions

The chimeric GFP-VWF was captured by the thiol-active beads (eluted from the beads by DTT) and detected by a polyclonal VWF antibody (**A**, reducing SDS-PAGE). Plasma VWF (**B** & **C**) or the chimeric GFP-VWF (**D** & **E**) was perfused over ULVWF strings secreted from histamine-stimulated HUVECs, together with regular beads coated with anti-VWF antibody and fluorescent beads coated with anti-GFP antibody for 6 min. Adhesion of VWF antibody beads to ULVWF strings (under regular light) was co-localized with fluorescent GFP-antibody beads (with a FITC filter). The figure is a representative of three separate experiments (bar = 100 μ m).

Figure 4. NEM blocked the formation of ULVWF strings under flow conditions

HUVECs were stimulated with histamine to release ULVWF and then perfused with Tyrode's buffer containing NEM for 3 min. After washing to remove free NEM, lyophilized platelets were perfused to mark ULVWF strings. **A.** NEM treatment dose-dependently reduced the numbers of ULVWF strings formed on endothelial cells. The first two bars represent controls of the number of strings formed in the absence of NEM (white bar) and on unstimulated HUVECs (gray bar), respectively (the Student's *t* test, $n = 4$, $* p < 0.01$ as compared to untreated). **B.** ULVWF strings with NEM perfusion were also more rigid and with less adherent platelets (bar = 100 μm). **C.** NEM also reduced the number of strings marked by beads coated with a VWF antibody (absence of platelets).

Figure 5. NEM-induced cytotoxicity

Cultured HUVECs were treated with NEM for 3 min and then examined for morphological changes and viability. NEM up to 10 mM did not induce morphological changes (**A-C**) or DNA dye uptake (**E**), whereas at 20 mM, it induced significant cell shrinkage and detachment (**D**).

Figure 6. ULVWF did not contain surface exposed free thiols

A. ULVWF and plasma VWF multimers were labeled with MPB and immunoprobed with HRP-streptavidin and a polyclonal VWF antibody. Both forms of VWF were labeled with MPB. **B.** ULVWF and plasma VWF multimers were incubated with the thiol active sepharose 6B beads and the bead captured VWF (containing free thiols) was released by DTT. VWF in supernatant and eluted from the beads was detected by immunoblotting with the polyclonal VWF antibody. The figure is a representative of 6 and 3 separate experiments for plasma VWF and ULVWF, respectively.

A. ULVWF

B. Plasma VWF

Figure 7. (UL)VWF re-multimerization after reduction

ULVWF freshly released from HUVECs and plasma VWF multimers were incubated with 200 μM of GSH for 15 min at 37°C. The GSH concentration was then gradually reduced by a series of dilutions with buffer. VWF multimer pattern in each fraction was evaluated by 1% SDS-agarose gel electrophoresis and immunoblotting with a polyclonal VWF antibody. ULVWF multimers were reduced to smaller forms by 200 μM of GSH, but re-multimerized to the original size upon removal of GSH by gradual dilution. The figure is a representative of 3- 20 separate experiments.