von Willebrand factor (VWF), the largest multimeric adhesion ligand circulating in blood. Its adhesion activity is related to multimer size, with the ultra-large forms freshly released from the activated endothelial cells being most active, capable of spontaneously binding to platelets. In comparison, smaller plasma forms circulating in blood bind platelets only under high fluid shear stress or induced by modulators. The structure-functional relationships that distinguish the two types of VWF multimers are not known. In this study, we demonstrate that some of the plasma VWF multimers contain surface-exposed free thiols. Physiological and pathological levels of shear stresses (50 and 100 dynes/cm²) promote the formation of disulfide bonds utilizing these free thiols. The shear-induced thiol-disulfide exchange increases VWF binding to platelets. The thiol-disulfide exchange involves some or all of nine cysteine residues (Cys889, Cys898, Cys2448, Cys2451, Cys2490, Cys2491, Cys2453, Cys2528, and Cys2533) in the D3 and C domains as determined by mass spectrometry of the tryptic VWF peptides. These results suggest that the thiol-disulfide state may serve as an important structural determinant of VWF adhesion activity and can be modified by fluid shear stress.

von Willebrand factor (VWF), the largest adhesion ligand in blood, is critical for bleeding arrest at the site of vessel injury, where it initiates platelet adhesion to subendothelium. This hemostatically essential molecule also plays an active role in platelet aggregation, especially when it is exposed to pathological high fluid shear stress. VWF is synthesized in endothelial cells and megakaryocytes as a monomer with a very large propeptide at its N terminus. In the endoplasmic reticulum and Golgi apparatus, two monomers form a dimer through the C-terminal disulfide bonds (1, 2), and dimers then form multimers through the N-terminal disulfide linkages (3, 4). Although the thiols involved in multimerization are in the mature VWF, the intrinsic protein-disulfide isomerase activity associated with the propeptide may be essential for the process (5, 6).

Once synthesized, VWF multimers are either constitutively released or targeted to storage in endothelial Weibel-Palade bodies and platelet α-granules (7). VWF multimers released through the constitutive pathway are relatively small, whereas those in storage granules are rich in the ultra-large (UL) forms (8, 9). Upon release, ULVWF multimers are rapidly but partially cleaved by the metalloprotease ADAMTS-13 into smaller forms that circulate in blood. The adhesiveness of VWF is determined by its multimeric size. The loss of large multimers of plasma VWF results in bleeding diathesis associated with a subtype of von Willebrand disease (7). In contrast, ULVWF multimers that are not cleaved by ADAMTS-13 are highly adhesive in binding and agglutinating platelets (10, 11). The accumulation of ULVWF in plasma is considered to be responsible for the systemic thrombosis in microvasculature associated with thrombotic thrombocytopenic purpura.

VWF multimers circulating in plasma do not bind the platelet GP Ib-IX-V complex but can be induced to do so by high fluid shear stress or modulators such as ristocetin. The ristocetin-induced VWF-GP Ib interaction shares many characteristics with shear-induced binding (12). These observations suggest that plasma VWF exists in an inactive conformation that can be activated by fluid shear stress. Consistent with this possibility, shear stress has been shown to change VWF multimers from a globular shape to an elongated rope-like structure (13). Fluid shear stress may also promote VWF multimerization in a cell-free system (14). However, the structural basis for shear-induced VWF activation has yet to be determined. If the adhesion activity of VWF is attributed to multimer sizes, then the thiol-disulfide state of VWF multimers may serve as a critical regulator of VWF activity. This possibility is supported by the experimental observations that partial reduction of VWF multimers decreases VWF binding to platelets (15–17).

We hypothesized that fluid shear stress activates plasma VWF multimers through changes in their thiol-disulfide states, and we have tested this hypothesis by determining the following: 1) if VWF multimers contain free cysteine thiols; 2) if these free thiols form disulfide bonds or are rearranged with existing disulfide bonds under fluid shear stress; and 3) if the thiol-disulfide exchange correlates with shear-induced VWF binding to platelets.
Production and Purification of Plasma VWF—Plasma VWF (pVWF) was purified from human cryoprecipitate (Gulf Coast Regional Blood Bank, Houston, TX) by glycine and NaCl precipitation and chromatography on a 2.5 × 50-cm Sepharose 4B column (bed volume of 3000 ml; GE Healthcare), as described previously (18). Briefly, 250 ml of cryoprecipitate was thawed at 37°C, diluted (1:20) with 55 mM sodium citrate, pH 7.4, and incubated with stirring at 37°C for 30 min. Glycine buffer (2.6 M glycine, 0.3 M NaCl, 25 mM Tris, pH 6.8) was then added to a final concentration of 2.0 M glycine (19). The mixture was centrifuged at 5000 × g for 30 min at 23°C. The supernatant was collected, mixed with NaCl (90 g/liter of supernatant) for 1 h at 37°C, and centrifuged again at 20,000 × g for 30 min at 23°C. The precipitate was dissolved in 10 ml of sodium citrate buffer (25 mM citric acid, 0.15 M NaCl, pH 6.15) and centrifuged at 10,000 × g for 5 min to remove the lipid fraction. It was then applied to the Sepharose 4B column and eluted with NaCl/citrate buffer at room temperature at a flow rate of 1 ml/min. Fractions were collected, and VWF antigen was determined by enzyme-linked immunosorbent assay (Ramco Laboratory, Houston, TX) and multimer pattern by 1% SDS-agarose gel electrophoresis followed by immunoblot with a rabbit polyclonal VWF antibody (Dako Corp, Carpinteria, CA).

Detection of Free Thiols in Plasma VWF—Plasma VWF multimers were incubated with 100 μM of maleimide-PEO₂−-biont (MPB, Pierce), which is a thiol-reactive agent with a hydrophilic polyethylene oxide spacer arm, at room temperature in phosphate-buffered saline. Maleimide reacts with the thiol group on a cysteine residue to form a stable carbon-sulfur bond. After 5 min of incubation, the reaction was quenched by 200 μM of GSH. The labeled VWF was then separated on 5% SDS-PAGE under reducing and nonreducing conditions and detected by immunoblotting using horseradish peroxidase-conjugated streptavidin (Pierce). For immunoblots, the protein loading was controlled at ~0.3 μg except experiments where the ratio of free thiol form to disulfide form of VWF was compared before and after shear exposure.

Detection of Surface-exposed Free Thiols by Covalent Chromatography—We used activated thiol-Sepharose 6B, a mixed disulfide formed between 2,2′-dipyridyl disulfide and glutathione coupled to CNBr-activated Sepharose 6B (with a fractionation range of 10,000–4,000,000 for globular proteins, GE Healthcare) (20), to distinguish free thiols exposed on the surface of the VWF multimeric quaternary structure from those buried within the structure that may require denaturation to be exposed. Reaction of activated thiol-Sepharose beads with thiol groups of VWF multimers yields mixed disulfide bonds that capture VWF onto the beads. Briefly, 2 mg of thio-2-propyl-Sepharose 6B beads were washed extensively with and suspended in 1 ml of binding buffer (0.2 M NaCl, 0.1 M Tris-HCl, pH 7.5). The washed beads were then incubated with pVWF (1–5 μg) or a recombinant VWF A1–3 polypeptide (5 μg, kindly provided by Dr. Miguel Cruz, Baylor College of Medicine) for 15 min at room temperature with constant rotation. To ensure beads capture all available VWF multimers, the amount of thiol beads used had a protein-coupling capacity of 20 mg, greatly exceeding the amount of VWF added (1–5 μg). The bead-bound VWF was washed with the binding buffer three times and released with 20 mM DTT (Sigma) or 50 μM β-mercaptoethanol (Sigma). The eluted VWF was separated on 5% SDS-PAGE under reducing conditions and detected by the polyclonal VWF antibody.

To verify results from thiol-active beads, VWF was also labeled with MPB (100 μM) and then incubated with streptavidin-coupled agarose beads (Invitrogen) for 15 min at room temperature. The beads were extensively washed with phosphate-buffered saline to remove unbound VWF, mixed with SDS sample buffer, and boiled for 5 min. The beads captured by VWF were detected by the VWF antibody (Dako).

To further determine whether the free thiol form of VWF is also present in plasma, we recruited three healthy donors 27–41 years old (2 females and 1 male). Citrate blood was centrifuged first at 150 × g for 15 min and then 800 × g for 10 min at 25°C to collect platelet poor-plasma. The thiol-activated beads were incubated with 1 ml of platelet poor-plasma for 15 min at room temperature and then washed with the binding buffer three times. The captured VWF was reduced by DTT, separated on 5% SDS-PAGE, and detected by the polyclonal VWF antibody (Dako).

Identification of VWF Free Thiols by Mass Spectrometry—To identify surface-exposed free thiols, we incubated VWF multimers (200 μg) with the thiol-reactive beads for 15 min at room temperature with constant rotation. The beads were washed five times with the binding buffer to remove unbound VWF and then incubated with 10 μg of trypsin (Roche Applied Science) for 17 h at 37°C in a 0.1 M Tris-HCl buffer. The beads were again washed five times with binding buffer and incubated with 20 μM DTT to release VWF tryptic peptides that remained bound (containing free thiols). Under this condition, VWF was completely cleaved because no uncleaved or partially cleaved VWF can be detected by protein silver staining (detection kit from Invitrogen) after 15% SDS-PAGE in reducing conditions (data not shown).

The solution of VWF tryptic peptides released from the beads was evaporated to ~50 μl, acidified with trifluoroacetic acid to pH ~3, and desalted on a C₁₈ ZipTip column (Millipore, Billerica, MA). The peptides were eluted from the ZipTip with 3–5 μl of 50% acetonitrile and 2% formic acid, spotted on a MALDI target plate with matrix (α-cyano-4-hydroxycinnamic acid), dried, and analyzed in reflector mode on an Applied Biosystems 4700 Proteomics Analyzer MALDI TOF/TOF mass spectrometer. The spectra were examined for protonated monoisotopic peptide masses of Cys-containing peptides predicted from a tryptic digest of VWF. Selected peptide precursor ions were subjected to high energy collision-induced dissociation to generate MS/MS fragmentation data that were analyzed to define the sequence and determine whether the peptides were from VWF.

Fluid Shear-induced Thiol-Disulfide Exchange of VWF—A cone and plate viscometer (RS1, Thermo Fisher Scientific, Newington, NH) was used to measure the effects of fluid shear stress on the thiol-disulfide state of VWF multimers in solution. The surface of cone and plate was first blocked with 0.2% of liquid silicon (Prosil-28®, PCR Inc., Gainesville, FL) to reduce
nonspecific VWF adhesion to the metal surface, which tends to capture larger VWF multimers. The coating reduced over 90% of VWF adhesion to the metal surface (data not shown). Plasma VWF multimers (5 μg/500 μl) were loaded onto the plate and subjected to a constant shear stress of 100 dynes/cm² for 3 min at 37°C in the presence or absence of 100 μM of MPB. The sheared VWF was immediately mixed with either the activated thiol-Sepharose beads for covalent chromatography or washed platelets to measure VWF binding. The time between shear cessation and sample processing was <30 s. Additional experiments were also performed to determine whether the thiol-disulfide exchange also occurs at a physiological level of shear stress (50 dynes/cm²) and after shorter shear exposure (0.5 min).

Shear-induced Binding of VWF to Washed Platelets—Blood was collected from healthy subjects using 10% acid/citrate dextrose buffer (ACD, 85 mM sodium citrate, 111 mM glucose, and 71 mM citric acid, pH 6.8) as an anti-coagulant. The use of human blood was approved by the Institutional Review Board of Baylor College of Medicine. All donors signed consent forms before blood was drawn. Blood was centrifuged at 150 g for 15 min at 3°C to obtain platelet-rich plasma, which was then centrifuged at 800 × g for 10 min to collect platelets (21). The platelet pellets were resuspended in a CGS buffer (13 mM sodium citrate, 30 mM glucose, and 120 mM sodium chloride, pH 7.0) and centrifuged again at 900 × g for 10 min. Washed platelets were resuspended in Ca²⁺- and Mg²⁺-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 dibasic sodium phosphate, pH 7.4) to a final density of 1–3 × 10⁹/μl for VWF binding studies. Platelets were used within 2 h after preparation to prevent spontaneous activation and aggregation.

To measure shear-induced VWF binding, washed platelets were immediately mixed with pVWF multimers that had been exposed to a high shear stress of 100 dynes/cm² (3 min at 37°C) for 10 min. A FITC-conjugated polyclonal VWF antibody (Dako) was then incubated with platelets for 20 min at room temperature, and the antibody binding was detected on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL). Two types of negative controls were used: FITC-conjugated mouse IgG for background fluorescence and unsheared VWF for baseline binding to platelets. The binding specificity was determined by treating platelets with the monoclonal antibody AK2 (Beckman Coulter, Miami, FL), which is known to block shear- and ristocetin-induced platelet aggregation (12).

Three techniques were used to determine whether MPB affects GP Ibα expression and function on the platelet surface. First, washed platelets were incubated with 100 μM of MPB for 20 min at room temperature and then with either a FITC-conjugated monoclonal GP Ibα antibody (clone AK2, Beckman Coulter) or integrin αIIb antibody (clone HIP8; Pharmingen) for 20 min at room temperature. Platelets were then fixed with 1% paraformaldehyde, and antibody binding was detected by flow cytometry. Because AK2 blocks GP Ib-VWF interaction (12), its binding will determine whether MPB alters the VWF-binding epitope and/or changes surface receptor density. Second, we conducted a separate set of experiments using lyophilized platelets to avoid potential effects of MPB on platelet activation. Third, after exposure to shear stress, free MPB in the sample solution was quenched with 1 mM of cysteine (Sigma) before incubation with washed platelets. A molar excess of free cysteine will prevent the potential effect of MPB on GP Ibα, which has been reported to contain free thiols and subjected to redox regulation (22).

Statistical Analysis—All data are presented as mean ± S.E. The Student’s t test was used for data analysis, and a p value less than 0.05 was considered statistically significant.

RESULTS

MPB Blocked Shear-induced VWF Binding to Platelets—Previous studies have demonstrated that fluid shear stress increases the adhesion and aggregation activity of VWF (23, 24) and possibly the size of VWF multimers (14). We were to determine whether shear-induced activation of VWF involves the thiol-disulfide exchange as measured by VWF binding to washed platelets (by flow cytometry) before and after exposure to a 100 dynes/cm² shear stress. Consistent with previous observations, binding of sheared pVWF multimers to platelets was significantly higher than unsheared pVWF (Fig. 1A, white bars). The binding was mediated specifically through GP Ibα because it was blocked by the GP Ibα monoclonal antibody AK2 (Fig. 1B), which blocks shear- and ristocetin-induced platelet aggregation (12). The shear-induced increase in VWF binding was significantly reduced when plasma VWF multimers were exposed to shear stress in the presence of 100 μM MPB, which blocks free thiols on plasma VWF multimers.

The observed effect of MPB on VWF binding to platelets was not because of MPB modifying cysteine thiols on the VWF receptor GP Ib-IX-V (22), because quenching free MPB with free cysteine did not alter the effect of MPB on VWF binding to platelets (Fig. 1C). Furthermore, incubation of washed platelets with 100 μM of MPB for 20 min did not change the following: 1) binding of conformation-dependent AK2 binding, and 2) the surface expression of GP Ib and GP Ib-IIIa (Fig. 1D). Finally, the blocking effect of MPB was also observed using lyophilized platelets (Fig. 1A, black bars). Together, these results demonstrate that pVWF multimers, which do not bind platelets in native forms in blood circulation, were induced to bind platelets by high fluid shear stress in a thiol-dependent manner.

Plasma VWF Multimers Contain Surface-exposed Free Thiols—Results presented in Fig. 1 indicate that pVWF contain free thiols that are critical for shear-induced binding to platelets. To demonstrate directly the presence of such free thiols, purified plasma VWF multimers were incubated with MPB, which reacts with free thiols of VWF in neutral pH. The incorporated MPB was detected by horseradish peroxidase-conjugated streptavidin on reducing and nonreducing SDS-PAGE, which was verified by blotting the membrane with VWF antibody (Fig. 2). Because of samples being quenched with 200 μM of GSH, which partially reduced VWF, VWF detected on immunoblots was the primary dimer on nonreducing gels. This observation was consistent with a previous study that showed that low doses...
One concern is that active thiols coupled to the beads could act as a reducing agent for VWF. If this is the case, the free thiols detected perhaps resulted from reduction of the native structure instead of being in the VWF native structure. To address this concern, the MPB-labeled VWF was captured by the streptavidin-coupled agarose beads and directly solubilized in SDS sample buffer. As shown in Fig. 3C, the streptavidin beads also captured VWF, indicating that pVWF multimers indeed contain free thiols in their native structure. Compared with the full-length VWF multimers, a recombinant polypeptide containing the three homologous A domains (A1–3) was not captured by the thiol beads (Fig. 4A), indicating that the free thiols are not located in the A domains.

To determine whether VWF multimers with surface-exposed free thiols can also be detected in plasma, three healthy donors were recruited, and citrate plasma was collected. The thiol-active beads (2 mg) were incubated with 1 ml of platelet poor-plasma for 15 min at room temperature. The bead-captured VWF was eluted by DTI reduction and detected by SDS-PAGE and immunoblot. As shown in Fig. 4B, the thiol-active beads captured plasma monomeric (250 kDa) and dimeric (500 kDa) VWF in all three donors. The ratio of free thiol form to disulfide form of VWF was estimated to be ~3 to 7.

Shear Stress Promoted Thiol-Disulfide Exchange of VWF Multimers—We have shown that free thiols are exposed on the surface of VWF multimers in plasma or purified forms. We then asked whether these free thiols are involved in the activation of VWF by high fluid shear stress, which is known to induce conformational changes and enhances the adhesive activity of VWF multimers. Plasma VWF multimers were exposed for 3 min at 37 °C to a shear stress of 100 dynes/cm², a pathological level found in stenosed arteries that promotes VWF to aggregate platelets (26). The sheared VWF was then incubated with thiol-reactive beads. As shown in Fig. 5A, VFW multimer with free thiols found in unshered samples were minimally detectable after shear exposure.

The similar phenomenon was also observed at a physiological shear stress of 50 dynes/cm² and after a shorter shear exposure of 0.5 min (Fig. 5B). However, measured by densitometry, the amount of reduction in VWF with free thiols under both conditions was significantly less as compared with a higher shear stress of 100 dynes/cm² for 3 min.

There are at least two possible explanations for the disappearance of free thiol(s) from the surface of VWF after shear...
exposure. First, shear stress promotes the formation of new disulfide bonds utilizing the free thiols. Second, shear stress drastically changes the conformation of VWF so that these free thiols become buried within the quaternary structure of VWF multimers, no longer accessible for binding to the active thiols coupled to large Sepharose beads. To distinguish the two possibilities, sheared plasma VWF multimers were incubated with either 8 M urea, which denatures VWF and exposes potentially buried free thiols, or 50 mM DTT, which reduces disulfide bonds in VWF multimers. As shown in Fig. 6A, the activated thiol beads captured sheared plasma VWF that was treated with DTT, but not with urea, indicating that the free thiols formed new disulfide bonds. As expected, the new disulfide bonds are stable, because the active thiol beads failed to capture sheared VWF in the absence of DTT at least 5 h post-shear exposure (Fig. 6B).

Taken together, these results indicate that the shear stress induces transition of soluble VWF from a free thiol form to a disulfide form. The thiol-disulfide exchange occurs mostly under pathological high shear stress (100 dynes/cm² as tested).

**Mass Spectrometry Identified the Exposed Free Thiols**—We have so far shown that shear induces disulfide formation utilizing the free thiols exposed on the surface of VWF multimers.

**FIGURE 2.** Plasma VWF multimers contained free thiols. VWF (3 μg) purified from human cryoprecipitate was incubated with MPB and separated on 5% SDS-PAGE (0.3 μg per sample loading) under reducing (R) and nonreducing (N) conditions. The incorporation of MPB into VWF multimers was detected by horseradish peroxidase-streptavidin. The MPB incorporation into VWF was verified by blotting the same membrane with a polyclonal VWF antibody. The figure is a representative of six separate experiments.

**FIGURE 3.** Plasma VWF contained surface-exposed free thiols. Plasma VWF multimers were incubated with the activated thiol beads to covalently capture VWF. A, thiol beads captured some but not all plasma VWF multimers that were released by 20 mM DTT and detected on immunoblot by a VWF antibody. B, thiol-active beads failed to capture VWF that was pretreated with 2 mM N-ethylmaleimide (NEM). C, streptavidin-coupled agarose beads, but not thiol-active beads, capture MPB-labeled VWF. Unlabeled VWF was not captured.

**FIGURE 4.** The thiol-active beads failed to capture recombinant A1–3 polypeptide. A, thiol-active beads were incubated with recombinant A1–3 polypeptides for 15 min at room temperature, washed extensively with the binding buffer, and then treated with 50 mM DTT for 10 min. No recombinant A1–3 was detected from the bead eluates, even after denaturing the polypeptide with 8 M urea. B, the thiol-active beads were incubated with platelet-poor plasma from three healthy human subjects and VWF bound to the beads eluted by DTT reduction. The eluted VWF was separated on 5% SDS-PAGE on a mild reducing condition. The figure represented 3–8 separate experiments.
We further show that this thiol-disulfide exchange results in enhanced VWF binding to platelets. The surface-exposed free thiols are therefore critical for VWF adhesion activity. To identify these free thiols, the bead-captured VWF was first digested with trypsin, and the bead-bound tryptic peptides were then released from the beads after treatment with 50 mM DTT (0.5 min at 37 °C). Exposure to 50 dynes/cm² of shear stress reduced the bead captured VWF (lane 3) as compared with unsheared sample. However, the amount of captured VWF was significantly greater than that at 100 dynes/cm². The figure is a representative of three separate experiments.

**DISCUSSION**

We have demonstrated that fluid shear stress at a pathophysiologic (100 dynes/cm²) level induces VWF binding to platelets, a process that is blocked by MPB, a thiol-reactive agent that binds and blocks the free thiols on VWF substrate (Fig. 1). Consistent with this observation, we find that plasma VWF multimers in solution contain the surface-exposed free thiols that reside outside the homologous A domains (Figs. 2 and 3). Mass spectrometry of VWF tryptic peptides identified two cysteines in the tryptic peptide of Tyr^883–Arg^906 in the D3 domain and seven additional cysteines in three clustered peptides of Ser^2435–Arg^2464, Ser^2479–Arg^2490, and Ser^2516–Arg^2535 in the C domain (Fig. 7). Because each peptide contains more than one cysteine residue, we have yet to determine whether all these cysteine residues are in free thiol forms and how many of these free thiols are induced to form disulfide bonds by high shear stress (Fig. 5). It is also possible that some of these cysteine residues are not in free thiol forms but rather form covalent association with free thiol-containing peptides and therefore are co-purified from the beads. Nevertheless, these data suggest that thiol-disulfide exchange associates with the shear-induced VWF activation.

Our results are partially consistent with a recent study suggesting that plasma VWF contains free thiols that are likely buried in the VWF quaternary structure (28). However, our data differ from an earlier study showing that free thiols exist in pro-VWF dimers but not in mature VWF multimers (27), implying that all free thiols in a pro-VWF dimer have been utilized during intracellular multimerization. The discrepancies could be due to different VWF preparations (VWF freshly released from endothelial cells versus that circulating in blood).
Redox Regulation of VWF Activity

However, to be consistent with the demonstrated disulfide-mediated process of VWF multimerization, thiols at least at the ends of a native VWF multimer should be free because they are no longer involved in multimerization, unless they are otherwise oxidized or forming a head-to-tail multimeric circle. Thus far, no experimental evidence suggests that this is the case. Furthermore, if free thiols at the end of a multimer are indeed consumed in ways other than multimerization, how and where does the multimerization process stop?

As a cysteine-rich glycoprotein, the disulfide bond structure in a VWF multimer has not been fully mapped as only 29 intramolecular disulfide bonds are predicted from 169 cysteine residues in a mature VWF monomer. In addition, 2–3 cysteine residues in the C-terminal region are involved in the VWF dimerization (29, 30) and Cys^{1099} and Cys^{1142} in the D/D3 region are involved in the N-terminal VWF multimerization (31).

We have identified nine cysteine residues in four tryptic peptides, which may exist in the form of free thiols; none are known to be involved in the VWF dimerization and multimerization. Two cysteines (Cys^{889} and Cys^{898}) found in the D3 domain are close to those involved in VWF multimerization (31). Interestingly, the majority of the potentially free cysteine thiols (Cys^{2448}, Cys^{2451}, Cys^{2453}, Cys^{2790}, Cys^{2791}, Cys^{2528}, and Cys^{2533}) are clustered in a sequence of 100 amino acids (Arg^{2435} to Arg^{2535}) in the C domain that includes the RGD sequence. The finding that most surface cysteine residues are clustered in this region suggests that the segment is exposed on the quaternary structure of a VWF multimer and is responsive to fluid shear stress. More importantly, this region may interact with the A1 domain to prevent the GP Ib-VWF interaction. By inducing the thiol-disulfide exchange (Figs. 5 and 6), pathophysiological shear stress may disrupt this inhibitory C-A1 interaction to expose the A1 domain. Our observation supports an early finding that the VWF C domain participates in GP Ib-VWF interaction under high shear stress (32). It remains to be determined as how the thiol-disulfide exchange involving primarily cysteine in the C domain alters or modifies the A1 domain. However, transition of multiple cysteine residues from free thiol to disulfide form is very likely to induce profound conformational changes of VWF. For example, formation of vicinal disulfide often results in a tight turn of protein backbone (33) and has been demonstrated previously as regulatory machinery of protein functions (34).

It also remains unclear as to how the new disulfide bonds are formed and what global structural changes they may bring. For the thiols in the D domain, they could contribute to VWF multimerization because they are adjacent to cysteines involved in multimerization. For those in the C domain, they may interact with the A1 domain in a globular conformation, which has been demonstrated previously through rotary shadowing microscopy (13). This globular conformation may be energetically favored during VWF transportation in solution. Shear stress extends VWF from globular to an elongated conformation to facilitate inter-chain disulfide bonds among VWF multimers by increasing molecular collision brought by high fluid shear stress. The formation of interchain disulfide bonds results in lateral, instead of linear, association among VWF multimers.

Fig. 8 schematically illustrates how shear stress may promote the lateral association. Although speculative, the illustrated transition of the VWF thiol-disulfide state under shear stress and lateral covalent association is supported by several lines of evidence. First, the free thiols are located in the C domain instead of regions known to be involved in the multimerization process (Fig. 7) and are unlikely to participate in linear elongation of a multimer. Second, the lateral association has previously been demonstrated between immobilized VWF and circulating VWF under physiological levels of shear stress (35). Although the thiol-disulfide exchange is observed under comparable shear stress, the most significant transition occurs under pathological high shear stress (Fig. 5). The difference may be that, in addition to wall shear stress, the lateral association of circulating VWF with immobilized VWF (immobilization activates VWF) also involves torque force. As a result, the force applied to VWF is greater than the bulk shear stress experienced by VWF in solution. Third, the possible lateral association of multiple VWF multimers is consistent with the shear-induced elongation of VWF from globular to a chain-like structure (13). Finally, it is also consistent with a recent report that fluid shear stress may promote the formation of larger VWF multimers (14). The proposed lateral association among VWF multimers could activate VWF by promoting the lateral alignment of multiple A1 domains to increase the binding avidity (Fig. 8) and by utilizing free thiols to remove potential inhibitory effects of C domains on the A1 domain.

Alternatively, the free thiols may also form intra-chain disulfide bonds that will drastically change VWF conformation, resulting in the A1 domain being exposed to bind the GP Ib-IX-V. Additional studies may delineate the structural changes induced by the thiol-disulfide exchange under shear stress.
Although the mechanism for shear stress-induced thiol-disulfide exchange is unclear, it is likely to be normally prevented in vivo by redox molecules (such as GSH) or substrate specific reductants that are present in plasma (36). Nevertheless, our results support a previous notion that, in addition to the lumen of the endoplasmic reticulum (37), disulfide bonds can also form in a cell- and enzyme-free system (6, 38, 39). In fact, extra-cellular thiol-disulfide exchange has increasingly been recognized as a way to regulate protein function. For example, vitronectin forms intermolecular disulfide bonds with thrombin (40, 41) and thrombospondin with thrombin-anti-thrombin complex (42). Both of these reactions result in functional variations. More recently, thiol-disulfide exchange has been cited as a key structural change to activate tissue factor (25).

Finally, thiol-reactive Sepharose beads do not capture all plasma VWF multimers, even though the binding capacity of the beads is in a great excess to the amount of added VWF multimers (Fig. 3). Plasma from normal human subjects contains more disulfide than free thiol forms of VWF (Fig. 4, an estimated ratio of 7:3). The observation suggests that plasma VWF exists in different thiol-disulfide states and, as a result, may have different adhesive activities. It is therefore tempting to speculate that environmental changes such as oxidative stress shift VWF multimers between different thiol-disulfide states with different adhesion activity. Future studies will delineate the differences among these states with respect to structure and function of VWF.

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