Shear-Dependent Changes in the Three-Dimensional Structure of Human von Willebrand Factor

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The three-dimensional tertiary structure of human von Willebrand Factor (vWF) on a hydrophobic surface under aqueous conditions and different shear stress regimes was studied by atomic force microscopy (AFM). vWF was imaged by AFM at molecular level resolution under negligible shear stress, under a local applied shear force (7.4 to 19 nN) using the AFM probe in contact mode scanning, and after subjecting vWF to a range of shear stress (0 to 42.4 dyn/cm²) using a rotating disk system. The results demonstrate that vWF undergoes a shear stress-induced conformational transition from a globular state to an extended chain conformation with exposure of intra-molecular globular domains at a critical shear stress of 35 ± 3.5 dyn/cm². The globular vWF conformation (149 nm by 77 nm and height 3.8 nm) is representative of native vWF after simple diffusion to the hydrophobic surface, followed by adhesion and some spreading. In a shear stress field above the critical value, protein unfolding occurs and vWF is observed in extended chain conformations oriented in the direction of the shear stress field with molecular lengths ranging from 146 to 774 nm and 3.4 nm mean height. The shear stress-induced structural changes to vWF suggest a close conformation-function relationship in vWF properties for thrombogenesis in regions of high shear stress.

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conditions using AFM. Quantitative shear forces are applied to vWF using the AFM probe tip and by using a modified rotating disk system. We use tapping mode AFM to minimize sample perturbation, modified scanning probe tips to improve lateral resolution of the protein, and mathematical morphology modeling techniques to account for tip broadening on measured lateral dimensions. We obtain accurate information on the three-dimensional structure of vWF under a range of applied shear conditions. Our results demonstrate and quantify the critical role of shear forces on the conformational state of vWF, and suggest there is a close structure-function relationship in vWF for platelet adhesion and thrombus formation in regions of high shear stress.

MATERIALS AND METHODS

vWF

Purified human vWF free of factor VIII (American Bioproducts, Parsippany, NJ) was reconstituted in phosphate buffered saline (PBS, 120 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L phosphate buffer, pH 7.4; Sigma Chemical Co, St Louis, MO) and stored at -70°C. The vWF (30 μg/mL) was analyzed for antigenic and functional vWF levels, residual factor VIII coagulant activity, and qualitatively by multimeric analysis. vWF antigen was analyzed by an enzyme-linked immunosorbent assay (ELISA; Asserachrom vWF, American Bioproducts) referenced against a standardized vWF plasma solution. vWF functional activity was assessed by the Ristocetin cofactor assay (Helena Laboratories, Beaumont, TX), performed on Helena PACKS-4 optical aggregometer by measuring the agglutination speed of fixed, lyophilized platelets when the sample was incubated with Ristocetin. Assayed, reference plasma (Cryo Check Normal; Precision Biologicals, Dartmouth, Nova Scotia, Canada) was used for the standard curve. Residual factor VIII coagulant activity in the vWF (diluted 1:1 with factor VIII deficient plasma) was determined using an activated partial thromboplastin time-based method using Actin FSL (Dade, Miami, FL) and an MLA 1600 automated coagulation analyzer (MLA, Pleasantville, NY). The vWF multimeric distribution was determined using a modification of the Laemmli discontinuous buffer system involving 1.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Type V-high gelling agarose, Sigma Chemical Co) with a 0.75% spacer-gel. Electrophoresis was carried out using a Biorad power supply (Richmond, CA) and LKB Multiphor 2117 chamber run at constant current (15 mA) for 90 minutes with a running buffer of 0.049 mol/L Tris, 0.38 mol/L glycine, and 1% SDS, pH 8.35. This was followed by Western blot detection with transfer of the multimers to nitrocellulose, reaction with a primary polyclonal rabbit antihuman antibody to vWF (Behring, Westwood, MA) and detection with a secondary goat antirabbit IgG alkaline phosphatase-conjugated antibody (Fisher Scientific, Pittsburgh, PA). The vWF multimeric pattern was compared with a normal human control sample.

Preparation of Hydropobic Substrates

To achieve high resolution atomic force microscopy (AFM) images of protein molecules under aqueous conditions, it is essential that the underlying substrate is adhesive with the protein and is sufficiently smooth so that protein can easily be detected. This was accomplished for vWF imaging using a hydrophobic self-assembled monolayer (SAM) prepared by depositing octadeucyltrichlorosilane (OTS) on to carefully cleaned glass coverslips. The coverslips were first cleaned by sonication in ACS grade chloroform (Fisher Chemical, Fair Lawn, NJ) for 30 minutes, followed by rinsing in a stream of chloroform. Coverslips were further cleaned by radiofrequency glow-discharge surface treatment using an Argon/H₂O discharge environment for 15 minutes on each side. The cleaned glass coverslips were placed into a 2 to 2.5 mmol/L solution of OTS (Aldrich Chemical, Milwaukee, WI) in dicyclohexyl (Aldrich Chemical) for 30 minutes reaction on each side. Both OTS and dicyclohexyl were vacuum distilled before use. Coverslips were removed with Teflon tweezers, rinsed in a stream of chloroform, sonicated in chloroform for 30 to 45 minutes, rinsed again with a chloroform stream, air dried, and stored in sealed fluoroware containers. Before use, OTS-SAM substrates were cleaned by sonication in chloroform for 15 minutes, followed by rinsing in chloroform and brief air-drying. The integrity and uniformity of the substrate was confirmed by contact angle analysis and AFM. The OTS-SAM substrates provide a highly hydrophobic (water contact angle ~110°) ultra-smooth surface (root mean square [RMS] roughness ~0.22 nm over 1 μm²).

AFM

All images of vWF were obtained in PBS using either contact mode or tapping mode AFM. A Nanoscope III Multimode AFM (Digital Instruments, Santa Barbara, CA) was used for contact mode imaging. Tapping mode imaging was performed using a Nanoscope III BioScope AFM (Digital Instruments) mounted on a Nikon Diaphot inverted microscope. Images were collected with 100 μm long silicon nitride (Si₃N₄) triangular cantilevers with integrated 5 μm tall pyramidal tips, or Si₃N₄ tips modified with an ultrasharp carbon spike. The manufacturer's reported spring constants for the cantilevers were 0.58 N/m or 0.38 N/m. We fabricated ultrasharp carbon tips by growing a carbon spike onto the apex of Si₃N₄ tips using a stationary focused electron beam at 20 to 25 kV accelerating voltage in a scanning electron microscope. The carbon spike tips have a high aspect ratio of ~10 and an ultrasharp tip radius of ~10 nm.

Contact mode AFM imaging of vWF. Protein samples were prepared in situ using a glass fluid cell attachment for the Multimode AFM. Before imaging, the cell was cleaned with an aqueous solution (0.5 g/L) of SDS (99%, Sigma Chemical) followed by rinsing with distilled water and anhydrous 100% ethanol (Pharmco products, Bayonne, NJ). The fluid cell was placed into an H₂SO₄/Nochomix (Godaix Laboratories, New York, NY) bath overnight, rinsed with tap water, distilled water, ethanol, and acetone (Fisher Chemical). OTS-SAM modified coverslips were attached to a 12-mm steel disk with a cyanoacrylate adhesive and dried overnight. The OTS-SAM was placed onto the AFM stage, and the fluid cell was placed over the top and sealed using a silicone rubber O-ring. PBS was injected into the cell through silicone tubing and polypropylene connectors. AFM images of the OTS-SAM substrate were obtained to confirm a smooth and uniform surface. vWF in PBS (24 ng/mL) was then introduced into the cell and allowed to adsorb for 60 minutes. The protein solution was then replaced by introducing fresh PBS into the fluid cell. AFM images were collected in constant force mode with 100-μm long triangular Si₃N₄ cantilevers with integrated pyramidal tips. In constant force mode operation, an angstrom-level deflection in the cantilever, caused by tip-sample surface contact, is detected by a laser beam reflected off the back of the cantilever onto a segmented photodiode. A three-dimensional image is obtained by rastering the probe tip across the sample surface (Fig 1). The tip is in constant contact with the surface during imaging. Typical adhesive forces acting down on the sample during imaging ranged from 14 to 36 nN, obtained by multiplying the cantilever spring constant by the distance required to cause the tip to snap free from the surface.

Tapping mode AFM imaging of vWF. Protein samples were prepared in glass petri dishes (50 mm diameter) mounted on the BioScope AFM sample stage. The petri dishes were cleaned with Isoclean and rinsed with tap water, distilled water, and ethanol. OTS-SAM substrates were attached to the petri dishes with a cyanoacry-
3D STRUCTURE OF vWF

Fig 1. Contact mode and tapping mode AFM imaging. In contact mode, a 3-D image is obtained by raster scanning the cantilever and attached probe, with respect to the sample surface, whereas changes in height are detected by a laser beam reflected off the cantilever onto a segmented photodiode. Shear forces are applied to the sample during scanning since the probe tip remains in constant contact. In tapping mode, the cantilever is oscillating vertically at high frequency during raster scanning. Interaction of the tip with the sample causes attenuation of the oscillation amplitude, which is used to monitor changes in sample height. Shear forces on the sample are negligible, because the lateral scanning movement of the probe occurs above the sample.

late adhesive and dried in a laminar flow hood. The petri dish was placed onto the Bioscope stage, and PBS (5 mL) was added. Images of the OTS-SAM substrate were obtained using a 100-μm triangular Si₃N₄ cantilever and integrated tip with an added carbon spike. After substrate imaging was completed, vWF was added to a final concentration of 24 ng/mL, and allowed to adsorb for 60 minutes. The protein solution was then replaced by sequential dilutions of PBS. After 60 minutes, AFM imaging was initiated.

In tapping mode (Fig 1), the cantilever is oscillating vertically at high frequency. The RMS amplitude of the cantilever oscillation is used as the feedback signal (analogous to the constant deflection used in contact mode). Interaction of the tip with the sample during scanning causes attenuation of the oscillation, which is referenced back to a control signal and used in a feedback loop that controls the sample position. A three-dimensional image of the sample is generated by recording the position of the sample required to keep the attenuation of the amplitude at a constant value. Shear forces on the protein are negligible in tapping mode and the chances of perturbing the sample significantly reduced because the probe is not in constant contact with the sample surface.

Controlled shear experiments. A modified rotating disk (RD) system (Pine Instruments, Grove City, PA), illustrated in Fig 2, was used to subject vWF to a well-defined range of applied shear stresses (0 to 42.4 dyn/cm²) representative of normal physiological conditions.44 The details of the basic system have been described previously.44 Under laminar flow conditions, the shear stress at the RD surface increases linearly with increasing radial distance from the center and is given by the vector sum of its radial and tangential components at any point on the surface, while the mass flux of protein is uniform across the entire disk surface.45

A clean, smooth OTS-SAM substrate (confirmed by AFM) was attached to a stainless steel disk (12-mm diameter) using cyanoacrylate adhesive and cured overnight. The sample disk was press-fit into a PTFE sample holder, and attached to the RD shaft by a polypropylene connector. The RD shaft was lowered into a flow chamber consisting of a 25 mL glass vessel with an attached upper fluid reservoir. PBS was added from the reservoir to submerge and equilibrate the disk. A stream of filtered (10 nm pore size) nitrogen was directed above the vessel to prevent deposition of atmospheric contaminants at the air-liquid interface. This was an important modification to our initial system that solved problems of particulate contamination observed on control OTS-SAM disks. After preliminary AFM experiments that confirmed a smooth and clean substrate,
vWF was injected into the PBS, yielding a final protein concentration of 50 ng/mL. Disk rotation of 1,000 RPM at 25°C was begun immediately and continued for 5 minutes. The calculated Reynolds number (Re) was ~8,000, well within the theoretical limits for laminar flow in this system. These conditions generated shear stresses ranging from 0 dyne/cm² at the disk center up to 42.4 dyne/cm² at a radial distance of 6 mm. The protein solution was gradually exchanged in fresh PBS (±250 mL) delivered from the reservoir. The sample disk was transferred from the vessel and immediately submerged in PBS in a petri dish with an attached Viton O-ring used to mount the disk. AFM images of vWF exposed to applied shear using the RD system were obtained at radial increments of 1 mm from the disk center, in tapping mode on the Bioscope AFM as described previously.

Data Analysis

AFM images were subjected to a 3 x 3 low-pass filter, zero order flattening, and a second order planefit. Dimensions of vWF molecules were measured by drawing a cross-sectional line along the molecular chain and recording the major axis length of each globular domain. The minor axis length was determined by drawing a line perpendicular to the major axis across the domain peak. Height measurements were determined by taking the average of the peak height measured in both the major and minor axis directions. Mean and standard deviation values of vWF molecule dimensions were calculated from measurements derived from a sampling number of not less than 8 for globular vWF and not less than 15 for extended vWF chains.

We used mathematical morphology to analyze AFM data and obtain improved resolution of vWF molecules. We have described the detailed methodology of this technique previously.20,26 AFM images were transferred in binary format to a Sparc 10 workstation (Sun Microsystems) for analysis using two image processing software systems (Dip-Station, HIPG, Boulder, CO; and Matlab, Mathworks, Natick, MA). The processing uses two basic functions: dilation and erosion. Assuming hard surface interaction between the sample and probe tip, dilation simulates the creation of an AFM image. This process substantially broadens the sample, but does not affect its height. Erosion reduces the effect of tip broadening and generates a higher resolution image. In order to analyze images of vWF, we first need to obtain information on the size and shape of the AFM probe tips. For this purpose, 18 nm gold beads (assumed ideal spheres) were imaged with Si3N4 and carbon fiber AFM probes, and using the measured height as the sphere diameter and the erosion function, we generated restored images of the probe tips. Figure 3 includes computer-generated reconstructions of Si3N4 and carbon fiber tip images used in AFM imaging. Three probes of each type were imaged and analyzed. Note the uneven surface of the Si3N4 tip as compared with the smooth, symmetrical, carbon tip which also has a smaller tip radius and a higher aspect ratio. The probe tip dimensions and asymmetric features such as those seen on the Si3N4 tip produce artifacts in AFM images, but these effects can be largely removed using mathematical morphology.

The computer-generated tip surfaces were used in the analysis of the AFM images of vWF. Initial models of vWF were generated using Interactive Data Language (IDL; Research Systems, Inc) on a Silicon Graphics workstation. Globular vWF was modeled as an ellipsoid. vWF dimer globular domains in extended chains were modeled as ellipsoids and connecting filaments as cylinders. To simulate AFM images, morphological dilations were performed on the models using the known probe tip surface. The model parameters were modified until an optimal fit was obtained between the predicted vWF dimensions and measurements obtained by AFM. Based on the information obtained from the iterated models, the dimensions of globular vWF, vWF dimer, and extended chains were obtained with improved resolution.

RESULTS

Functional analysis of vWF

The vWF at 30 μg/mL showed a Ristocetin Cofactor level of 0.21 U/mL, where 1.0 U/mL is equivalent to a plasma vWF activity of 100%. The vWF antigen level was 0.1 U/mL, where 1.0 U/mL is equivalent to a plasma vWF antigen level of 100%. Residual factor VIII coagulant activity was negligible, with less than 0.03 U/mL detected. vWF multimeric analysis is shown in Fig 4, in which the upper band in each lane is vWF dimer (n = 2), followed by bands n = 4, n = 6, etc. The vWF sample used in the AFM experiments consisted of middle molecular weight range multimers, with only sparse levels of low (n < 8) and high (n > 26) molecular weight multimers.

Molecular Scale Images of vWF

The data obtained directly from the AFM images overestimates the lateral dimensions of vWF, because the observed image is a nonlinear combination of both the protein molecule and the probe tip. In order to obtain more accurate dimensions for the imaged vWF, it is necessary to remove the contribution of the probe tip. This is accomplished using mathematical morphology42,46 (see Materials and Methods). By this approach, we analyzed the dimensions of globular vWF imaged under negligible shear force, vWF after probe tip-induced chain unfolding, and vWF after flow field induced chain unfolding. The improvement in resolution compared with the lateral dimensions of vWF measured directly from the AFM images ranged from ~4% for globular aggregates, and up to ~57% for smaller intra-molecular globular domains. The results for the dimensional analysis of the vWF molecules under the different experimental conditions are summarized in Table 1.

Globular vWF. Figure 5 shows AFM images of vWF obtained in fluid tapping mode using a carbon fiber probe. In order to clearly distinguish vWF molecules, the underlying substrate must be sufficiently smooth so that protein features, only a few nanometers in height, are not confused with features of the background, as shown by the ultra smooth and featureless OTS-SAM substrate, imaged under PBS (Fig 5a). Objects less than 1 nm tall can be easily detected on this surface by AFM. Figure 5b shows the same substrate region after vWF (24 ng/mL) adsorption from PBS solution. Numerous large and small globular structures of vWF are observed on the surface.

Qualitative observations of the images suggested two different size populations of globular vWF, which we confirmed by statistical analysis (F-test, P < .05) of the normal distributions of the major axis dimensions. Further calculations based on predicted vWF dimer volume compared with the volumes of imaged vWF indicate that the larger globular structures seen in Fig 5 are molecular aggregates (see Table 1 for dimensions). The smaller globular structures are single vWF molecules with average corrected dimensions (n = 25) of 149 ± 46 nm (major axis), 77 ± 28 nm (minor axis) and
Fig 3. Computer generated reconstructions of (a) Si$_3$N$_4$ and (b) carbon probe tips used in AFM imaging. To create the images, mathematical morphology is used to reconstruct the probe surface by taking an original AFM image of a known object (an ideal sphere) and eroding by the actual structure imaged. For this purpose, 18-nm gold beads (assumed ideal spheres) were imaged with the AFM probes, and the measured height used as the sphere diameter. The computer reconstructions shown are averages of three separate tips. Note the asymmetry of the Si$_3$N$_4$ tip as compared with the smooth and symmetrical carbon tip, which also has a smaller radius at the tip and a high aspect ratio.

A height of $3.8 \pm 1.6$ nm, consistent with multimer sizes ranging up to 26 (as seen in Fig 4) in isolated vWF molecules. These data are comparable with reported dimensions for native vWF conformation in solution determined by quasi-elastic light scattering$^{24}$ and for vWF sprayed on cleaved mica, vacuum dried, and measured by electron microscopy.$^{23-24}$ Slayter et al$^{24}$ reported an average maximal diameter of $140 \pm 80$ nm for vWF in the conformational state referred to as the “ball of yarn” form, corresponding to the native (latent) vWF structure in solution and the most commonly observed structure by electron microscopy. Our AFM images of vWF are obtained under aqueous buffer conditions, which lead us to suggest that the globular vWF conformation is representative of an unperturbed native state that has undergone simple diffusion to the hydrophobic substrate, followed by some spreading which is suggested by the small height.

**Probe tip induced unfolding of vWF.** One of the vWF molecules shown in Fig 5b (arrow) is in an extended conformation. We believe this structure resulted from the protein being “caught” by the scanning probe tip and pulled across the surface causing protein unfolding. A higher resolution scan of this extended chain, shown in Fig 5c, reveals the presence of individual globular domains (arrows). Some asymmetry in the shape of the globular domains is evident. We assume that this is caused by motion of the protein across the surface, since it follows the probe’s scanning direction. This observation suggests that the globular domains possess relatively high dimensional stability, which is not unexpected considering the protein’s numerous intramolecular disulfide linkages. A cross-section of three globular domains in the extended vWF conformation is shown in Fig 5d and indicates a measured inter-globular domain distance of 74.6 nm.

Figure 6 shows a series of AFM images similar to those shown in Fig 5, of vWF adsorbed on the hydrophobic OTS-SAM surface under PBS buffer, but imaged in contact mode AFM using a Si$_3$N$_4$ probe. The smooth and featureless hydrophobic OTS-SAM substrate before vWF adsorption is shown in Fig 6a. Figure 6b shows this same surface area after vWF adsorption. Extended chains of vWF are evident on the sample, confirmed by an increase in surface RMS roughness from 0.22 nm to 0.71 nm. The extended vWF were stable over repeated scanning and at 90° scan angles, which validated the images we obtained. In contrast to the previous tapping mode AFM experiments, all the vWF are now shown as individual molecules in extended chain conformations, formed by the shear force applied by the probe tip during contact mode imaging. A higher resolution image (250 nm full scale) of one vWF extended chain molecule is shown in Fig 6c, in which individual globular domains are seen (arrows). A cross-section of three globular domains is shown in Fig 6d, indicating the measured width (59.6 nm) and height (3.5 nm) of a single vWF globular domain.

We developed a model of vWF dimer based on measurements of over 100 globular domains in vWF molecules imaged with a Si$_3$N$_4$ probe in contact mode AFM. Our initial model of vWF dimer consisted of two globular outer domains linked to a smaller central domain modeled as ellipsoids, connected by fibrous rods modeled as cylinders. In Fig 7, the structure on the left is the predicted AFM image after dilation with the Si$_3$N$_4$ probe using mathematical morphology. Iteration of the model yielded predicted lateral dimensions of vWF globular domains that were within ±5% of the mean dimensions measured directly by AFM. Molecular height is not affected by morphological dilation with the AFM probe. Standard deviations in the dimer dimensions (see Table 1) were calculated based on analyzing the propagation of errors in the globular domain measurements obtained by AFM.$^{27}$ Figure 7 (right side) illustrates the vWF
Fig 4. Multimeric composition of the vWF sample determined by SDS-PAGE (1.5%) followed by Western blot detection with transfer of multimers to nitrocellulose, reaction with a primary antibody to vWF and detection with a secondary alkaline phosphatase-conjugated antibody. The specimen application point is at the bottom. Low molecular weight multimers migrate to the top of the gel, while high molecular weight forms are at the bottom. The pattern on the right is from a normal individual. The topmost band is vWF dimer (n = 2), subsequent bands are n = 4, n = 6, etc. The multimeric pattern of vWF used in AFM experiments shows mid-range multimers, and a relative absence of low (n < 8) and high molecular weight multimers (n > 26).

Table 1. Dimensions of vWF Conformations Imaged by AFM

<table>
<thead>
<tr>
<th></th>
<th>Length (nm)</th>
<th>Major Axis (nm)</th>
<th>Minor Axis (nm)</th>
<th>Height (nm)</th>
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<tbody>
<tr>
<td><strong>Globular vWF</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Single molecules</td>
<td>—</td>
<td>149 ± 46</td>
<td>77 ± 28</td>
<td>3.8 ± 1.6</td>
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<tr>
<td>Molecular aggregates</td>
<td>—</td>
<td>373 ± 74</td>
<td>279 ± 33</td>
<td>10.3 ± 3.2</td>
</tr>
<tr>
<td>vWF dimer†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Outer domains</td>
<td>—</td>
<td>73 ± 13</td>
<td>33 ± 12</td>
<td>2.8 ± 1.0</td>
</tr>
<tr>
<td>Central domain</td>
<td>—</td>
<td>38 ± 5</td>
<td>15 ± 2</td>
<td>2.9 ± 1.0</td>
</tr>
<tr>
<td>Fibrous rods</td>
<td>—</td>
<td>8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Unfolded vWF multimers‡</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Single molecules</td>
<td>373 ± 184</td>
<td>—</td>
<td>64 ± 23</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>146-774 range</td>
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Results shown are mean ± standard deviation, with n values in parentheses. Major and minor axis dimensions are corrected using mathematical morphology to account for the size and shape of the probe tip. Heights are not affected by morphological processing.

* Static adsorption, carbon probe tip, imaged in tapping mode AFM.
† Static adsorption, SiO₂N probe tip, imaged in contact mode AFM.
‡ Dynamic flow (35 ± 3.5 dyne/cm²), carbon probe tip, imaged in tapping mode AFM.
Fig 5. AFM tapping mode images of vWF on OTS-SAM substrate adsorbed under static aqueous conditions. Height scale range is from 0 nm (dark) to 10 nm (light). (a) Surface of smooth OTS-SAM hydrophobic substrate (5 μm scan) before vWF adsorption. (b) vWF on OTS-SAM substrate (same substrate as in [a]) after 1 hour vWF adsorption from solution (24 ng/mL) followed by exchange of protein solution with PBS and 1 hour adhesion time. vWF is observed primarily in globular conformations. The image also shows a single extended chain (arrow) that has been unfolded by the scanning probe tip. (c) High resolution scan of the extended chain shown in (b), showing the presence of individual globular domains (arrows). (d) Cross-section of three of the globular domains in the extended vWF chain, showing observed lateral dimension (74.6 nm) and height (2.5 nm).

Fig 6. AFM contact mode images of vWF on OTS-SAM substrate adsorbed under static aqueous conditions. Height scale range is from 0 nm (dark) to 10 nm (light). (a) Surface of smooth OTS-SAM hydrophobic substrate (2.5 μm scan) before vWF adsorption. (b) vWF on OTS-SAM substrate (same substrate as in [a]) after 1 hour adsorption with vWF solution (24 ng/mL) followed by exchange for PBS. Extended chains of vWF are evident, which are formed by the shear force (7.4 to 19 nN) applied by the silicon nitride probe. (c) High resolution image (250 nm full scale) showing individual globular domains (arrows) in an extended vWF chain. (d) Cross-sectional analysis of three globular domains in the extended chain shown in (c), showing observed lateral dimension (59.6 nm) and height (3.5 nm) for one globular domain.
lateral force is applied—the vWF molecule is extended. In all other observations no lateral perturbation to vWF molecules are evident. Comparison of the data obtained in tapping mode and contact mode AFM indicates that the applied shear force in contact mode was sufficient to cause a conformational change in the vWF molecules, from the large globular structures seen in Fig 5 to the extended chain structures seen in Fig 6.

Shear-induced unfolding of vWF. The contrasting AFM data of vWF we obtained under negligible and finite shear force applied by the probe tip suggested experiments under controlled shear conditions with imaging by tapping mode AFM. These experiments were carried out using a modified RD system which provides well defined shear stress conditions that increase linearly with distance from the disk center to the periphery, and in our experiments corresponds to 0 to 42.4 dyn/cm² (0 to 4.2 N/m²). We examined numerous areas on the disks in 1.0-mm increments extending from the center to the periphery. We obtained qualitative evidence for decreasing amounts of adsorbed proteins as our analysis progressed toward the disk periphery, but we found no evidence for extended vWF molecular chains, until we reached a radial distance of 5 mm. These data are shown in Fig 8. The image in Fig 8A was obtained at a radial distance from the disk center of 5.0 ± 0.5 mm corresponding to an applied shear stress of 35 ± 3.5 dyn/cm². The shear stress increases with radial distance (right to left in Fig 8), but the shear stress field is approximately in the perpendicular direction (top to bottom). The AFM image shows the presence of extended vWF chains (arrows) distributed among large vWF aggregates. The expanded region, shown in Fig 8B, clearly reveals several shear-induced extended vWF chains. The extended chains appear oriented in the direction of the shear stress field, with molecular lengths ranging from 146 nm to 774 nm. Cross-sectional analysis performed at numerous points within the chains indicated mean widths of 64 ± 23 nm and heights of 3.4 ± 0.7 nm (Table 1). The vWF chains have meandering conformations, and individual globular domains could not be identified in sufficient numbers for statistical analysis. In all lower shear stress regimes only aggregates of vWF were observed with no evidence for the single extended chain conformations. These data suggests a critical shear stress for vWF unfolding on a hydrophobic surface of $35 \pm 3.5 \text{ dyn/cm}^2$.

DISCUSSION

The functional properties of vWF in the regulation of hemostasis and thrombosis are surface dependent. Subendothelial collagen and the platelet membrane are two examples of important substrata. Furthermore, binding to platelet GP receptors under conditions of high shear stress is a known unique function of vWF. The results presented in this report on shear dependent changes in the three-dimensional structure of human vWF were all obtained under aqueous buffer conditions using AFM. Our results provide some appreciation for the physical properties of vWF and for conformational events influenced by substrate properties, aqueous media, and applied shear stress that may accompany known functional changes in the protein.

Of primary interest in this study was to determine the effects of applied shear stress on the conformation of vWF. The possibility of a conformational change in vWF after surface adsorption has been suggested to explain subsequent platelet adhesion. Similarly, a shear-dependent conformational change in vWF may increase the number of vWF-platelet interaction sites and the overall binding strength, and thus account for the unique function of vWF under high shear conditions. However, hypotheses concerning surface and/or shear-induced conformational changes in vWF have lacked supporting experimental evidence.

The results presented in this report provide the first illustration of shear-induced conformational changes in vWF. In Fig 9, we present a schematic illustration, using actual AFM images, that summarize the observed shear-induced changes in vWF on a hydrophobic surface under PBS media. Under negligible shear conditions (left side), vWF has a globular conformation comparable in mean maximal dimension with the native solution conformation. We suggest that this conformation is representative of unperturbed native state vWF that has undergone simple diffusion to the hydrophobic sub-
Fig 8. AFM images of vWF adsorbed on OTS-SAM under aqueous conditions in the presence of known applied shear stress and imaged in tapping mode. Height scale range is from 0 nm (dark) to 10 nm (light). We used a modified rotating disk system to apply known shear stresses (0 to 42.4 dyn/cm²). (a) This image was obtained in the shear stress region of 35 ± 3.5 dyn/cm² and shows the presence of extended vWF chains (arrows) distributed among large vWF aggregates. The shear stress increases with radial distance (right to left) but the shear stress field is approximately in the perpendicular direction (top to bottom). This shear stress regime is the lowest in which extended chains are observed. (b) Expanded region showing higher resolution image of several shear-induced extended vWF chains seen in (a). The extended vWF molecules are oriented in the general direction of the shear stress field.

Fig 9. Schematic model using actual AFM images of vWF illustrating shear-induced conformational changes in vWF under aqueous conditions. The three images of vWF are on the same scale so they are directly comparable. Under negligible shear (left side), vWF has a globular conformation on the hydrophobic surface, comparable in maximal dimension with the native solution conformation. Shear forces (center) (7.4 to 19 nN) applied by the AFM probe tip, cause protein unfolding, and the vWF has a short extended chain conformation in which individual globular domains are visualized. After exposure to a shear stress field of 35 ± 3.5 dyn/cm² vWF is observed in extended conformation (right side), with molecular length ranging from 146 to 774 nm.
strate, followed by some spreading. Under the effect of shear forces (7.4 to 19 nN) applied by the AFM probe (center), protein unfolding occurs and vWF exhibits an extended chain conformation in which individual globular domains are visualized. Under the effect of a shear stress (right side) greater than 31.5 dyn/cm², protein unfolding occurs and vWF is observed in a meandering extended conformation, oriented in the general direction of the shear stress field. Thus, the shear force applied by the probe tip or a known shear stress field applied by the RD system leads to vWF unfolding and extended chain conformations.

It is clear from our experiments that vWF binds well to hydrophobic surfaces through noncovalent, nonspecific forces. This observation is a unique consequence of imaging vWF in aqueous media. The OTS molecules in the SAM substrate are uniformly terminated by methyl groups, which produces a nonionized, highly hydrophobic surface. Hydrophobic amino acid residues in vWF will adhere to the surface if the energy gain in the configurational entropy of water is favorable. This is the case for vWF, as shown by the protein’s stability under the different imaging conditions. The adhesive interaction is quite strong, sufficient to withstand the applied shear force of the probe or the applied shear stresses of the RD system, thus permitting three-dimensional molecular scale imaging by AFM. Hydrophobic interaction is nonspecific, and in the present context, this means that vWF will show similar adhesive behavior to any exposed surface that is hydrophobic, including artificial surfaces used in a range of cardiovascular devices and components of atherosclerotic plaque or subendothelium that exhibit hydrophobic or lipid-like properties. The overall strength of the interaction will be proportional to the summed area contained in all protein-surface contacts. Our results indicate that adhesive interaction with hydrophobic surfaces is an intrinsic physical property of vWF. This may provide a plausible nonspecific adhesive mechanism for augmenting specific binding interactions of vWF with substrate such as subendothelial collagen.

A second key property of vWF that is critical for maintaining surface adhesion under applied shear is the protein’s high molecular weight. This provides a mechanism for increasing the number of hydrophobic-hydrophobic contact points that contribute to its adhesion and resistance to applied shear forces. Since the vWF subunit structure repeats down the multimeric protein, the number of contact points, and hence, adhesion stability, will be in proportion to the number of dimer subunits in the vWF multimer. The nature of the intermolecular adhesion is different from binding to the subendothelial matrix in vivo, but the consequences of applied shear should be similar. In other words, changing the adhesion mechanism (eg, hydrophobic to electrostatic) will not change the outcome—that unfolding can occur once the critical shear stress is exceeded. The fact that vWF is multimeric increases the probability of the critical shear stress values being similar, because there are multiple potential binding sites by either mechanism.

The elongated form of vWF should be stable, although minor changes (eg, spreading) may continue as the protein approaches the interfacial free energy minimum with the surface. Only if all of the multiple adhesive bonds with the surface were broken simultaneously, would this permit the protein to return to solution. The probability of this occurring increases with decreasing molecular weight of the vWF molecule (ie, the smaller multimers will detach more easily). One would then expect the lowest energy solution form (ie, globular) to be the dominant conformation. This critical role of molecular weight is further exemplified by AFM experiments we have performed with fibrinogen under similar conditions (data not shown). Images of fibrinogen are not obtained in the presence of an applied shear force as in contact mode AFM, because the fibrinogen is easily swept away by the probe tip. Under similar applied shear forces, vWF exhibits much greater adhesive stability than fibrinogen.

During the RD experiments, vWF is transported in uniform flux to the entire disk surface. The transported protein will experience shear stress only in a thin layer of fluid immediately adjacent to the RD surface given by the vector sum of its radial and tangential components at any point on the surface. The shear stress decays rapidly away from the surface. Thus, under these experimental conditions, the conformational change does not occur in the bulk solution, but only after the vWF has adsorbed to the surface and is subjected to a shear stress above the critical value.

In relating the possible significance of our results to the physiological situation in vivo, it is important to appreciate the effect of solution viscosity, because it is substantially higher in whole blood compared with the PBS used in our in vitro experiments. The absolute viscosity (µ) of PBS is 0.00682 poise, while the value for platelet rich plasma (PRP) is 0.011 poise and for whole blood is 0.035 poise. Thus, if the RD experiments could be performed at a higher solution viscosity analogous to PRP, we should observe the conformational change at a decreased radial distance, but the critical shear stress value should not change. What would change is the critical wall shear rate (γ), which, for a Newtonian fluid, is related to the shear stress (τ) by the relation: τ = γµ. Thus, we can estimate the critical shear parameters for the conformational transition in vWF at an adhesive (thrombogenic) surface in PRP to be a shear stress of 35 ± 3.5 dyn/cm² with a corresponding wall shear rate of 3,181 ± 318 sec⁻¹. This wall shear rate drops to ~1,000 sec⁻¹ in whole blood, a non-Newtonian fluid. These shear rate values are in the range in which vWF becomes predominant over fibrinogen as the principal mediator of platelet adhesion. This provides us with some confidence in the physiological significance of the critical shear stress value we obtain for the vWF conformational transition.

While perturbations such as substrate properties and media conditions can cause significant alterations in the conformation of a large polymeric globular protein such as vWF, it is also important to recognize possible concomitant temporal effects. Our images were obtained at least 1 hour after surface adsorption, which allowed time for the vWF to maximize adhesive interactions. Adsorption to a surface is a dynamic process that inevitably will involve time-dependent conformational changes in the vWF. In our images, this is manifested as evidence for spreading of the vWF over the surface, a process that is constrained by disulfide linkages.

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and intra-molecular hydrogen bonding that impart dimensional stability. One consequence of temporal effects is that our results show deviation from the dimensions of vWF determined by electron microscopy.\textsuperscript{21-24} Globular vWF we observed under negligible shear force shows some evidence for spreading, although the mean maximal dimension of 149 nm is not statistically different from results obtained by electron microscopy. However, our images of unfolded vWF observed under applied shear force show marked deviation from electron microscopy results, which we attribute to protein spreading. In addition, we do not detect the 2 nm diameter fibrous inter-globular domain region in images of extended vWF. The fibrous regions may well be present, as our model dimer (Fig 7) indicates, but the AFM tip radius is too large to probe between spreading globular domains.

The results presented in this report demonstrate that vWF undergoes a shear stress-induced structural transition from a globular state to an extended chain conformation with exposure of individual globular domains. On a hydrophobic surface in PBS, the conformational transition occurs in the critical shear stress regime of >31.5 dyn/cm\textsuperscript{2}. vWF exhibits strong adhesion with hydrophobic surfaces through nonspecific interactions, which suggest a possible structurally dependent adhesive mechanism that may aid specific vWF binding to biological substrata. Intra-molecular globular domains visualized in extended chain conformations exhibit dimensional stability, but undergo spreading on the hydrophobic surface. A model of surface-adsorbed vWF dimer was developed using measurements obtained after exposure of vWF to high shear force. The shear stress-induced conformational changes in vWF suggest a close structure-function relationship in vWF for platelet adhesion and thrombus formation in regions of high shear stress. It remains to be determined if the vWF extended chains we observe, and indeed which specific regions of the chain, exhibit the expected functional properties that facilitate binding to platelet GP receptors. However, the ability to determine three-dimensional protein structure at a submolecular level under aqueous conditions is provided by AFM and this approach offers unique opportunities for direct correlation of structural and functional events. The results of the present study may provide new insights in progress toward elucidating vWF structure-function relations under shear and in developing a comprehensive understanding of the pathophysiology of thrombogenesis.

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