Translational Science

Direct Observation of von Willebrand Factor Elongation and Fiber Formation on Collagen During Acute Whole Blood Exposure to Pathological Flow

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Objective—In severe stenosis, von Willebrand Factor (vWF) experiences millisecond exposures to pathological wall shear rates ($\gamma_w$). We sought to evaluate the deposition of vWF onto collagen surfaces under flow in these environments.

Methods and Results—Distinct from viscometry experiments that last many seconds, we deployed microfluidic devices for single-pass perfusion of whole blood or platelet-free plasma over fibrillar type I collagen (<50 ms transit time) at pathological $\gamma_w$ or spatial wall shear rate gradients (grad $\gamma_w$). Using fluorescent anti-vWF, long thick vWF fibers (>20 μm) bound to collagen were visualized at constant $\gamma_w$>30 000 s$^{-1}$ during perfusion of platelet-free plasma, a process enhanced by EDTA. Rapid acceleration or deceleration of EDTA platelet-free plasma at grad $\gamma_w$=±1.1×10$^7$ to ±4.3×10$^7$ s$^{-1}$/cm did not promote vWF deposition. At 19 400 s$^{-1}$, EDTA blood perfusion resulted in rolling vWF–platelet nets, although blood perfusion (normal Ca$^{2+}$) generated large vWF/platelet deposits that repeatedly embolized and were blocked by anti-glycoprotein Ib or the $\alpha_{\mathrm{IIb}}\beta_3$ inhibitor GR144053 and did not require grad $\gamma_w$. Blood perfusion at venous shear rate (200 s$^{-1}$) produced a stable platelet deposit that was a substrate for massive but unstable vWF–platelet aggregates when flow was increased to 7800 s$^{-1}$.

Conclusion—Triggered by collagen and enhanced by platelet glycoprotein Ib and $\alpha_{\mathrm{IIb}}\beta_3$, vWF fiber formation occurred during acute exposures to pathological $\gamma_w$ and did not require gradients in wall shear rate. (Arterioscler Thromb Vasc Biol. 2013;33:105-113.)

Key Words: glycoprotein Ib ■ hemodynamics ■ platelet ■ stenosis ■ von Willebrand Factor

Severe arterial stenoses are sites of extreme flow acceleration and deceleration, where pathological wall shear rates ($\gamma_w$) can range from 5000 to >100 000 s$^{-1}$ (wall shear stresses of 150 to >3000 dyne/cm$^2$). More than 75% stenosis is required before a significant flow resistance causes symptoms of angina.$^8$ Severe stenosis or occlusion can result in a flow recirculation zone in the poststenotic region, with a transition to turbulence and vortex shedding during the deceleration phase of diastole.$^5,8$

Sites of flow disturbance resulting from complex secondary flows, oscillatory slow flow, reversing flows, or extreme pathological shear rate gradients are correlated with endothelial dysfunction.$^9$ These sites are also predisposed to atherosclerosis, plaque rupture, platelet activation, and thrombosis. Peak spatial gradients in $\gamma_w$, grad $\gamma_w$=600 000 s$^{-1}$/cm (grad $\tau_w$=20 000 dyne cm$^{-2}$ cm$^{-1}$), are predicted for the inlet to a human coronary artery stenosis.$^1$ At the exit of a human coronary stenosis, maximal grad $\gamma_w$ approaches ~3×10$^9$ s$^{-1}$/cm.$^3$ Similarly, Schirmer and Malek$^4$ reported grad $\tau_w$=±20 000 dyne cm$^{-2}$ cm$^{-1}$ in human carotid stenosis (grad $\gamma_w$=±570 000 s$^{-1}$/cm).

At sites of arterial injury, von Willebrand factor (vWF) greatly facilitates platelet capture via tethering through glycoprotein Ib (GPIb) binding.$^{10}$ After binding collagen type I or type III, vWF can undergo a conformational change that enhances exposure of the A1 domain to promote GPIb interaction with platelets.$^{11}$ The binding of vWF A1 domain to platelet GPIb is essential for platelet adhesion at physiological arterial wall shear rates (>1500 s$^{-1}$). In patients with von Willebrand disease (vWD) type 3, a condition in which almost no detectable vWF is present, a severe bleeding phenotype is observed.$^{12}$

When sheared continuously in a cone-and-plate viscometer, high concentrations of soluble vWF (100 μg/mL) will undergo aggregation into enormous fibrous aggregates of 32×10$^4$ molecular weight (MW) (2155 s$^{-1}$ for 30 seconds) to 847×10$^6$ MW (6000 s$^{-1}$ for 120 seconds).$^{13}$ Similarly, shearing in a cone-and-plate viscometer at >2200 s$^{-1}$ for 3 minutes will result in shear-induced platelet activation, a phenomenon that requires vWF and platelet release of ADP.$^{14-17}$ Ultralarge vWF (uLvWF) is particularly potent in mediating shear-induced platelet activation as observed in a cone-and-plate viscometer.$^{18}$ Recirculation of a vWF solution in an air-exposed, piezodriven recirculation results in vWF unfolding and extension at a critical shear rate >5000 s$^{-1}$, but the time of exposure needed to observe this response is unknown, and the effect of the air–liquid interface is potentially problematic in the study of
proteins. Steppich et al used 10 to 20x physiological levels of vWF (200 μg/mL) exposed to high shear for a few minutes to create vWF fibril aggregates. Similarly, Barg et al detected vWF fiber formation on collagen after 1-minute perfusion of 10x physiological levels (50 μg/mL) of vWF at 35 dyne/cm² (∼5000 s⁻¹), which was not observed at physiological levels of 5 μg/mL vWF.

In contrast to a viscometer or closed-loop recirculation systems, blood is never continually experiencing pathological shear stresses in vivo for many seconds or minutes. Rather, the exposures are extremely brief, and the unfolding or fibrillar forming behavior of vWF during exposure to pathological flows is not well studied under such conditions. Using a stenosis microfluidic device, Nesbitt et al reported a role for shear rate gradients as a causative mechanism in local platelet accumulation, which was GPIb-dependent; however, the underlying role of vWF structural changes was not explored.

Interestingly, platelet display of P-selectin downstream of a human stenosis can increase relative to the upstream position. Also, patients with severe aortic stenosis (wall shear stress of 118 dyne/cm² or ∼4000 s⁻¹ shear rate) display a depletion of the largest vWF multimers to yield an acquired von Willebrand syndrome. Similarly, left ventricular assist device patients can experience an acquired vWD. As a shear-sensitive molecule, vWF undergoes a conformational change in these extreme hemodynamic environments, enhancing exposure of a concealed site, the A2 domain, which is susceptible to cleavage by the metalloprotease, ADAMTS13. At shear rates exceeding 15000 s⁻¹, rolling aggregates of discoid platelets have been observed in flow chambers on surfaces of vWF and collagen. These aggregates were independent of integrin function and required soluble vWF. In vivo aggregates of discoid platelets have also been generated downstream of an artificial stenosis in the mouse mesenteric arteriole. In addition to GPIbα/vWF interactions, these thrombi were dependent on αIIbβ3 interactions, suggesting a role for integrin activation. These prior studies, however, did not investigate the structure of vWF during the creation of thrombotic entities.

The present study explores platelet-free plasma (pPF) and whole blood exposure to pathological shear rates during millisecond exposures in a microfluidic injury model of surface fibrillar collagen. We observed that vWF, detected via epifluorescence microscopy, forms massive fiber aggregates on collagen type I surfaces under pathological wall shear rate conditions in plasma and whole blood. When platelets are present, platelet-vWF deposits with elongated and aggregated vWF can be achieved at pathological shear rates even in the absence of Ca²⁺. Unstable and rolling aggregates that contain significant amounts of vWF as fibrous aggregates were also observed. We demonstrate that high shear rate environments lead to vWF deposition, as well as massive and unstable platelet aggregation on platelet surfaces in the presence of Ca²⁺. These results represent the first real-time visualization of massive vWF fiber deposition under pathophysiologically relevant conditions supported by collagen. Platelet interactions with these vWF aggregates range from firmly adhered monolayers to massive thrombi with embolic potential.

Materials and Methods

Blood Collection and Preparation

Blood was drawn via venipuncture from healthy men, who self-reported as free of any disease or bleeding disorders, as well as any oral medication, for at least 10 days. All blood donors provided informed written consent in accordance with the Internal Review Board of the University of Pennsylvania. Blood was anticoagulated with sodium citrate (Sigma, St. Louis, MO) or d-Phe-Pro-Arg chloromethylketone (PPACK, 100 μmol/L; Haematologic Industries, Essex, VT) to inhibit thrombin. PFP was generated by centrifugation of whole blood at 1000g for 10 minutes. Severe vWD plasma was from George King Biomedical (Overland Park, KS) and contained <1% detectable vWF. For fluorescent detection of vWF, all samples were treated with a fluorescein isoionicate-conjugated polyclonal anti-vWF antibody (1 μg/mL; AbD Serotec, Kidlington, United Kingdom). Some samples were treated with 5 mM EDTA (Sigma) to chelate calcium or 40 μg/mL of function-blocking anti-GPIb antibody AK2 (Abcam, Cambridge, MA). When platelet deposition was measured in real-time epifluorescent microscopy, 0.125 μg/mL of fluoresecin conjugated monoclonal antibody HIP8 (BD Biosciences, San Jose, CA) was added. In some experiments, the αIIbβ3 antagonist, GR144053 (Tocris Biosciences, Bristol, United Kingdom) was added (final concentration, 2 μmol/L) to block αIIbβ3 function.

Microfluidic Flow Experiments

Two types of microfluidic devices were used in this study. First, the straight-channel microfluidic device had a 100-μm wide×60-μm high cross section generated in poly(dimethylsiloxane) (Sylgard; Dow Corning, Midland, MI) as previously described (Figure IA in the online-only Data Supplement). The second type of microfluidic device, the stenosis channel, consisted of a 500-μm wide×60-μm high channel that rapidly constricted to a 15-μm wide×1000-μm long channel (Figure IB and IC in the online-only Data Supplement), before expanding again to a 500-μm wide channel. The entrance and exit to the stenosis region tapered and expanded over a 100-μm distance in an identical nonlinear fashion to prevent boundary layer separation. Whole blood or PFP samples were perfused through either device via withdraw using a syringe pump (Harvard Apparatus, Holliston, MA). Surfaces of equine collagen type I (Chronopar; Chronolog, Havertown, PA) were presented to the flow by using a previously described surface-patterning technique (Figure II in the online-only Data Supplement). Briefly, a microchannel, 250-μm wide by 5-cm long (which runs perpendicular to flow channels), was affixed to a glass substrate and was filled with a 1 mg/mL collagen solution and immediately rinsed with 0.5% BSA (Sigma) in HEPES-buffered saline (30 mMol/L HEPES, 150 mMol/L NaCl, pH 7.4; Sigma). The patterning device was removed, and the microfluidic device for blood or plasma perfusion was placed on top of the collagen strip. The flow channels were blocked with BSA for at least 30 minutes before sample perfusion. Real-time deposition of vWF fibers was visualized using epifluorescence microscopy. In plasma experiments, vWF fibers were deposited and subsequently washed in BSA, stained with the fluorescently labeled anti-vWF, and visualized.

In whole blood experiments, vWF fiber formation was visualized in real time by direct addition of the fluorescent antibody to the perfusion solution. Microfluidic chambers were mounted on an Olympus IX81 inverted microscope (Olympus America, Center Valley, PA) equipped with a charge-coupled device camera (Hamamatsu, Bridgewater, NJ).

Wall shear rates were calculated by evaluating the analytic solution to flow in a rectangular channel using a custom MATLAB (Mathworks, Natick, MA) script. Furthermore, a 3-dimensional model of the stenosis channel was generated in COMSOL (Burlington, MA) to determine the flow field and wall shear rate in the more complicated entrance and exit regions. COMSOL simulations were confirmed to reproduce the analytic results for steady flow in the straight rectangular regions of the stenosis microfluidic device. The spatial gradient of wall shear rate along the centerline is defined as grad γ = γ|y=0 = γ|y=0 + |y=0|∂y/∂|y=0| where the centerline wall shear rate is γ = γ = γ|y=0 + |y=0|∂y/∂|y=0|.
Quantification of vWF Fiber Density and Length
Density of vWF fibers on patterned collagen surfaces was assessed by measuring the number of fibers intersecting a line drawn perpendicular to the flow direction (we refer to this as a line scan). Unless noted, vWF density represents the average of 3 line scans drawn at 10%, 50%, and 90% of the height of the image. Fiber length was assessed by measuring the length of all fibers that intersect the line scan drawn at 50% of the image height.

Results

vWF Fibers Deposit on Collagen Type 1 Surfaces Under Plasma Flow: Effect of Spatial Gradients
Fibrillar collagen type 1 (250 μm wide) was patterned across the inlet to the 15 μm region of the stenosis channel or across the outlet from the 15 μm region of the stenosis channel. A total of 20 μL of PFP was treated with 5 mmol/L EDTA and perfused through the channels at 3 flow rates: 0.5, 2, or 20 μL/min. These plasma flow rates resulted in maximum centerline wall shear rates of 3000, 12 500, and 125 000 s⁻¹, respectively. Based on the velocity at a height of 150 nm above the surface, plasma transit time across the 250μm collagen patch was 231 ms for a channel cross section of 500 μm and 38 ms at a 15 μm cross section for a 20 μL/min perfusion. After the 20 μL of plasma was perfused, the surface was rinsed with 0.5% BSA in HEPES-buffered saline and treated with fluorescently labeled anti-vWF and rinsed again for visualization.

Computational fluid dynamics revealed a very steep centerline wall shear rate gradient (grad γw) into the constricting and out of the expanding regions of the stenosis microfluidic device (Figure 1A–1C). For a 20 μL/min perfusion, this gradient (grad γw) was calculated to be ±4.3×10⁷ s⁻¹/cm. At 2 μL/min perfusion, the centerline gradient was ±4.3×10⁶ s⁻¹/cm and at 0.5 μL/min, the gradient was ±110 000 s⁻¹/cm.

For centerline wall shear rates of 3000 (Figure 2A and 2D) and 12 500 s⁻¹ (Figure 2B and 2E), <5 fibers per line scan were observed, and grad γw of ±110 000 to ±4.3×10⁶ s⁻¹/cm did not enhance fiber formation in the entrance or exit regions of the stenosis (Figure 2G and 2H). At 20 μL/min, the maximum centerline gradient in wall shear rate was ±4.3×10⁷ s⁻¹/cm (Figure 2C and 2F). In the shear gradient region, fiber generation was restricted to regions where the local wall shear rate was >±30 000 s⁻¹ (Figures 1A, 2C, and 2F). There was no marked difference (Figure 2G and 2H) between vWF deposition in the inlet region, the outlet region, or the constant width region (distal to a narrowing or distal to an expansion). The elevated shear rate environment produced long vWF fibers (>100 μm). A constant wall shear rate of 125 000 s⁻¹ was sufficient to cause vWF elongation and fiber formation on the surface collagen. An extreme wall shear rate gradient of −4.3×10⁷ s⁻¹/cm for a rapidly decelerating flow actually quenched vWF deposition (Figure 2F).

vWF Fibers Deposit on Collagen Type 1 Surfaces Under Plasma Flow: Effect of Calcium
Because vWF and its cleaving protease, ADAMTS13, have calcium-sensitive domains, we sought to understand the role of Ca²⁺ in the fiber-deposition process. We compared citrated plasma treated with an additional 5 mmol/L EDTA (≈2 mmol/L free Ca²⁺) to recalcified, citrated PFP treated with PPACK to prevent thrombin generation. In these experiments, collagen patterning was restricted to the region of 15 μm channel width (Figure 3H, inset). We observed that vWF fibers formed under EDTA conditions were continuous strands >300 nm in width and 100 μm in length at the highest shear rate tested (125 000 s⁻¹; Figure 3C, 3H, and 3I). At lower shear rates (31 200 and 62 400 s⁻¹), the deposition of vWF was significantly reduced, as was fiber length (Figure 3A, 3B, 3H, and 3I). In recalcified, citrated PFP with PPACK, we observed a significant increase in the number of deposited vWF fibers between the lowest and highest shears tested.

Figure 1. Large wall shear rate gradients are generated in the inlet and outlet of a novel microfluidic model of stenosis. A, Computational fluid dynamics defined the wall shear rates in the outlet of the stenosis channel. B, A representation of the stenosis channel in COMSOL. The colors indicate local wall shear rate and are equivalent to the scale bar in A. C, The local wall shear rate along the centerline of the stenosis channel (dotted white line in B) indicates a steep gradient in centerline wall shear rate (1000–125 000 s⁻¹) at the inlet and outlet of the stenosis (grad γw of ±4.3×10⁷ s⁻¹/cm).
At the highest shear rate tested (125 000 s⁻¹), the fiber morphology was disjointed compared with EDTA-treated samples, consisting of many strings of different lengths resulting in an average fiber length of 50 μm (Figures 3F and 3I). Citrated PFP (≈40 μm Ca²⁺) performed identically to PPACK plasma in this experiment (not shown). These results demonstrate that vWF elongated and formed fibers on collagen in a platelet-independent manner, but high wall shear rates (those >30 000 s⁻¹) were required. Removal of calcium enhanced the deposition process. As expected, no deposition of vWF fibers was detected at 125 000 s⁻¹ in vWD plasma (Figure III in the online-only Data Supplement).

To confirm that accelerating flows at the stenosis inlet were not strictly necessary for vWF fiber deposition, these experiments were repeated using a constant width device lacking a stenotic region. For both EDTA-treated plasma and EDTA/anti–GPIb-treated whole blood, vWF elongation on collagen was observed (Figures IV and V in the online-only Data Supplement), demonstrating that exposure to an elongational flow was not strictly required for vWF fiber formation on collagen.
In the presence of Ca\(^{2+}\), functional integrins led to rapid platelet adhesion and embolization (Figure 4A, PPACK). Fibers were observed, although large masses of platelets also stained positive for vWF (Movie II in the online-only Data Supplement). To block \(\alpha_{\text{IIb}}\beta_{3}\) platelet–platelet interactions via fibrinogen, a small-molecule inhibitor of \(\alpha_{\text{IIb}}\beta_{3}\) was used (2 \(\mu\)mol/L, GR144053; Figure VII in the online-only Data Supplement). This allowed us to replicate the integrin-blocking effects of EDTA without chelating Ca\(^{2+}\). When \(\alpha_{\text{IIb}}\beta_{3}\) was inhibited in the presence of Ca\(^{2+}\), large platelet masses were no longer present and faint fibers were seen adhering to the surface at 19.400 s\(^{-1}\) (Figure 4A, PPACK+GR144053). The vWF fiber formation (normal Ca\(^{2+}\)) was more evident when the wall shear rate was increased to 38.800 s\(^{-1}\) (Figure 4A). The dependency on \(\gamma_{c}\) of vWF deposition in the presence of PPACK and GR144053 is shown in Figure 4B. At the lowest shear rate tested (11.600 s\(^{-1}\)), few fibers were observed, although several collagen fibers did stain positively for vWF. Increasing the shear rate to 15.300 s\(^{-1}\) revealed a modest increase in vWF fibers, although fibers >50 \(\mu\)m required shear rates of 19.400 s\(^{-1}\). When the wall shear rate was increased to 38.800 s\(^{-1}\), many vWF fibers were generated on the surface, as well as rolling clumps of vWF and platelets.

Figure 4C presents time course data of vWF fluorescence on the collagen surface under perfusion of whole blood with normal Ca\(^{2+}\) (PPACK), PPACK plus GR144053, and PPACK plus 40 \(\mu\)g/mL of a function-blocking antibody against GPIb (AK2). In the absence of inhibitors, large platelet aggregates that were highly vWF-positive (Figure 4C, PPACK) were repeatedly generated and embolized rapidly. With \(\alpha_{\text{IIb}}\beta_{3}\) antagonism (2 \(\mu\)mol/L GR144053), larger fibers, as well as nets of vWF and platelets, were detected (Figure 4C, PPACK+GR144053); however, large thrombi were not, resulting in a significant reduction in vWF signal (Figure 4C). In the presence of AK2, formation of vWF fibers was not blocked; however, the large platelet masses and rolling nets of vWF and platelets were completely abolished. This resulted in a significant reduction in vWF signal, as well (Figure 4C, PPACK+ab[AK2] and Figure VIII in the online-only Data Supplement). The presence of platelets in whole blood enhanced the formation of vWF fibers on collagen surfaces under conditions of normal Ca\(^{2+}\) (Figure 3B and 3D) for single-pass transit time exposures of 3.4 to 61.5 ms.

### vWF Mediates Rapid and Embolic Platelet Adhesion at Pathological Shear Rates

Whole blood anticoagulated with PPACK (normal Ca\(^{2+}\)) and treated with fluorescently conjugated anti-vWF and anti-CD41a to label platelets was perfused over a collagen type 1 surface at a venous wall shear of 200 s\(^{-1}\). Platelet adhesion had reached a steady state after 1500 seconds of perfusion (Figure 5A[a] and 5C). Very little vWF immunofluorescence and...
no elongated vWF fibers were observed in the platelet mass formed at venous shear rate (Figure 5B[a] and 5D). At ≈1600 seconds, the flow rate was suddenly increased such that the nominal wall shear rate would have been 7800 s⁻¹ in an empty channel, but was several fold higher here because of channel obstruction resulting from the platelet mass. Within 100 seconds of the shear rate increase, massive platelet accumulation had occurred, embolized, and rebounded (Figure 5A[b–d]). However, upon shear increase, the large platelet masses formed on top of the steady-state surface incorporated vWF (Figure 5B[b and d]). The vWF fluorescence data presented in Figure 5D were normalized to the 1530-second time point for all conditions. The large platelet masses seen with PPACK vanish with the addition of either inhibitor.

Figure 4. von Willebrand Factor (vWF) fibers deposit on collagen type 1 surfaces during whole blood flow perfusion in a straight-channel geometry. A, Whole blood was perfused in the presence of EDTA or normal Ca²⁺ with or without GR144053 to block integrin function at the indicated wall shear rates. At 120 seconds in the absence of Ca²⁺, long fibers of vWF are detected on the surface, as well as rolling aggregates of vWF and platelets. In the presence of Ca²⁺, large platelet aggregates form, which embellished rapidly and incorporated vWF. In the absence of integrin function, large aggregates no longer formed, but faint fibers of vWF were seen on the surface, consistent with our previous results indicating that normal Ca²⁺ led to less vWF than EDTA conditions. At 2-fold elevated shear rates, vWF was observed both in platelet aggregates (functioning integrins) and on the surface (blocked integrins). B, vWF fiber deposition was a shear-dependent process under normal Ca²⁺ in the absence of integrin function. At the lowest shear tested, firmly adherent platelets were observed (arrow), although vWF was not detected. With increasing shear, more vWF was observed on the collagen surface. At the highest shear rate tested, rolling aggregates of vWF and platelets were observed (arrow). C, vWF fluorescence was measured over a 150-second perfusion of untreated whole blood (α-Phe-Pro-Arg chloromethylketone [PPACK]) at 38 800 s⁻¹ or samples treated with GR144053 and a glycoprotein Ib (GPIb) function-blocking antibody (AK2). The results indicate that the large platelet masses formed on the collagen surface enhance vWF capture and require both active αIIbβ₃ integrin and GPIb. The representative images (right) demonstrate the vWF fluorescence at the 120-second time point for all conditions. The large platelet masses seen with PPACK vanish with the addition of either inhibitor.

Discussion
Long and thick fibers of vWF were visualized as they formed on collagen type 1 surfaces under pathological shear rate conditions. These fibers were present, regardless of the use of fluorescent antibody during the experiment or after perfusion. The observed fibers were collagen-bound. Given their fluorescence width of several pixels (>300 nm), the observed elongated vWF fiber bundles were highly unlikely to be individual extended vWF molecules (30 nm width <1 pixel). Importantly, we observed that these vWF fibers were in a quenched state (ie, bound to collagen), such that they did not relax when flow was stopped. We report that the fiber-deposition process was not sensitive to the elongational flows present at the inlet or the outlet of the stenosis microfluidic device. We conclude that acute exposure to an extreme wall shear rate >30 000 s⁻¹ is a sufficient criteria for vWF fiber formation on collagen, independent of elongational forces in accelerating or decelerating flows. High wall shear rate and elongational flows are not mutually exclusive, however, and either or both may be operative in various situations. For
as the fiber-generation process described here occurs on the channel wall via collagen (see Discussion in the online-only Data Supplement).

Formation of these fibrils was evident in PPACK-treated whole blood at shear rates of 19 400 s$^{-1}$ but was best visualized at 38 800 s$^{-1}$. In PFP, the phenomenon had a threshold of $\approx$30 000 s$^{-1}$. The absence of Ca$^{2+}$ greatly enhanced the visualization of the fibers. The role of Ca$^{2+}$ may be 2-fold in preventing the aggregation of vWF, as it supports refolding of the vWF A2 domain and is required for ADAMTS13 function.33,34 We have demonstrated that platelets rapidly accumulate and embolize under conditions of high pathological shear with a mechanism likely related to vWF unfolding and did not require a shear rate gradient. The large and unstable masses incorporate vWF and seem to be nucleated by vWF fibers aggregating on collagen or platelet surfaces. Figure 4C demonstrated that vWF fiber formation and deposition during high-shear whole blood flow was dependent on GPIb and $\alpha$IIb$\beta$3. However, platelet aggregation also requires these receptors, and it is difficult to delineate collagen--vWF receptor mechanisms from platelet aggregation--vWF mechanisms under these flow conditions. Because significant vWF fluorescence is associated with the platelet aggregates beyond that formed on the collagen surface, multiple flow-dependent processes may coexist (platelet aggregation, wall-directed platelet fluxes, etc) beyond the direct effect of flow on vWF fiber formation on collagen. The experiment shown in Figure 6 was designed to understand whether high shear rate can cause vWF fiber extension, as platelet mass accumulates. Indeed, elongated fibers were detected at high wall shear rate (Figure 6C), when plasma was perfused over a preexisting platelet deposit. However, this result does not address whether platelet receptors on flowing platelets potentiate vWF accumulation in the growing platelet aggregates. After 1530 seconds of perfusion, no detectable vWF was present. After the shear rate increase, the massive platelet aggregates that form are highly vWF-positive. C, Platelet accumulation was measured by fluorescence at the microfluidic injury site. The gray shading indicates the SD of 4 experiments. Upon shear rate increase (a), the fluorescence intensity briefly drops as platelets were torn from the surface, and then recovered in a rapid burst of adhesion. Note the difference in time scales. D, vWF accumulation in the growing thrombus as flow was increased. The gray shading indicates the SD of 4 experiments. The fluorescent intensity was normalized to the 1530-second time point. Upon shear rate increase (a), vWF was incorporated into the rapidly growing thrombus.

Pathological wall shear rates exceeding 30 000 s$^{-1}$ are predicted in severe stenoses, as well as in left ventricular assist devices.1–5 Patients with severe aortic stenosis or a left ventricular assist device often present with a bleeding tendency that is linked to loss of the largest vWF multimers. On valve replacement or heart transplant, the distribution of vWF multimers is corrected.25,37 Recently, Yong et al23 have demonstrated that platelets respond to the acute exposure of elevated shear, as well. By sampling blood proximal and distal to a variety of coronary stenoses, the authors reported a significant increase in P-selectin exposure downstream of the occlusion, a result that correlated with stenosis severity. In contrast to soluble and globular plasma vWF in physiological hemodynamic conditions, elongated and aggregated vWF fibers formed under the flow conditions in this study may be particularly well suited for multivalent engagement of platelet GPIb, a receptor whose clustering by closely situated A1 domains in the vWF fiber may help drive GPIb-mediated signaling. Generation of vWF fibers may participate in this P-selectin display.

![Figure 5](https://example.com/figure5.jpg)

**Figure 5.** Onset of pathological shear rates triggered formation of massive aggregates that were unstable. A, Platelet deposition from whole blood (5–Phe-Pro-Arg chloromethylketone [PPACK]) onto a collagen type 1 surface was monitored over an $\approx$1530-second time period at an initial venous wall shear rate of 200 s$^{-1}$. At the 1530 seconds, a stable nongrowing platelet deposit had formed (a). The flow rate was suddenly increased such that in an empty channel the local wall shear rate would have been 7800 s$^{-1}$. Within 100 seconds, massive platelet adhesion had initiated, which resulted in a cycle of thrombus growth and embolization (b–d). The images are representative of multiple experiments. B, von Willebrand Factor (vWF) content was also measured in the growing platelet aggregates. After 1530 seconds of perfusion, no detectable vWF was present. After the shear rate increase, the massive platelet aggregates that form are highly vWF-positive. C, Platelet accumulation was measured by fluorescence at the microfluidic injury site. The gray shading indicates the SD of 4 experiments. Upon shear rate increase (a), the fluorescence intensity briefly drops as platelets were torn from the surface, and then recovered in a rapid burst of adhesion. Note the difference in time scales. D, vWF accumulation in the growing thrombus as flow was increased. The gray shading indicates the SD of 4 experiments. The fluorescent intensity was normalized to the 1530-second time point. Upon shear rate increase (a), vWF was incorporated into the rapidly growing thrombus.

![Figure 4](https://example.com/figure4.jpg)

**Figure 4.** Visualization of vWF fibrils. A, Phase contrast and fluorescence images of fibril formation in whole blood (PPACK) at a wall shear rate of 200 s$^{-1}$, before (a) and after (b–d) shear rate increase to 7800 s$^{-1}$. B, vWF accumulation in the growing platelet aggregates. After 1530 seconds of perfusion, no detectable vWF was present. After the shear rate increase, the massive platelet aggregates that form are highly vWF-positive. C, Platelet accumulation was measured by fluorescence at the microfluidic injury site. The gray shading indicates the SD of 4 experiments. Upon shear rate increase (a), the fluorescence intensity briefly drops as platelets were torn from the surface, and then recovered in a rapid burst of adhesion. Note the difference in time scales. D, vWF accumulation in the growing thrombus as flow was increased. The gray shading indicates the SD of 4 experiments. The fluorescent intensity was normalized to the 1530-second time point. Upon shear rate increase (a), vWF was incorporated into the rapidly growing thrombus.

![Figure 3](https://example.com/figure3.jpg)

**Figure 3.** Formation of pathological shear rates triggered formation of massive aggregates that were unstable. A, Platelet deposition from whole blood (5–Phe-Pro-Arg chloromethylketone [PPACK]) onto a collagen type 1 surface was monitored over an $\approx$1530-second time period at an initial venous wall shear rate of 200 s$^{-1}$. At the 1530 seconds, a stable nongrowing platelet deposit had formed (a). The flow rate was suddenly increased such that in an empty channel the local wall shear rate would have been 7800 s$^{-1}$. Within 100 seconds, massive platelet adhesion had initiated, which resulted in a cycle of thrombus growth and embolization (b–d). The images are representative of multiple experiments. B, von Willebrand Factor (vWF) content was also measured in the growing platelet aggregates. After 1530 seconds of perfusion, no detectable vWF was present. After the shear rate increase, the massive platelet aggregates that form are highly vWF-positive. C, Platelet accumulation was measured by fluorescence at the microfluidic injury site. The gray shading indicates the SD of 4 experiments. Upon shear rate increase (a), the fluorescence intensity briefly drops as platelets were torn from the surface, and then recovered in a rapid burst of adhesion. Note the difference in time scales. D, vWF accumulation in the growing thrombus as flow was increased. The gray shading indicates the SD of 4 experiments. The fluorescent intensity was normalized to the 1530-second time point. Upon shear rate increase (a), vWF was incorporated into the rapidly growing thrombus.

![Figure 2](https://example.com/figure2.jpg)

**Figure 2.** Visualization of vWF fibrils. A, Phase contrast and fluorescence images of fibril formation in whole blood (PPACK) at a wall shear rate of 200 s$^{-1}$, before (a) and after (b–d) shear rate increase to 7800 s$^{-1}$. B, vWF accumulation in the growing platelet aggregates. After 1530 seconds of perfusion, no detectable vWF was present. After the shear rate increase, the massive platelet aggregates that form are highly vWF-positive. C, Platelet accumulation was measured by fluorescence at the microfluidic injury site. The gray shading indicates the SD of 4 experiments. Upon shear rate increase (a), the fluorescence intensity briefly drops as platelets were torn from the surface, and then recovered in a rapid burst of adhesion. Note the difference in time scales. D, vWF accumulation in the growing thrombus as flow was increased. The gray shading indicates the SD of 4 experiments. The fluorescent intensity was normalized to the 1530-second time point. Upon shear rate increase (a), vWF was incorporated into the rapidly growing thrombus.

![Figure 1](https://example.com/figure1.jpg)

**Figure 1.** Visualization of vWF fibrils. A, Phase contrast and fluorescence images of fibril formation in whole blood (PPACK) at a wall shear rate of 200 s$^{-1}$, before (a) and after (b–d) shear rate increase to 7800 s$^{-1}$. B, vWF accumulation in the growing platelet aggregates. After 1530 seconds of perfusion, no detectable vWF was present. After the shear rate increase, the massive platelet aggregates that form are highly vWF-positive. C, Platelet accumulation was measured by fluorescence at the microfluidic injury site. The gray shading indicates the SD of 4 experiments. Upon shear rate increase (a), the fluorescence intensity briefly drops as platelets were torn from the surface, and then recovered in a rapid burst of adhesion. Note the difference in time scales. D, vWF accumulation in the growing thrombus as flow was increased. The gray shading indicates the SD of 4 experiments. The fluorescent intensity was normalized to the 1530-second time point. Upon shear rate increase (a), vWF was incorporated into the rapidly growing thrombus.

![Figure 6](https://example.com/figure6.jpg)

**Figure 6.** Visualization of vWF fibrils. A, Phase contrast and fluorescence images of fibril formation in whole blood (PPACK) at a wall shear rate of 200 s$^{-1}$, before (a) and after (b–d) shear rate increase to 7800 s$^{-1}$. B, vWF accumulation in the growing platelet aggregates. After 1530 seconds of perfusion, no detectable vWF was present. After the shear rate increase, the massive platelet aggregates that form are highly vWF-positive. C, Platelet accumulation was measured by fluorescence at the microfluidic injury site. The gray shading indicates the SD of 4 experiments. Upon shear rate increase (a), the fluorescence intensity briefly drops as platelets were torn from the surface, and then recovered in a rapid burst of adhesion. Note the difference in time scales. D, vWF accumulation in the growing thrombus as flow was increased. The gray shading indicates the SD of 4 experiments. The fluorescent intensity was normalized to the 1530-second time point. Upon shear rate increase (a), vWF was incorporated into the rapidly growing thrombus.
In a recent study, Nesbitt et al.\(^2\) suggested that shear gradients drove the formation of a loosely packed discoid platelet aggregate in a microfluidic model of stenosis lacking an adhesive protein surface. The authors demonstrated that no aggregates formed in a channel of constant cross section under the same hemodynamic conditions. They reported that GPIb was required for initial platelet adhesion at the stenosis apex, whereas GPIb and αIIbβ₃ were required for thrombus propagation. In the present study, we have found that rapid formation of loosely packed aggregates did form in channels of constant cross section but required a surface of vWF fiber-coated collagen or platelets. In agreement with the authors, we demonstrate that GPIb can support platelet firm adhesion on coated collagen or platelets. In agreement with the authors, we demonstrate that GPIb can support platelet firm adhesion on coated collagen or platelets.

Last, we present a role for platelet-bound vWF in thrombogenesis at pathological shear rates. Using a microfluidic channel of constant 100-μm cross section, we generated steady-state thrombi whose ability to capture platelets was restored by a sudden increase in shear rate. We suggest that platelet recruitment was reestablished by enhanced GPIb/vWF tethering resulting from increased vWF self-aggregation at high shear on the thrombus surface. Before the sudden increase in shear rate (Figure 5), the thrombus had grown to create hemodynamic conditions that prevented further platelet attachment and was insufficient to cause vWF fiber formation. After the flow rate increase, vWF fibers formed and were able to support platelet tethering and subsequent activation required for stable adhesion. A similar process was proposed by Kulkarni et al.\(^1\) who illustrated the importance of soluble vWF in platelet adhesion to an immobilized platelet monolayer in the absence of an activating surface, even at venous shear rates. They suggest that without soluble agonists tethering platelets have less active α₃β₁ and require more GPIb/vWF interactions to achieve stable adhesion. Our results are consistent with these earlier findings, and we have visualized the fundamental event of vWF fiber formation that may underlie these prior observations.

During arterial thrombosis at physiological wall shear rates (eg, in mild stenosis with plaque rupture), vWF elongation and fiber formation may initially be minimal. However, as the thrombus growth begins to occlude the vessel and before a significant pressure drop is created by the nonocclusive thrombus (<75% stenosis), the wall shear rates will increase dramatically with a consequent transition to elongated and fibrous vWF-mediating platelet capture. At high pathological shear rates, these nets of vWF and platelets may be less stable and subject to embolism, consistent with a condition of unstable angina or cyclic thrombosis. This study defines precise experimental conditions in which to detect and measure the dynamics of vWF unfolding, vWF fiber elongation, and aggregate formation at pathological shear rates on collagen.

**Acknowledgments**

Thomas V. Colace performed the experiments, analyzed data, and contributed to the manuscript. Scott L. Diamond designed the research, analyzed data, and contributed to the manuscript.

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**Disclosures**

None.

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Direct Observation of von Willebrand Factor Elongation and Fiber Formation on Collagen During Acute Whole Blood Exposure to Pathological Flow

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SUPPLEMENTAL MATERIAL

von Willebrand Factor unfolding and elongation on collagen during acute whole blood exposure to pathological flow

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Supplemental Discussion

A velocity field \( \mathbf{v}(x,y,z) \) can be represented as the superposition of two components: its vorticity \( \omega \) (which only rotates a collapsed polymer) and deformation \( \mathbf{D} \) (which extends and compresses a collapsed polymer) where the elongation rate \( \dot{\varepsilon} = \text{grad} \ \mathbf{v} = \omega + \mathbf{D} \).1 In theory, a cone-and-plate viscometer creates an ideal linear shear field that is both rotational and deformational. In cone-and-plate viscometers, we note that the collision rate between vWF molecules and the unavoidable generation of secondary flows due to fluid inertia are both greatly enhanced when pathological shear rates are obtained.2 In contrast to a linear shear field, flow acceleration into and deceleration out of a stenosis creates an elongational flow with significant hydrodynamic forces that are expected to cause coil-stretch transitions in polymers in the bulk flow.3,4 Impinging and contracting flows, boundary layer separation, and vortexing secondary flows may also generate significant elongational forces to alter vWF conformation in a flow field. Distinct from these situations is unidirectional and parabolic viscous flow over a surface as encountered in pipe flow or flow between parallel-plates. Very near the surface (eg. < 400 nm for globular vWF with a prolate ellipsoid shape of 175 x 28 nm for multimeric vWF),5 the boundary layer flow approximates a linear shear field. In this region very near the wall, a strong linear shear field can cause vWF to rotate and experience oscillatory compression and extension as it rotates. However, a linear shear field of sufficient strength is likely to only cause flow-alignment, but is unlikely to generate significant stretching of polymer molecules.1 In contrast, an accelerating flow that is highly elongational is best suited to cause an extended and stretched vWF conformation in the bulk flow away from the wall.6

At sufficiently high \( \gamma_w \), the process of vWF unfolding on the wall occurs for plasma or whole blood flow where the wall shear rate is not changing in the direction of flow (\( \text{grad}_x \gamma_w = 0 \)) for a constant-width microfluidic device completely lacking a stenotic inlet (Fig. S4-5). Theoretical treatments of adsorbed polymer extension on a wall are just emerging7,8 and indicate fundamental differences from processes in bulk flow. The vWF adsorption problem include difficult aspects of: (1) adsorption-driven annealing of vWF to multimeric collagen; (2) sub-micron geometries and flow fields (with non-zero \( \omega \) and \( \mathbf{D} \)) around molecules of finite submicron size such as globular vWF and fibrillar collagen; (4) hydrodynamic interactions of the partially adsorbed molecule with the wall, and (4) quantifying sub-nanometer water movement around and through a porous and extending vWF protein. Such theories also require an estimate of the vWF relaxation time \( \lambda \), however, it is unclear if relaxation rates measured for vWF bundles apply to single vWF multimers.9 While micron-scale gradients in a microfluidic flow field are not required for elongated vWF fiber formation on collagen, we show that pathological wall shear rates are highly predictive of their formation. The role of sub-micron flow fields that change on the length scale of a single protein or collagen fiber (or individual platelet) and may create elongational forces remains an area of investigation.
Supplemental References

Supplemental Figure I, The straight channel microfluidic device used in this study had a constant cross section of 100 μm wide by 60 μm tall. The large circles at the ends of the channel are the inlet/outlet ports. B, A bright field micrograph of the inlet to the stenosis in the stenosis channel. At the top of the image the channel cross section is 500 μm wide by 60 μm high. The channel rapidly tapers over a 100 μm length to a 15 μm wide by 60 μm high region. C, This representation of the stenosis channel illustrates that the 15 μm wide stenosis region was 1 mm in length.
Supplemental Figure II A, A representative bright field image of the collagen type 1 coated surface used in this study. B, A representative fluorescence microscopy image of a collagen type 1 surface treated with a biotinylated polyclonal anti-collagen antibody and fluorescently labeled streptavidin.
Supplemental Figure III, Plasma from a patient with severe von Willebrand disease does not deposit vWF fibers. A, PFP (EDTA) was perfused over a collagen type 1 surface in the stenosis chamber at the indicated wall shear rate. vWF fibers are detected using a fluorescently labeled polyclonal antibody. B, Plasma from a patient with severe vWD was also treated with EDTA and perfused in a similar manner as A. No vWF fibers are detected.
**Supplemental Figure IV**, Whole blood (EDTA) treated with a function blocking antibody against GPIb (A) or plasma (EDTA) was perfused over a collagen type 1 surface in a microfluidic channel of constant cross section (100 μm by 60 μm). Given the width of the channel, collagen was briefly fixed for 30 s with 2 % glutaraldehyde to help attach it to the surface. The centerline wall shear rate was 48,000 s⁻¹ for the whole blood sample and 125,000 s⁻¹ for the plasma sample to maintain constant wall shear stress (τᵢ = 2125 dyne/cm²). The surfaces were washed after sample perfusion and stained for vWF, images are representative of at least 3 experiments per condition. **A,B** Fibers of vWF are observed in both conditions in these representative images. **C,** The mean number of fibers (n=3) intersecting a line scan drawn horizontally through the center of the image was counted for each condition. **D,** The length of each of the fibers intersecting the line scan from **C** was averaged for each condition.
Supplemental Figure V, vWF fluorescence for whole blood (EDTA) treated with a function blocking antibody against GPIb (A) or plasma (EDTA) (B) perfused over a collagen type 1 surface in a channel of constant cross section (100 μm by 60 μm). The centerline wall shear rate was 48,000 s⁻¹ for the whole blood sample and 125,000 s⁻¹ for the plasma sample to maintain constant wall shear stress (τw = 2125 dyne/cm²). The vWF fluorescence over the channel width was averaged over the length of the viewing window for 3 replicates (blue line) and was mathematically smoothed (black line). The dashed red lines indicate the local wall shear rate along the 100 μm width as calculated from the analytical solution for the velocity profile in a rectangular duct. A, vWF fluorescence is elevated in the middle of the channel where the wall shear rate is at a maximum. In the corners, high signal was also observed (especially in the case of whole blood flow), which may be due to the non-physiologic corner-flow geometry of the device. B, In plasma samples, the highest signal was observed along the center of the channel.
Supplemental Figure VI A, EDTA-treated whole blood was perfused over a collagen type 1 surface in a microfluidic channel with a 100 μm x 60 μm cross section at the indicated wall shear rate. Within 30 sec, long fibers of vWF were seen on the surface. Shortly after, rolling aggregates of platelets and vWF were observed. B, PPACK-treated whole blood was perfused in an identical manner as A. vWF fibers were detected after 30 sec, although there was less deposition. By two minutes massive platelet aggregation had occurred. C, The αIIbβ3 inhibitor, GR 144053, was used to prevent massive platelet aggregation in an identical perfusion experiment to A. Reduced vWF deposition was observed under these conditions, suggesting a role for αIIbβ3-mediated platelet adhesion in supporting vWF fiber elongation and aggregation. D, E, Identical experiments to B and C were performed at 2-fold increased wall shear rate. The deposition of vWF was markedly increased. The time to platelet aggregation was also reduced.
Supplemental Figure VII, Compound GR 144053 inhibits secondary platelet aggregation on collagen type 1 surfaces. A, Fluorescently labeled platelets in WB (PPACK) were perfused over a collagen type 1 surface at 200 s⁻¹ in the presence of the indicated amount of GR 144053. B, The perfusion experiment was performed in an 8-channel microfluidic device that allowed for 8 different inlet concentrations of the inhibitory compound. C, A representation of the chemical structure of GR 144053 (provided by Tocris Bioscience). D, The platelet fluorescence after 5 minutes of perfusion over a collagen type-1 surface in the 8 channel microfluidic device was used to obtain an IC₅₀ value for GR 144053, 26.0 nM.
Supplemental Figure VIII, The formation of stable platelet adhesion and platelet aggregates required GPIb/vWF interaction. Whole blood in the presence or absence of 40 μg/mL of function blocking anti-GPIb antibody, AK2, was perfused at the indicated wall shear rate over a collagen type 1 surface. Under both conditions vWF deposition was observed. In the presence of the neutralizing antibody, few platelets firmly adhered to the surface while many were seen in its absence. Furthermore, large platelet aggregates were present in the absence of AK2. The images are representative of multiple experiments.
**Supplemental Movie M1** vWF fiber deposition under whole blood flow in the absence of Ca$^{2+}$ (EDTA) at 19,400 s$^{-1}$ on a collagen type 1 surface, without stenosis. Flow is from top to bottom.
Supplemental Movie M2 vWF fiber deposition under whole blood flow in the presence of Ca\(^{2+}\) and PPACK at 38,800 s\(^{-1}\) on a collagen type 1 surface, without stenosis. Flow is from top to bottom.