Flow-driven assembly of VWF fibres and webs in in vitro microvessels

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Several systemic diseases, including thrombotic thrombocytopenic purpura, manifest much of their pathology through activation of endothelium and thrombotic occlusion of small blood vessels, often leading to multi-organ failure and death. Modelling these diseases is hampered by the complex three-dimensional architecture and flow patterns of the microvasculature. Here, we employ engineered microvessels of complex geometry to examine the pathological responses to endothelial activation. Our most striking finding is the capacity of endothelial-secreted von Willebrand factor (VWF) to assemble into thick bundles or complex meshes, depending on the vessel geometry and flow characteristics. Assembly is greatest in vessels of diameter \( \leq 300 \mu m \), with high shear stress or strong flow acceleration, and with sharp turns. VWF bundles and webs bind platelets, leukocytes and erythrocytes, obstructing blood flow and sometimes shearing passing erythrocytes. Our findings uncover the biophysical requirements for initiating microvascular thrombosis and suggest mechanisms for the onset and progression of microvascular diseases.

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Willebrand factor (VWF), a very large, multimeric, blood protein, has a pivotal role in initiating haemostasis and thrombosis and has emerged as an important risk factor and therapeutic target for many vascular diseases. VWF is primarily secreted from the endothelium, either constitutively or in a regulated fashion from Weibel–Palade bodies after endothelial stimulation. Much of the secreted VWF remains bound to the endothelial surface until it is proteolytically removed by the metalloprotease ADAMTS13. Endothelium-attached VWF unfolds under fluid shear stress and flow acceleration, becoming more adhesive to bind platelets, and more susceptible to ADAMTS13 proteolysis. Failure to remove endothelium-bound VWF allows individual multimers to self-associate to form long strands that facilitate platelet adhesion and thrombus formation, which promotes microvascular occlusion in a group of life-threatening disorders that include thrombotic thrombocytopenic purpura (TTP), haemolytic uraemic syndrome and other vascular diseases. These pathologies, VWF multimers in plasma are often abnormally large and abundant, and, in TTP, terminal arterialies and capillaries become occluded by platelet-and VWF-rich thrombi. However, the mechanisms of these diseases are not fully understood. In particular, it is not known why only the small vessels are affected, or whether platelets themselves are always necessary for the development of occlusive thrombi in TTP, which often worsens clinically even in the face of severe thrombocytopenia.

Fluid shear stress is an important regulator of VWF’s ability to bind platelets, as it unfolds the VWF molecule and renders it more adhesive to bind platelets. Nevertheless, how flow and vessel characteristics modify the structure and functions of VWF strands bound on the vessel walls have not been well studied, because it is difficult to directly image VWF strands with high resolution in small vessels in vivo.

Here, using a fully endothelialized system of in vitro microvessels that recapitulate the complex architectures and flow characteristics found in vivo, we examined the effects of haemodynamics and vessel geometry on the assembly of thin VWF strands into thicker strands or fibres and on their interactions with platelets and other blood cells. We found that the extent of strand formation and thickening depends on vessel architecture, flow and the proteolytic activity of ADAMTS13. As vessels become smaller, VWF strands become thicker and longer. If the turn is abrupt, VWF strands are thinner and more discrete. VWF is also more adhesive to bind platelets as it unfolds the VWF molecule and renders it more susceptible to proteolysis. A minimal flow shear stress appeared to be required in the microvessels, of ~0.3 dyn cm⁻² for VWF strand formation. Below this shear, VWF remained undissociated (Supplementary Fig. 1a–c).

In vessels under 200 μm in diameter containing multiple turns, which simulate tortuous vessels, the secreted VWF not only formed strands on the wall in the direction of flow, the strands also lifted away from the wall, and assembled into thick fibres as they narrowed towards the centre of the flow stream (Fig. 2g–k). Near the wall (Fig. 2h,i,k), a meshwork of 1–2-μm-thick VWF strands formed; these strands coalesced to form much thicker fibres and bundles at the centre of the flow stream (Fig. 2j), which anchored at the inside corners of the turns (asterisks, Fig. 2j). The thick VWF bundles occupied roughly 15% of the vessel cross-sections at the thickest region (arrowhead, Fig 2j, with a maximal cross-sectional area of ~1,400 μm²), and spanned the vessel lumen in the shortest path between turns (Fig. 2g). Numerical simulation of flow in these regions predicted that peak flow would shift toward the inside corners where the shear stress is highest (Fig. 2l), consistent with previous studies. Pairs of counter-rotating vortices developed in cross-sectional planes located immediately before and after the turns, which could serve as the driving forces for VWF accumulation in the centre stream of the tortuous vessel. The magnitude of the effect of these secondary flows on VWF self-association depended on parameters of vessel geometry, including the curvature, diameter and aspect ratio. In the vessels with continuous turns and low aspect ratios (ratios of segment length between turns to diameter ≤ 4), the VWF strands formed continuous transluminal fibres throughout the entire vessel, reaching lengths of over 5 cm (Fig. 2m).

When the vessels were stimulated with an endothelial secretagogue, the endothelium released VWF, of which a significant fraction remained bound to the endothelial surface and formed strands under flow. The pressure applied to each vessel ranged from 10 to 1,000 Pa between the vessel inlet and outlet during stimulation to generate an average wall shear stress of 5 dyn cm⁻². In vessel regions that approximated straight tubes, individual VWF strands from adjacent streamlines assembled to form thicker fibres in the direction of flow (Fig. 2a,b), visible fibres ranging in thickness from 1 to 6 μm. This phenomenon occurred in all vessels with diameters between 100 and 1,000 μm (Fig. 2a–c). Near the vessel wall, most of the VWF strands followed the direction of bulk flow, but a few strands deviated from this pattern, likely because of small local flow disturbances induced by the surface irregularities on the endothelial monolayer (Fig. 2d). This effect was more prominent in vessels < 200 μm in diameter. In larger vessels (> 500 μm) with one turn, the VWF strands followed the direction of flow and formed thicker strands near the inside corner of the turn (Fig. 2e). In larger vessels with multiple turns, the VWF strands (Fig. 2f) followed the streamlines and remained close to the vessel walls, within a distance of 20 μm from the vessel wall (~5% of the vessel diameter). More and thicker VWF strands appeared at regions of high shear stress, some reaching a thickness of up to 20 μm at regions of peak shear stress (arrowheads, Fig. 2f). In regions where the shear stress was below 0.5 dyn cm⁻², as in vessel concavities, no VWF strands were visible, the VWF remained globular (asterisks, Fig. 2f). A minimal flow shear stress appeared to be required in the microvessels, of ~0.3 dyn cm⁻² for VWF strand formation. Below this shear, VWF remained as punctate globules (Supplementary Fig. 1a–c).

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fibres in the region of acceleration (Fig. 3b). The accumulation of VWF at regions of vessel narrowing could be further enhanced by increasing curvature. For example, in the U-shaped vessel depicted in Fig. 3c,d, flow accelerated as it approached the region of maximum curvature (2.5 mm⁻¹) and decelerated once past this region. VWF fibres converged at this region of maximum curvature, which was also the narrowest, and formed clumps that occluded almost 80% of the cross-sectional area (Fig. 3d). Downstream of this region, the VWF fibres diverged into thinner strands, some of which attached to the opposite wall of the vessel (Fig. 3d,e).

Thus, VWF self-association was influenced both by vessel diameter and curvature, and by shear stress and flow acceleration. VWF strands were thickest in smaller vessels with sharp turns, and at regions of narrowing.

Flow-induced 3D VWF webs. Native vessel plexuses are highly branched as blood passes from arterioles to capillaries and to venules. In these structures, blood flow first diverges into a region of high total luminal cross-sectional area and decelerates, then converges and accelerates as it approaches the efferent vessel. To approximate this flow pattern, we used a grid vessel network with 13 × 13 vessel branches between one inlet and one outlet (Fig. 1d). When the endothelium in the grid was activated, secreted VWF formed strands and transluminal fibres in many vessel branches, particularly in the branches near the inlet and the outlet (Fig. 4a), where the flow rate and shear stress were both high (Fig. 4b). No transluminal fibres were observed at the branches near off-diagonal corners, where the wall shear stress was nearly 1/50 of the shear stress at the inlet. In the grid, flow first accelerated as it left the large inlet and entered the first two branches in the plexus. Thereafter, flow decelerated as branching increased (Fig. 4b). Flow speed increased again as the branches merged and flow approached the outlet. The number of transluminal fibres and the quantity of visible VWF were both proportional to the local wall shear stress: more VWF strands and transluminal fibres formed at high-shear regions near both the
inlet and outlet (Fig. 4c, Supplementary Fig. 2a,b). A minimum shear rate of 50 s$^{-1}$ was required for the formation of trans-luminal fibres and VWF strands along the vessel wall, consistent with the data shown in Fig. 2f. In high shear regions with multidirectional flow, the assembled fibres formed complex meshes that could potentially trap blood cells (Fig. 4d,e).

At the entrance to the grid (Fig. 4f,g and Supplementary Fig. 2a), where the vessel first bifurcated, VWF strands extended ~2 mm downstream along the outer walls of the vessel and multiple VWF strands merged to form thick fibres (arrowheads in Fig. 4f,g). Near the crotch of the first bifurcation, VWF strands from single cells or multiple cells extended downstream, and split where the flow stream diverged (asterisks in Fig. 4g). Near the outlet, long VWF fibres extended continuously across several branch points, traversing the vessel lumen in the shortest path between branch corners and becoming progressively thicker as
they approached the grid outlet (some strands were as thick as 40 μm at the outlet; Fig. 4h). The thickness of the transluminal fibres and the abundance of VWF in the webs corresponded with extensive depletion of VWF stores in Weibel–Palade bodies (compare Fig. 1c and Supplementary Fig. 2c, right panel).

**Effect of platelet adhesion on ADAMTS13 cleavage.** We perfused platelets only, suspended in buffer, through a grid vessel after it was activated by phorbol myristate acetate (Supplementary Fig. 3). The platelets bound the preformed VWF strands and fibres (a process involving the platelet membrane glycoprotein (GP) Ib-IX-V complex) producing mural and transluminal thrombi, which were larger in regions where the VWF was most abundant. The platelet–VWF strings/thrombi persisted throughout the 15 min of platelet perfusion, with minimal strand breakage.

VWF contains a single site for ADAMTS13 cleavage within each VWF subunit (in the A2 domain)\(^\text{12}\), cleavage of VWF by this enzyme produces smaller and less adhesive VWF multimers, thereby reducing platelet accumulation. When we perfused whole blood from normal donors (containing active ADAMTS13) instead of isolated platelets through the stimulated grid vessels, the number and size of platelet thrombi were greatly decreased (Fig. 5a and Supplementary Movie 1), and only a few VWF-platelet strands were apparent. Antibody inhibition of platelet GPIbα (the VWF-binding subunit of the GPIb-IX-V complex) almost completely prevented platelet adhesion to the VWF strands (Fig. 5b–d and Supplementary Movie 2). Many VWF strands appeared to have detached from the vessel wall and embolized as intact strands, and some of these were trapped at bifurcation crotches (Fig. 5b,c). It appears that in the absence of platelets, ADAMTS13 cleaves the VWF strands on the vessel wall more slowly. The attachment of platelets apparently creates a drag force that opens the VWF A2 domain and exposes the cleavage site for ADAMTS13, allowing the enzyme to degrade the VWF strands into smaller pieces, consistent with previous observations that ADAMTS13 preferentially acts on platelet-VWF complexes under fluid shear stress\(^\text{18}\). In addition, the binding of platelets to VWF strands prevents the strands from forming tightly packed fibres, which further facilitates ADAMTS13 cleavage. When platelets cannot bind to the strands this allows the strands to self-associate into thicker strands that take longer to cut, but when they are cleaved embolize as large pieces.

**Effect of ADAMTS13 on VWF strand structure.** Deficiency of ADAMTS13 activity due to genetic mutation\(^\text{19,20}\) or neutralizing autoantibodies\(^\text{21,22}\) promotes microvascular thrombosis and development of TTP. We examined how ADAMTS13 deficiency affects the structure of VWF strands and webs. The microvessels were stimulated in the absence or presence of recombinant ADAMTS13 at a concentration of 1 μg ml\(^{-1}\) (a physiologic enzyme concentration, Fig. 4a and Supplementary Fig. 4), or in the presence of normal human plasma (Fig. 6a) or plasma from a TTP patient with <5% ADAMTS13 activity (Fig. 6b). Plasma with normal ADAMTS13 activity had the same effect as recombinant ADAMTS13, cleaving nearly all of the VWF strands during activation. Only small globules of VWF remained on the endothelial surface, likely representing the ‘stumps’ of VWF strands that remained at the sites of endothelial attachment (Fig. 6a and Supplementary Fig. 4c,d). In contrast, when TTP plasma was present during vessel stimulation, numerous VWF strands and complex webs accumulated on the vessel walls, and some of the strands spanned the vessel lumen (Fig. 6b). Vessels stimulated in the absence of ADAMTS13 had the longest VWF strands (Fig. 6c), with sizes ranging from globules of 1–2 μm to strands longer than the size of the field of view (>1.2 mm). The presence of ADAMTS13, either recombinant or in plasma, eliminated all of the long fibres on the wall or lumen; the maximum length of VWF strands left was ~30 μm. In vessels activated in the presence of TTP plasma, the VWF strands were significantly longer than in the presence of normal plasma, spanning lengths between 1 and 800 μm. These strands were even thicker than those formed in the absence of plasma, sometimes even partially occluding the vessel lumens. The thickening of VWF fibres in the presence of TTP plasma was likely a result of association of fluid-phase VWF from the plasma with the immobilized endothelium-bound VWF strands\(^\text{23}\).

Next, we perfused stimulated vessels with blood containing inhibitory antibodies, blocking either ADAMTS13 alone or both ADAMTS13 and platelet GPIbα. In blood with control mouse IgG, the number of bound platelets plateaued after 1 min of perfusion (Fig. 6d,e). When the ADAMTS13 inhibitory antibody...
A10 (ref. 24) was added to the blood, platelet adhesion increased continuously over the 10 min that the vessel was monitored (Fig. 6d,e and Supplementary Movie 3), consistent with persistence of thick VWF strands (Fig. 6f,g). In addition to the thick strands, some VWF bundles appeared as loosely associated assemblies of several thin fibres connected to each other through bound platelets (Fig. 6h). Blood treated with both A10 and the GPIbα inhibitory antibody AK2 showed almost no platelet binding (Fig. 6d,e and Supplementary Movie 4), even though VWF strands were still present (Fig. 6i). In the vessels perfused with ADAMTS13-inhibited blood, flow resistance increased threefold at the 10 min time point compared with the control or double-blocking condition. This resistance was accompanied by a marked drop in the platelet count in the vessel effluent. Accompanying these changes, the platelet thrombi were much more prominent at the inlet of the grid (Fig. 6f) than at the outlet (Fig. 6g) despite the fact that the two regions had similar quantities of VWF fibres.

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The platelet–VWF strands also bound erythrocytes (Fig. 6f). Curiously, it appeared that several layers of platelets accumulated at some sites before erythrocytes were able to attach (see arrowheads in Fig. 6f). There was also evidence of erythrocyte fragmentation (schistocytes) in the vessel effluent (Fig. 6j). In the presence of both A10 and AK2 (Fig. 6i), very few platelets or erythrocytes bound the VWF strands, suggesting that erythrocytes primarily adhere to immobilized platelets. Leukocytes also accumulated on the platelet/VWF strings when proteolysis of VWF was inhibited (Fig. 7 and Supplementary Movie 5).

**Discussion**

Our studies have uncovered four important biophysical parameters that influence the development, thickness and length of VWF strands and webs in stimulated blood vessels: (1) vessel diameter, (2) vessel geometry (changes in curvature and diameter,
bifurcations and convergences), (3) fluid shear stress and (4) flow acceleration. Vessel diameter influences two important phenomena: the ability of adherent platelets to occlude the vessel lumen, and the capacity of VWF strands to span the vessel lumen at turns. Within the limits of the vessel diameters we studied (40–1,000 μm), VWF strand thickness and length increased as vessel diameter decreased at equivalent shear stresses. In large vessels, VWF strands remained near the vessel wall and the influence of geometry was minimal. Therefore, bound platelets and other blood cells attached to the wall are more likely to exert local biological effects on the vessel wall rather than to occlude the lumen, an example being to initiate atherosclerotic lesions. In smaller vessels with frequent turns, secreted VWF forms continuous strands that can span the vessel lumen and whose length is limited only by the length of the vessel. The longest we observed was ∼5 cm long. This is much longer than the longest VWF strands previously reported (several millimetres), which were formed on planar endothelial monolayers. We do not mean to propose that VWF strands 5 cm in length commonly occur in vivo in pathologic situations, but rather to demonstrate the potential of VWF to form very long strands under the appropriate biophysical conditions. As opposed to the situation in larger vessels, platelet thrombi that form on smaller vessels are much more likely to occlude the vessel lumen, a process of pathophysiologically important in systemic diseases such as TTP, sepsis and malaria.

Another interesting observation we made relates to the pattern of platelet adhesion in grid vessels. In the absence of blood components, more VWF accumulates near the inlet and outlet of the grid, and when platelets are perfused through the vessel, the pattern of platelet adhesion reflects this pattern of VWF accumulation. However, the pattern of platelet adhesion was strikingly different when whole blood containing an ADAMTS13 inhibitor was perfused through the vessel. In this situation, platelets accumulated extensively near the inlet, partially obstructing flow and increasing the upstream pressure, while the platelet thrombi that formed near the outlet were much smaller, being insufficient even to coat all of the VWF strands. The reason for this difference is not readily apparent, but may have to do with the depletion of platelets near the inlet, leaving fewer to attach at the outlet. This phenomenon may have an in vivo correlate, as the hyaline thrombi in TTP are seen in arterioles and capillaries, but rarely, if ever, are seen in venules, despite the fact that these vessels have plentiful VWF.

This study highlights the mechanisms underlying the extremely low platelet counts observed in human TTP. The endothelium has a tremendous capacity to bind platelets. With a total surface area of ∼700 m² in an adult human and abundant VWF stores,
the activated endothelium would consume the entire quantity of platelets in the blood to cover this surface area with a monolayer of platelets, if even a fraction of the VWF was released at once and the mechanisms for its removal (ADAMTS13) were absent.

In summary, we have used a recently developed in vitro microvessel system to investigate the properties of VWF secreted from the vessel wall. This microvessel system is very useful, yet has a number of limitations in its ability to mimic the situation...
These include, (a) the experiments described here have only one cellular component, endothelial cells. In vivo, vessels interact with a variety of cell types, including those of the organ in which the vessel resides, cells of the blood, and perivascular cells such as pericytes and smooth muscle cells; (b) although the vessels in our in vitro system can have very complex geometry, they are produced within only a single two-dimensional plane, whereas in vivo the vessels travel within 3D; (c) the system also differs from the in vivo situation in that the vessels are constantly perfused by blood that is circulating and continually being replenished in its content of hormones and small molecule mediators. Nevertheless, this system allows us to vary a number of parameters independently, such as geometry, diameter, flow and blood components, allowing us to uncover the physical parameters that drive the formation of hyperadhesive VWF strands and webs, and to provide tenable explanations for previously unexplained clinical phenomena. In addition, this system allows the use of entirely human components, making our findings more immediately relevant to human disease.

Methods

Patients and normal blood donors. Plasma was collected from a TTP patient with written informed consent approved by the Institutional Review Board of the University of Washington. Blood collections from healthy normal donors with written consent were under protocols approved by either the Institutional Review Board of the University of Washington or the Western Institutional Review Board.

Microvessel fabrication and culture. Type I collagen was prepared from rat tails to a stock concentration of 15 mg ml\(^{-1}\) (ref. 29), which was further diluted and neutralized to 7.5 mg ml\(^{-1}\) on ice before microvessel fabrication. The microvessels were fabricated via soft lithography and injection moulding\(^{13}\), which includes four major steps: (1) defining a microstructured silicone stamp, (2) injecting collagen in housing devices (both microstructured and flat pieces) and allowing for gelation at 37\(^\circ\)C, (3) sealing the two collagen layers to form the enclosed fluidic structure and (4) seeding human umbilical vein endothelial cells (Lonza) through the microchannels between the inlet and outlet and allowing them to attach before long-term culture with perfusion of medium. The microvessels were cultured with growth medium (single quote kits from Lonza) comprising endothelial base media with 2% fetal bovine serum, 1% penicillin/streptomycin, epidermal growth factor, bovine brain extract with heparin, hydrocortisone, ascorbic acid, gentamicin and amphotericin B.

Live imaging of VWF secretion and whole blood perfusion. In the studies of VWF secretion, the microvessels were activated with phorbol myristate acetate (50 ng ml\(^{-1}\) in serum free medium) for 40 min at a pressure drop between the inlet and the outlet varying between 10 and 1,000 Pa, depending on vessel geometry, to generate an average wall shear stress of \(~ 5\) dyn cm\(^{-2}\). The vessels were then washed with PBS buffer before being perfused with buffer containing a FITC-conjugated VWF polyclonal antibody (1:100 dilution, Abcam). The accumulation
of VWF in the microvessels was monitored with bright field and fluorescence imaging using an inverted microscope (Olympus IX81). After the live-imaging experiment, the microvessels were immediately fixed, stained for an endothelial marker (CD31, 1:50 dilution, Abcam) for further imaging. VWF was quantified in the stimulated vessels using Matlab software, with paired t-tests were carried out to compare the mean intensity of VWF staining between the experimental groups.

**Numerical simulation**. The flow characteristics within 3D tubes and networks were simulated with COMSOL Multiphysics software, package ver. 4.0. The Navier–Stokes equation were immediately used as pre-defined in COMSOL. The stationary solver for laminar flow was chosen for the Navier–Stokes equation. The fluid properties were defined as follows: viscosity of 1 \( \times 10^{-3} \) kg m \(^{-1} \) s \(^{-1} \) and density of 1 \( \times 10^{3} \) kg m \(^{-3} \) for water. The inlet boundary conditions are laminar flow with constant pressure of 10–1,000 Pa, and the outlet boundary conditions are laminar flow with zero pressure.

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Author contributions

Y.Z., J.C. and J.A.L. designed the project. Y.Z. performed experiments and computational simulation, and analysed data. Y.Z. and J.C. performed experiments involving blood. Y.Z., J.C. and J.A.L. interpreted the data. Y.Z. and J.A.L. wrote the manuscript. J.C. edited the manuscript.

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