The function of ultra-large von Willebrand factor multimers in high shear flow controlled by ADAMTS13

A. J. Reininger
Medical Affairs EMEA, Baxalta Innovations GmbH

Keywords
Von Willebrand factor, ADAMTS13, shear stress

Summary
The paradigm that platelet aggregation, which contributes to bleeding arrest and also to thrombovascular disorders, initiates after signaling-induced platelet activation has been refuted in past recent years. Platelets can form aggregates independently of activation when soluble von Willebrand factor (VWF) is present and the shear rate exceeds a certain threshold where active A1 domains become exposed in soluble VWF multimers and can bind to platelet glycoprotein Ib. Subsequently – fostering each other – VWF can self-assemble into large nets combining with platelets into large conglomerates, which are entirely reversible when they enter a flow region with shear rates below the threshold. In addition the threshold changes from approximately 20 000 s^{-1} in wall parallel flow to approximately 10 000 s^{-1} in stagnation point flow. VWF containing ultra-large multimers – as when just released from endothelial storage sites – has been shown to have the highest binding potential to platelets and to each other, thus facilitating rapid platelet accrual to sites of vessel injury and exposed subendothelial structures, i.e. collagen. The VWF nets as well as the platelet-VWF conglomerates are controlled by the cleaving protease ADAMTS13 within minutes under high shear flow. Therewith the hemostatic potential is delivered where needed and the thrombogenic potential is highly controlled twofold: by flow and enzymatic proteolytic cleavage.

Correspondence to:
Prof. Dr. med. Armin Reininger, MD, PhD
Head Medical Affairs Europe, Middle East, Africa, Hemophilia and Blood Disorders, Baxalta Innovations GmbH, Industriestrasse 67, 1221 Vienna, Austria
Tel. +43/1/20100247-2511, Fax -5733
E-mail: Armin_Reininger@Baxalta.com

Schlüsselwörter
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Zusammenfassung
In den vergangenen Jahren wurde das Paradigma infrage gestellt, dass die Initiierung der Plättchenaggregation – die zur Blutstillung und zu thrombovaskulären Störungen beiträgt – rein durch signalinduzierte Plättchenaktivierung erfolgt. In der Anwesenheit von löslichem von-Willebrand-Faktor können Thrombozyten unabhängig von ihrer Aktivierung Aggregate bilden. Dazu muss die Scherrate einen gewissen Schwellenwert überschreiten, sodass die A1-Domänen des VWF aktiviert, d. h. in löslichen VWF Multimeren exponiert werden und den Plättchenrezeptor Glykoprotein Ib binden. In der Folge sich gegenseitig verstärkend kann VWF zu großen Netzen selbstassemblieren und sich mit Plättchen zu großen Konglomeraten verbinden, die komplett reversibel sind, sobald sie in Flussregionen mit Scherraten unterhalb der kritischen Schwelle eintreten. Diese kritische Schwelle verringert sich von etwa 20 000 s^{-1} auf etwa 10 000 s^{-1} wenn statt wandparalleler Strömung eine Staupunktströmung vorliegt. Enthält VWF ultra-große Multimere – so wie nach seiner Freisetzung aus den endothelialen Speichern – haben diese das höchste Bindungspotenzial für Plättchen und untereinander und fördern dadurch die schnelle Ansammlung von Thrombozyten an verletzten Gefäßen und exponierten subendothelialen Strukturen wie Kollagen. Unter Flussbedingungen mit sehr hohen Scherraten kontrolliert die Protease ADAMTS13 die VWF-Netze sowie die Plättchen-VWF-Konglomerate innerhalb von Minuten durch enzymatische VWF-Spaltung. Somit wird das hämostatische Potenzial am Bedarfsort realisiert, und das thrombogene Potenzial wird zweifach reguliert: durch die lokale Flussbedingung und die proteolytische VWF-Spaltung.

Die Funktion von ultra-großen von-Willebrand-Faktor-Multimeren und ihre Regulation durch ADAMTS 13 unter Flussbedingungen mit sehr hohen Scherraten
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Von Willebrand factor (VWF) is a multimeric protein which consists of identical subunits, connected by disulphide bonds into dimers of approximately 500 kD, which, in turn, link the dimers into multimers of various sizes that can exceed 10 000 kD or 1 µm in length (1, 2). VWF is normally found in plasma, α-granules of platelets and endothelial cell organelles known as the Weibel-Palade bodies.

Following injury to a blood vessel, primary hemostasis is the assembly of a platelet-VWF plug, essentially a (bio)polymer-colloid composite that is driven by shear flow. Counterintuitively, stronger shear forces accrue more platelets not less, due to their activation of VWF and its interplay with non-activated platelets. The formation of such a plug is reversible and highly controllable, and depends mainly on the shear rate and the strength of the polymer-col-

loid binding potential (3). In primary hemostasis, VWF mediates the binding of platelets to sites of vascular injury by interacting with the specific receptors on the platelet surface and components of the extracellular matrix such as collagen. VWF also serves as a carrying and stabilizing protein for procoagulant FVIII (1, 4), protects FVIII from proteolytic inactivation, and prevents premature elimination of FVIII from the circulation. When secondary hemostasis occurs and the clotting factors are assembled to complexes on the activated platelet membrane surface, VWF has already delivered FVIII to its site of action. Platelets form thrombi via aggregation at sites of vascular injury that contribute to arrest bleeding but also may occlude atherosclerotic arteries causing cardiac or cerebrovascular complications (5, 6).

Platelet thrombus formation has been shown to occur in successive stages (7). Individual platelets first adhere to altered vascular surfaces where they are activated by physical and chemical agonists. The prerequisite for this is that VWF immobilizes, for example on collagen via its A3 domain, and thus captures platelet from flow onto its A1 domain, binding to the platelet GPIbα receptor, which is functional and does not require activation. Without the layer of immobilized VWF, the non-activated platelets in the flow would not arrest directly on collagen. The platelets' receptors requiring activation would not be operational. Once activated through intracellular signaling pathways, the platelet receptors can then bind plasma proteins, notably fibrinogen, VWF, and fibronectin to reinforce either the binding of the platelets to the vessel wall or to each other within a platelet aggregate. However, the next layer of platelets that needs to be captured from flow onto the aggregate surface to make it grow and plug the vessel leak again requires pre-bound VWF – now to the platelet surface – and its A1 domain as an "arresting wire" (▶Fig. 1) (1, 6). Again these platelets will undergo conformational change and engagement of additional receptors, e.g. integrin αIIbβ3, and the presentation of phosphatidylserine on the platelet membrane, a blood coagulation acceleration factor that is not found on the surface of resting platelets. The link to coagulation occurs on the activated platelet membranes, which foster assembly and activation of components of the coagulation cascade, such as tissue factor-factor VII, into the tenase complex and the prothrombinase complex. Secondary hemostasis refers to these later events of stimulated clotting, which generate thrombin and then lead to fibrin formation and polymerization. The platelet aggregate is finally stabilized by fibrin, which occludes the vessel injury and prevents deleterious leakage of blood (1).

Current knowledge is that platelets can keep in contact with a surface or with one another for a limited time with rapidly forming but short-lived VWF bonds until additional bonds, established mostly through integrin receptors, stabilize ad-

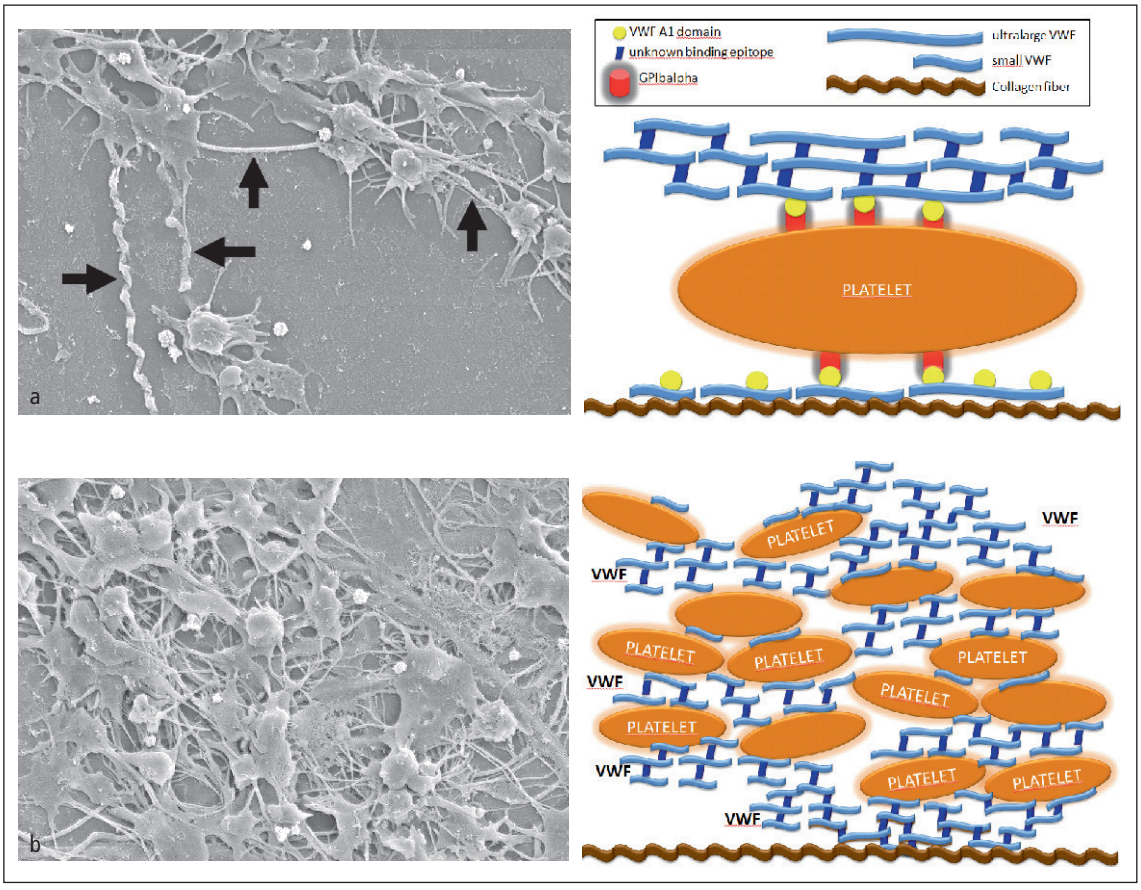

Fig. 1
Initial steps of platelet aggregation on collagen: The first layers (a) of adherent and spread platelets on collagen fibers (arrows) formed under flow as seen with scanning electron microscopy (left). The initial platelet(s) arrest on the collagen fiber mediated via pre-bound VWF; b) subsequent layers of VWF bound on the platelet surface accrue more platelets from the flow to form an aggregate (right).

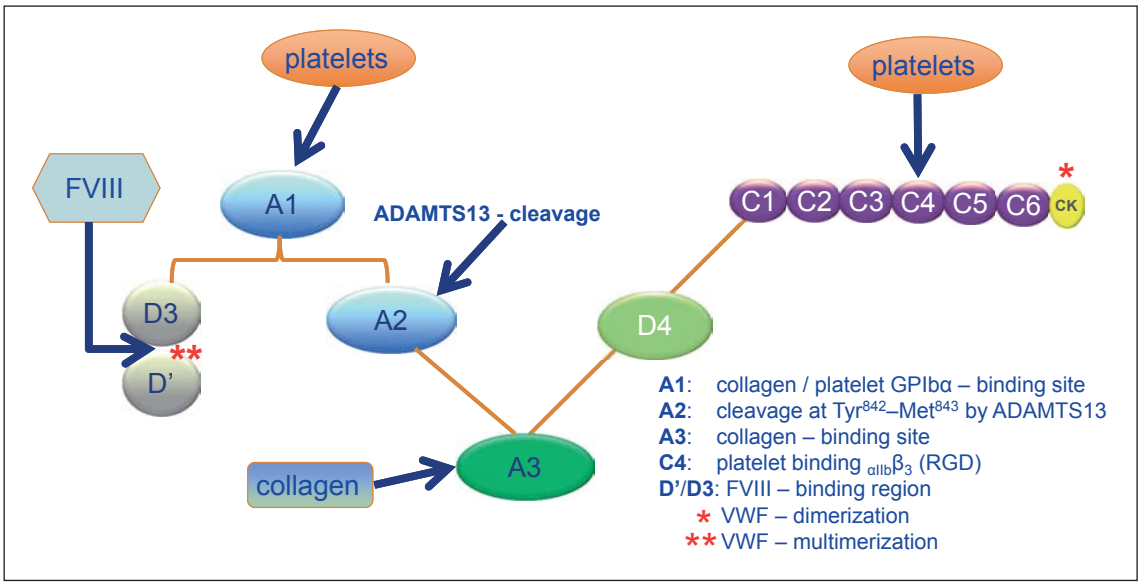

Fig. 2
Mature VWF subunit: Von Willebrand factor, delineating its active binding domains for platelets (A1 and C4), collagen (A3) and factor VIII (D'/D3) as well as its proteolytic site for ADAMTS13 (A2) according to Reininger AJ (Haemophilia 2008; 14 Suppl 5: 11–26) and Zhou YF et al. (Blood 2012; 120: 449–458).

hesion and aggregation (1, 8, 9, 10). These events are driven by shear forces acting in the blood flow. Physiological wall shear rates can reach as high as 4000 s^{-1} in the human circulation. Platelet deposition on various plasma proteins has been described as low as 50 s^{-1} for both fibrinogen and VWF but quickly decreases for fibrinogen surfaces and is literally absent when shear rates exceed 1500 s^{-1}; whereas VWF is the only plasma protein fully operational below and above these shear rates. Indeed VWF even works well at shear rates of 50 000 s^{-1} and probably far higher (11), a flow condition which can occur in vessels for example when a growing thrombus narrows the lumen to blood flow and thus dramatically increases the local shear rate (1, 7, 9).

VWF: the importance of ultra-large multimers

Subendothelial deposits of VWF can only be found in arteries, arterioles and large veins, according to immunohistological data (12), and basolateral secretion of VWF occurs only after stimulation in capillary endothelial cells *in vivo* (13). In response to several agonists of physiological relevance, such as histamine, estrogens, thrombin and fibrin, regulated secretion of stored VWF from endothelial cells occurs (14–17). It is generally assumed that VWF stored within the endothelial cell organelles – the Weibel–Palade bodies – is composed of the largest multimeric species (18, 19), i.e. ultra-large (UL) VWF, which is not usually observed in the blood of healthy individuals (20). Their controlled release at sites of injury would deliver the most thrombogenic forms of VWF.

As much as 20% of the total VWF present in blood may be found in the second storage site for VWF: platelet α-granules. Additional molecules important for hemostasis, such as fibrinogen, thrombospondin and fibronectin are also found in the platelet α-granules (21). The VWF of platelet α-granules characteristically also consists of the UL VWF multimers (22, 23). Upon stimulation by agonists such as adenosine diphosphate (ADP), collagen and thrombin, platelets release their α granule contents (24). Simultaneous release of UL VWF multimers from adjacent endothelial cells ensures the presence of the hemostatically most effective UL multimers at the vessel wall and in the fluid phase in the immediate vicinity of vascular lesions.

The metalloproteinase ADAMTS13 cleaves all VWF multimers including UL VWF, and thus functions as a physiological control mechanism to reduce their appearance in circulation (23). Therefore, UL VWF multimers can be detected in normal plasma only transiently, for example after induction of their secretion from endothelial storage sites with the therapeutic agent I-deamino-8-D-arginine vasopressin (DDAVP) (1, 23, 25). The VWF acutely released from storage sites is unlikely to have factor VIII (FVIII) bound to it and will bind it in the circulation. Thus it may serve to localize FVIII at a site of vascular damage and its subsequent detachment from VWF may be a prerequisite for FVIII activation and function as a clotting factor (26).

Specific functions have been identified for several domains on the VWF subunit (▶Fig. 2). The D' domain exhibits a binding site for FVIII and for heparin. In addition, the D'-D3 domains are possible binding sites for P-selectin, which has been found to anchor newly released UL VWF to the surface of activated endothelial cells, where ADAMTS13 can carry out proteolysis (27–29). The only known binding site for the platelet receptor GPIbα is the A1 domain, which also contains additional binding sites for heparin, sulphated glycolipids and the snake venom botrocetin, and disputably a binding site for collagen (30). The cleavage site for the metalloproteinase ADAMTS13 is located in the A2 domain. The A3 domain is the binding site for fibrillar collagens type I and III. The C4 domain with its RGD sequence is the binding site for integrin αIIbβ3. Both the A1 and A3 binding sites and their accessibility have been observed to rely on hemodynamic forces exerted on the molecule as well as its immobilization on a surface (1).

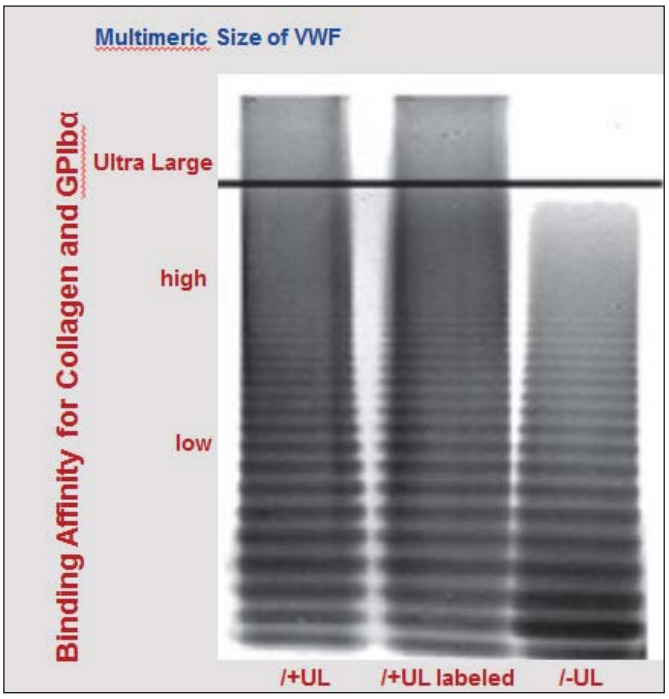

Fig. 3 Profile of VWF multimers: The binding affinity of VWF molecules for collagen and GPIb increases with the size of its multimers with ultra-large molecular weight multimers having the highest number of collagen and platelet binding sites per molecule and thus the largest hemostatic potential. VWF multimers are composed of disulfide bond-linked dimers that can exceed 1 µm in length, weighing approximately 500 kDa.

VWD is the most frequently inherited bleeding disorder caused by a deficiency of VWF, affecting approximately 1% of the general population. The incidence of clinically relevant cases is lower – about 100 cases per million people – characterized by quantitative (types 1 and 3) or qualitative (type 2) defects of VWF (31–33). The aim of VWD treatment is to correct the dual defect of hemostasis: abnormal coagulation expressed by low levels of FVIII and abnormal platelet adhesion expressed by the prolonged bleeding time. Plasma derived (virus inactivated) VWF/FVIII factor concentrates, initially developed for the treatment of hemophilia A, also contain large amounts of VWF and are often used to treat desmopressin-unresponsive patients (34–38). However, the plasma-derived VWF/FVIII concentrates are subject to several limitations such as variation in multimer composition, lack of UL VWF multimers due to cleavage by ADAMTS13 prior to isolation, and donor dependency (34).

Human VWF produced by recombinant technology could offer a new perspective in treatment of VWD (39, 40). Baxalta Innovations GmbH, Deerfield, IL, USA, has developed rVWF containing intact UL VWF multimers which is synthesized by a genetically engineered CHO cell line that expresses the VWF gene (41). This rVWF preparation has properties with respect to platelet mass accrual in the form of rVWF-platelet conglomerates (42), platelet adhesion and aggregation only occurring under high shear flow conditions and highly regulated by ADAMTS13 compared to preparations without UL multimers (▶Fig. 3).

The presence of these UL multimers in rVWF may result in improved platelet and collagen binding and therefore provide more effective treatment outcomes. However, without shear force, VWF remains in a globular form, its A1 receptors (which bind to the platelets' GPIbα-binding site) concealed. At increasing rates of shear force, the VWF protein is extended into a chain (▶Fig. 4), revealing its receptor sites and enabling it to form UL multimer aggregates with platelets, which may form the basis for the rapid formation of blood clots (1, 43, 44). This extension as well as the VWF-platelet interaction is entirely reversible in case of entry into a lower shear force environment.

Shear force-dependent VWF: self-assembly and formation of platelet conglomerates

Blood flow has an often-neglected role in hemostasis and thrombosis: it brings the reaction components together or dissipates them. In addition to localizing blood components, blood flow also exerts forces on the vessel wall, the blood cells and the plasma proteins and in doing so may influence their properties and function. The velocity of flowing blood, driven by the heartbeat, differs not only in the various provinces of the vasculature but also within the same blood vessel: at a maximum in the center, decreasing to zero at the vessel wall, the blood moves in discrete fluid layers, which exhibit differential velocities. A force is thereby generated between these layers termed fluid shear stress and expressed in Pascal (1 Pa = 1 N m^{-2} = 10 dynes cm^{-2}).

The viscosity of the blood is proportional to the shear force as well as the shear rate (s^{-1}), which is the difference in flow velocity as a function of distance from the vessel wall. As the viscosity of the non-Newtonian fluid "blood" changes with the shear force and since hemostasis and

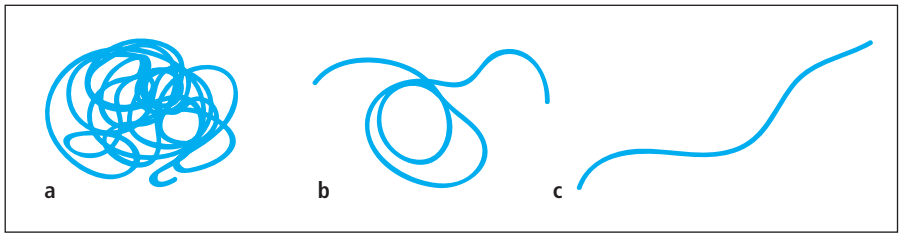

Fig. 4 Von Willebrand factor, the extendable protein: typical VWF polymer configurations as would be observed in VWF ultra large multimers in various solvent conditions and increasing shear rates; adapted from Schneider et al. (PNAS 2007) and Siedlecki et al. (Blood 1996)

a) coiled; b) partially coiled; c) fully extended

thrombosis occur at the boundary layer adjacent to the vessel wall or the surface of a growing thrombus, wall shear rate is often used to indicate the shear stress without the need to calculate the viscosity. Shear rates in arteries are usually higher than in veins, with the highest wall shear rates reached in small arterioles of 10–50 µm diameter: levels here have been estimated to vary between 500 and 4 ks^{-1}.

The rheological or hemodynamic phenomenon of erythrocyte axial migration refers to increasing flow velocity, which is the force that seems to cause erythrocytes to concentrate as bulk towards the vessel axis. This leaves a red-cell-free boundary layer adjacent to the vessel wall, in which platelets are enriched (plasma skimming): the distribution of platelets near vessel walls, also known as lateral migration or platelet margination (45–54). In cases of low red cell counts these dynamics can result in a reduced platelet concentration in this boundary layer, which may increase bleeding time (55).

A single platelet rolling over a rVWF-coated surface and interacting via its GPIbα receptor with the A1-domain of immobilized VWF can simultaneously interact with soluble VWF flowing in the adjacent fluid layer. If the shear rate exceeds a critical threshold, the previously globular, or coiled, VWF molecule can be stretched into an extended chain via the shear force (▶Fig. 4) (43, 44). This enables it to capture more platelets with the A1-domain exposed on the stretched molecule. Platelets attached to the elongated VWF molecule can then act in a sail like fashion to increase the stretching of the molecule, thus exposing more A1-domain. More A1-domain can then bind to more GPIbα receptors either on the same platelet or on newly captured ones. More VWF can bind on the surface of the platelets with a repetition of the above sequence of steps. Single molecule strands would thus bind to platelet surfaces and act as glue between non-activated platelets. In addition a shear dependent VWF self-assembly mechanism between the individual VWF molecules appears to be present, the biochemistry of which has not yet been analyzed.

We recently investigated the role of VWF molecules in the formation of rolling

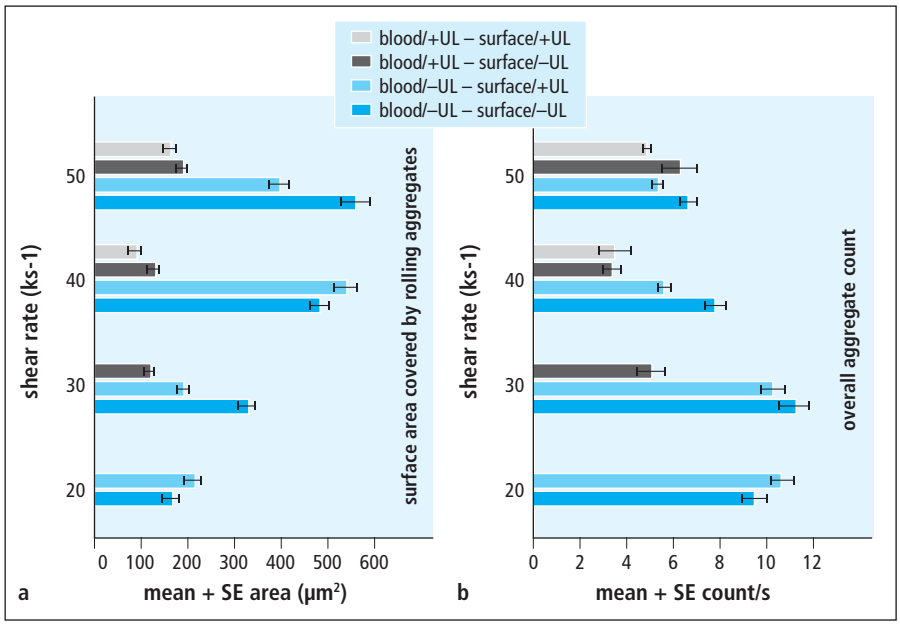

Fig. 5 Dependency of rolling aggregate formation on ultralarge VWF multimers in solution and on the surface; adapted from Kragh et al. (Thromb Res 2014): Rolling aggregate formation in washed blood flowing over rVWF coated surface (bright field). Perfusion (n = 3 for each condition) was performed at high shear rates (20 ks^{-1} to 50 ks^{-1}) with washed blood cells suspended in Hepes/Tyrode buffer (pH 7.4) over immobilized rVWF either with (rVWF/+UL) or without (rVWF/-UL) ultralarge VWF molecules (20 µg/ml coating concentration); the buffer solution also contained soluble rVWF with or without ultralarge VWF molecules, respectively. SE: standard error

a) The surface area (µm²) covered by rolling aggregates for shear rates of 20 ks^{-1}, 30 ks^{-1}, 40 ks^{-1}, and 50 ks^{-1}. Higher shear rates resulted in increased coverage and larger rolling aggregates. rVWF without UL multimers resulted in aggregate formation only at shear rates above 30 ks^{-1}.

b) The overall aggregate count was to be inversely related to increasing shear rates. The surface-bound rVWF was of greater impact at 50 ks^{-1} in terms of aggregate count per second. The highest aggregate counts were found with rVWF/+UL in both fluid and surface at 30 ks^{-1} and decreasing at higher shear rates.

platelet aggregates *in vitro* (42). In our wall parallel flow chamber experiments, the rVWF, which contained UL VWF multimers (+UL), was fluorescence-labeled but a single labeled molecule was not strong enough for video detection in flow. However when the shear rates increased above 20 ks^{-1}, the rVWF molecules self-assembled, and VWF fibers and networks could be detected either rolling over or immobilized on the surface. rVWF-networks and platelet aggregates were co-localized in conglomerates as detected with double illumination (bright field + fluorescence). Rolling rVWF networks steadily increased in length correlative with rising shear rates, but this effect was completely reversible below the threshold of 20 ks^{-1}. For the shear rates investigated, the main frequency range of the rVWF network length was between 10 and 70 µm, possibly indicating an optimum net length. Longer networks were observed, but these disaggregated within seconds. The shift of the peak frequency from 30 µm rVWF net length at 30 ks^{-1} to 55 µm length at 40 ks^{-1} seems to indicate the existence of higher binding forces between the rVWF molecules as well as rVWF and platelets at higher shear force.

When we coated the surface with collagen type I, the rVWF-platelet conglomerates no longer rolled but immobilized on this surface at high shear (42). Fluorescence-labeled rVWF molecules adhered from the fluid onto the coated collagen type I fibers and self-assembled to microscopically visible long strands oriented parallel to the flow streamlines. On top of these initial strands large rVWF-networks formed that caught platelets from the flow

Fig. 6 Stagnation point flow: Liquid flowing onto a flat surface creates a situation of stagnation point flow, with shear forces increasing radially from the center point. Stagnation point flow can be found in vessel curvatures and branchings, i. e. the common carotid artery bifurcation into the internal and external carotid artery.

and accumulated them to large surface bound aggregates. These stable rVWF-platelet conglomerates were either oriented in the flow direction or perpendicular to it in half-moon forms. Mobile aggregates rolled over those stable conglomerates but not over the collagen surface itself. Comparing the effect of rVWF/+UL presence and absence (rVWF/-UL) in the fluid showed significant differences (▶Fig. 5). We observed that at higher shear rates, the surface area (μm²) covered by rolling aggregates was much higher and the conglomerates larger. rVWF without UL led to aggregate formation only at shear rates above 30 ks^{-1}. The overall aggregate count was shown to be inversely related to increasing shear rates, with surface-bound rVWF having more impact at 50 ks^{-1} in terms of aggregate count per second. However, the highest aggregate counts were found with rVWF/+UL in both fluid and surface at 30 ks^{-1} and decreased at higher shear rates. In experiments lacking UL multimers, the collagen surface was covered with a larger number of smaller rVWF-strands that hardly bound platelets from the flow. This suggests that UL multimers were necessary in the initial attachment and growth phase.

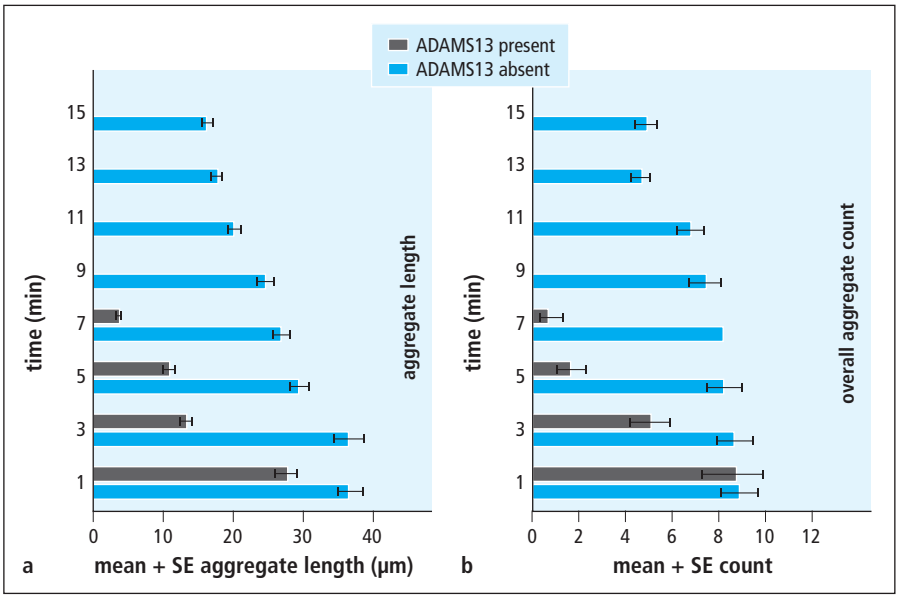

Fig. 7 Dependency of rolling aggregate formation on ultralarge VWF multimers in solution and on the surface; adapted from Kragh et al. (Thromb Res 2014)

a) The effect of rADAMTS13 kinetics on rVWF network formation under high shear rates. Perfusion (n = 3) was performed over immobilized rVWF/+UL at a shear rate of 40 ks^{-1} with a washed blood cell suspension containing fluorescence labeled rVWF.

b) Even at one minute of flow, a diminishing effect is detectable in the bar graphs. The normal distribution of the network lengths positively skewed and ended in a small peak between 10 and 20 μm, before they diminished completely. Surface-adherent rVWF-strands were reduced and had disappeared after 15 minutes.

The rVWF-platelet structures not only resisted the high shear stress but were actually fostered by it, which indicated the existence of catch bonds (57) between the platelet GPIbα receptor and the VWF A1 domain. This suggests that strong intermolecular cysteine bonds favor the interaction between single VWF molecules and that high shear rates activate the VWF molecule by uncoiling it (43, 44).

Stagnation point flow

In addition to wall parallel flow we also examined VWF-platelet interaction and conglomerate formation in stagnation point flow, which occurs in vessel bifurcations and curvatures, as well as downstream of a stenosis (42). Stagnation point flow has a flow component directed perpendicular to the wall (▶Fig. 6). Therefore, a wall shear gradient is generated that is zero in the center, has a peak at a distance (in our experiments at R = 241 μm) and decreases again further outwards. Despite principal hemodynamic differences between both flow conditions, the same phenomenon of rolling aggregates as with wall parallel flow was observed using rVWF/+UL in the fluid and coated on the surface. With increasing peak shear rates the average aggregate length steadily augmented. However, the first rolling platelet aggregates were already detectable in stagnation point flow at a shear rate of 8 ks^{-1} as compared to 20 ks^{-1} in wall parallel flow. This strongly indicates that shear rates do not yet give us the full picture and other still unknown rheological or biophysical parameters have to be added to fully explain platelet-VWF interactions under high shear flow.

ADAMTS13 as regulatory counterpart of VWF

It has been shown that VWF UL multimers are highly hemostatic, and thrombogenic risk under certain circumstances needs to be considered. The UL multimers present *in vivo* are, however, transient, due to rapid proteolysis of the multimers by the plasma metalloprotease ADAMTS13, which de-

grades VWF multimers under elevated shear stress by high affinity binding to VWF and by cleaving at the Tyr1605-Met1606 bond in the A2 domain (▶Fig. 2).

It is likely that the UL VWF molecules are the natural substrate for ADAMTS13 and that their proteolysis is partial and regulated. Because UL VWF multimers are very large in size and hyperactive in binding the platelet receptor GPIb-IX-V complex, they should be quickly removed from the plasma of healthy individuals (1, 58).

Upon stimulation, UL VWF is suggested to be released and immediately anchored onto the surface of endothelial cells via binding to P-selectin (27, 29, 59), stretched by fluid shear stress and the A2 domain – ADAMTS13 cleavage site – is exposed (this domain is usually sandwiched between two much larger A1 and A3 domains). The stretching of VWF molecules has been visualized under flow conditions and the necessary shear threshold was shown to range from 3 to 5 ks^{-1} (1).

Experimental data also demonstrated that shearing in the fluid phase without immobilization on a surface is sufficient to stretch the molecule (44). Once cleaved, the regular VWF fragments adopt a globular shape with limited access for ADAMTS13, rendering them resistant to further degradation. Quick cleavage of UL VWF when stretched while inactive when coiled minimizes potential adverse prothrombotic effects of even a brief appearance of UL VWF multimers in plasma.

Perfusion of normal plasma containing ADAMTS13 (or partially purified ADAMTS13) results in almost immediate cleavage of platelet/UL-VWF strings that were released in response to endothelial agonists, but this effect is not seen when ADAMTS13-deficient plasma from patients with TTP is used. These results support the notion that ADAMTS13 indeed may function to cleave newly secreted UL VWF multimers from the surface of endothelial cells, which otherwise may have persisted long enough to induce platelet adhesion (29).

In other *in vitro* experiments we could show that labeled rVWF-networks rolled over the VWF coated surface within seconds after flow start at a shear rate of 40 ks^{-1}. When rADAMTS13 was present in the fluid, after 1 min of flow a diminishing effect was detectable (▶Fig. 7). It led to complete disappearance of VWF-networks at 8–9 minutes of flow. The normal distribution (10–90 µm) of the network lengths became positively skewed and ended in a small peak between 10 and 20 µm, before they diminished completely. Accordingly surface-adherent rVWF-strands were reduced and had disappeared after 15 minutes.

In a recent prospective phase 1 clinical trial using rVWF in 32 patients with VWD, all patients were tested for ADAMTS13 levels at enrollment, and none had abnormally low baseline ADAMTS13 levels (min-max was 89% to 244% in VWD type 1 patients, and 134% to 156% in type 3 patients, with a normal range from 50% to 160% in healthy adults) (62), which confirms previously published reports that subjects with type 3 VWD and, to a lesser extent, type 1 VWD have higher ADAMTS13 levels compared to healthy individuals (63). No apparent differences in proteolysis of UL multimers over time could be linked to the differences in ADAMTS13 concentration in the clinical study subjects.

The study also demonstrated *in vivo* proteolysis of the multimers by ADAMTS13: cleavage products were detected in 21 of 22 type 3 VWD patients at 15 minutes post-infusion (the earliest time point measured), indicating rapid enzymatic degradation (64). As shown (▶Fig. 8), enzymatic degradation was rapid, as indicated by the appearance of typical VWF cleavage products 15 minutes post-infusion, which was the earliest time point examined.

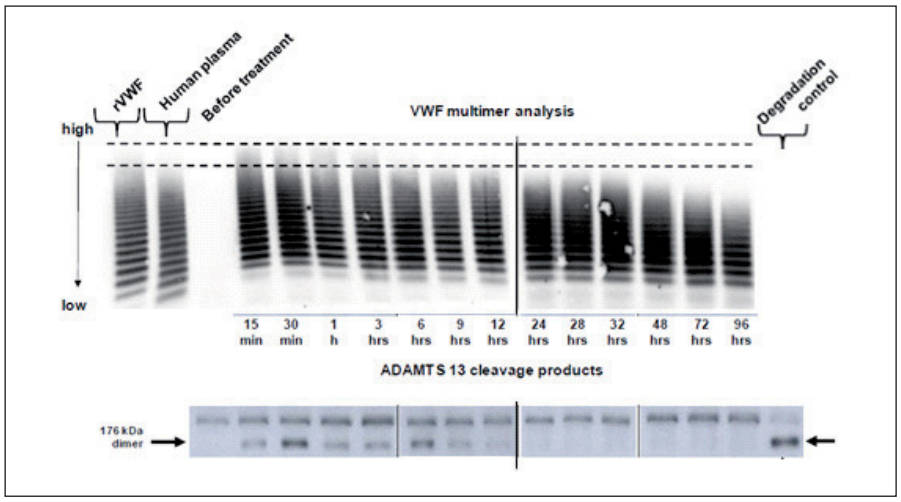

Fig. 8 VWF multimers and degradation products post-infusion of rVWF-rFVIII, adapted from ©Mannucci et al. (Blood 2013)

Electrophoretic analysis: qualitative analysis of VWF multimers was performed using SDS-agarose gel electrophoresis followed by Western blotting and sensitive luminescence two step detection. VWF degradation products generated by ADAMTS13-mediated cleavage were measured by SDS-PAGE under reducing conditions followed by Western blotting and immune-staining with a horseradish peroxidase (HRP)-labeled polyclonal rabbit antihuman VWF antibody with enhanced chemiluminescence detection.

Dotted horizontal lines indicate the zone where bands of UL multimers appear. Due to limitations in space, it was not possible to run all samples taken from a single patient during the PK study on 1 gel. Therefore, to show the distribution of multimers and fragments over time, it was necessary to combine different gels into 1 picture. Vertical lines have been inserted to indicate repositioned gel lanes.

Typical VWF multimer pattern (upper gels; low-resolution agarose [1% Seakem]; samples adjusted to VWF:Ag content) and fragments cleaved by ADAMTS13 (lower gels; SDS-PAGE followed by immunoblot with polyclonal anti-VWF antibody; samples were applied undiluted) post-infusion of rVWFrFVIII.

Conclusion

Several properties of VWF that had not been described previously were recently

identified: its ability to self-assemble into large networks of VWF strands under conditions of high shear stress and disassemble when entering below a certain shear threshold, its (also reversible) mutual reinforcement with non-activated platelets to form large conglomerates, and its dependency on rheological conditions that differed at least two-fold in shear rate – wall parallel versus stagnation point flow. In recent *in vitro* experiments mimicking the shear forces in blood vessels the UL VWF multimers appear to increase binding functions more than all other fractions of VWF. However, the presence of these UL VWF multimers in blood flow is only transient due to the metalloprotease ADAMTS13, which has been shown *in vitro* and *in vivo* to cleave large VWF multimers within minutes, serving as an effective regulatory counterpart to the highly reactive UL VWF.

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Conflict of interest

The author is an employee of Baxalta Innovations GmbH.

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