Regulation of von Willebrand factor-platelet interactions

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Summary
The formation of thrombi is a multistep process involving several components, including von Willebrand factor (VWF). VWF is an adhesive multimeric protein, which acts as a molecular bridge between the subendothelial matrix and the glycoprotein Ib/IX/V receptor complex. Furthermore, VWF promotes the expansion of the platelet plug by cross-linking platelets via binding to integrin αIIbβ3. In terms of thrombus formation, it is essential that VWF-platelet interactions occur timely; that is: it should happen not too early or too late. Given the co-existence of VWF and platelets in the circulation, this implies that there must be regulatory mechanisms that prevent premature formation of VWF-rich platelet aggregates that could occlude the vasculature. Indeed, several mechanisms have been identified at the level of VWF, which are dedicated to the prevention of excessive VWF-platelet interactions following endothelial release of VWF (which may include limited exposure to shear stress, the presence of Mg2+ ions, inhibition of VWF-platelet interactions by endothelial proteins, ADAMTS13-mediated proteolysis) and of circulating VWF-platelet aggregates during normal circulation (shielding of the platelet-binding A1 domain by other regions of the VWF molecule, inhibition of VWF-platelet interactions by β2-glycoprotein I). In the present review an overview of these mechanisms will be discussed.

Keywords
Von Willebrand factor, thrombosis, von Willebrand disease

Introduction
Cardiovascular complications such as myocardial infarction, stroke and other ischaemic events, are caused by impaired blood circulation due to the obstruction of vessels by platelet-rich thrombi. The formation of these thrombi is a multistep process involving several components, including von Willebrand factor (VWF). In view of its critical role in thrombus formation, it is not surprising that several epidemiological studies have revealed that increased VWF levels predispose to an increased risk of coronary heart disease (1, 2).

VWF is an adhesive multimeric protein, which acts as a molecular bridge between the subendothelial matrix and the platelet glycoprotein (Gp)-Ib/IX/V receptor complex. VWF-mediated recruitment of platelets to the site of injury is initiated by interactions between VWF and components of the subendothelial matrix, like collagen, that are exposed in the damaged vessel wall (3). VWF-collagen interactions allow the exposure of the platelet-binding site in the VWF molecule. It has been generally accepted that VWF is particularly important in the process of platelet adhesion under conditions of high shear rates (exceeding 500–800 s⁻¹ in human blood; [3]), shear rates that are normally found in the arterial vessels. This view is mostly based on in vitro perfusions experiments, in which platelets are found to adhere to collagen surfaces in a VWF-independent manner at shear rates below but not above this threshold. However, recent studies using VWF-deficient mice revealed that VWF is relevant for the recruitment of platelets in venous vessels as well (4). Indeed, VWF has been found to be present in ilio-femoral venous thrombi and in pulmonary thromboemboli of patients who died of venous thromboembolism (5).

It is important to realise that before participating in the formation of platelet-rich thrombi, VWF and platelets co-exist in the circulation without interacting with each other (Fig. 1). This lack of interaction is obligatory to avoid spontaneous occlusion of the vessels, and implies that mechanisms exist that prevent premature formation of VWF-rich platelet aggregates. Such regulatory mechanisms may act at the level of the GpⅡb/Ⅲa receptor complex via modulation of interactions between the GpⅡb receptor complex and cytoskeletal proteins like filamin A and 14–3–3ζeta (6, 7). The aim of the present review is to focus on the regulatory mechanisms at the level of VWF that are designed to prevent premature binding of VWF to platelets.

Platelet binding to freshly secreted VWF
The majority of circulating VWF originates from endothelial cells, where it is synthesised as a series of heterogeneously sized multimeric proteins, which can contain as many as 50 covalently linked subunits. Following its synthesis, VWF is targeted to endothelial storage granules, the Weibel-Palade bodies, and released in the cir-
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... culation. This release may occur in a constitutive manner or following stimulation of endothelial cells, and provides the first encounter between VWF and platelets in the circulation. Agonist-mediated stimulation of endothelial cells results in the rapid formation of strings that can be several hundred micrometers long (8, 9). By using high-resolution scanning electron microscopy, Huang et al. revealed that such strings consist of multiple VWF multimers, assembled in twisted bundles and networks (8). One of the properties of these freshly secreted VWF bundles is that they constitute an efficient adhesive platform for platelets, a property that has been elegantly visualised both in vivo and in vitro (10–12).

Interestingly, despite the full exposure of freshly secreted VWF to platelets, it seems that only part of the VWF bundles are covered with platelets. Moreover, Huang et al. were able to identify platelet-free VWF strings, even after prolonged exposure to these platelets (8). This indicates that not all of the GpIbα-binding sites within the VWF A1 domains are available for platelet binding.

Why only part of the GpIbα-binding sites is occupied is currently unclear, but a number of possibilities could be considered. First, it is possible that exposure of binding sites might correlate with the extent of shear to which the VWF bundles are exposed. This would imply that upon increased shear, the fraction of available GpIbα-binding sites should increase. Second, recent studies revealed that VWF-platelet interactions are affected by Mg2+ ions (13). In the presence of increasing Mg2+ concentrations, Dong et al. observed a dose-dependent inhibition of VWF-platelet interactions. In addition, the number of ultra-large VWF strings was reduced two-fold in the presence of micromolar concentrations of MgSO4. These data suggest that local changes in Mg2+ concentrations may interfere with the adhesion of platelets to freshly secreted VWF. Another possibility concerns endothelial proteins. When targeted to the endothelial Weibel-Palade bodies, VWF functions as a chaperone molecule to target several other proteins to the Weibel-Palade bodies. At present, this list of Weibel-Palade cohabitants includes over 10 different proteins, including P-selectin, interleukin-8 and osteoprotegerin (OPG) (for review see [14]). For at least one of these proteins, i.e. OPG, it has been shown that it remains associated to VWF following its secretion into the circulation (15, 16). Moreover, OPG binds selectively to the VWF A1 domain, suggesting that OPG may potentially interfere with platelet binding. We are currently investigating which proteins of the Weibel-Palade body proteome and those present in the endothelial cytosol have the capacity to bind to the VWF A1 domain and subsequently modulate VWF-platelet interactions.

Proteolytic processing of VWF by ADAMTS13

The VWF molecule is exposed to circulatory shear stress while being attached to the endothelial surface following its secretion, resulting in the molecule being at least partially unfolded. As mentioned above, this may result in the unmasking of platelet-binding sites within the VWF A1 domains. Simultaneously, exposure to shear stress makes VWF also susceptible to proteolysis by the VWF-cleaving protease ADAMTS13 (17). ADAMTS13 is a circulating metalloprotease of the “A Disintegrin And Metalloprotease with Thrombospondin type 1 motif”-family and proteolyses VWF within the A2 domain between residues Y1605-M1606 in a shear-dependent manner (18). Of note, ADAMTS13-mediated proteolysis is facilitated when the elongated VWF molecule is covered with platelets (19). Cleavage of VWF by ADAMTS13 results in the loss of the very large VWF multimers that are secreted from the endothelial cells and the generation of the typical satellite...
bands that are visible upon VWF multimer analysis. The multimeric profile of VWF present in the supernatant of PMA-stimulated endothelial cells contains more than 25 distinct bands (20, 21). Assuming that each band consist of dimeric building blocks, this number corresponds to the presence of multimers containing more than 50 individual subunits of 250 kDa each. In plasma of healthy individuals, however, the number of detectable bands does usually not exceed 20 bands (40-mers, when following the same reasoning), indicating that a substantial part of the high-molecular-weight VWF multimers is eliminated under normal conditions. In plasma of patients that have severely reduced ADAMTS13 activity, often an increase in multimer size is observed (22, 23). The size of these multimers is intermediate between those found in normal plasma and in the supernatant of stimulated endothelial cells (21). Nevertheless, the presence of these ultra-large VWF molecules in the circulation may be potential dangerous in view of their capacity to form platelet-rich thrombi. Indeed, the physiological importance of ADAMTS13 is illustrated by the notion that its absence is associated with the occurrence of platelet-rich thrombi that can occlude the microvasculature, a pathological condition known as thrombotic thrombocytopenic purpura (TTP) (24).

Apart from the removal of circulating ultra-large VWF multimers, ADAMTS13-mediated proteolysis also coincides with the loss of the spontaneous platelet-binding capacity. The mechanism by which ADAMTS13 cleavage results in loss of platelet adhesion to VWF has not yet been solved. Most likely, cleavage of VWF in its shear-induced, stretched conformation by ADAMTS13, results in its release from the endothelial surface. The drop in shear stress allows the molecule to adopt a globular conformation in which platelet accessibility is limited. A number of experimental approaches have been reported in which shear stress-dependent changes in VWF conformation have been monitored (25–27). For instance, Schneider et al. exploited a microfluidic flow chamber to monitor VWF’s response to hydrodynamic stress (26). Dependent on solvent conditions and different values of shear rate, different polymer conformations could be observed. The VWF molecule was found to adopt a closed globular configuration at low shear rates, while at high shear rates an open stretched structure was found. Of importance, the process of shear-dependent conformational changes appears to be fully reversible, in that the closed globular shape can re-adopt the open stretched conformation when shear rates increase.

**Intramolecular shielding of VWF platelet-binding domain**

Although not formally proven, it seems conceivable that shear stress-induced changes from an open elongated structure into a closed globular conformation results in shielding of the platelet-binding sites located within the VWF A1 domain. This in turn raises of course the question, how the A1 domain in this closed globular conformation is protected from interactions with GpIbα?

One of the curious properties of VWF is that it has a tendency to self-associate (28). This may involve homotypic interactions between immobilised and circulating soluble VWF molecules, but also shear-dependent self-association of VWF in suspension has been described (28, 29). By using a series of VWF fragments, it was found that these homotypic interactions involve multiple domain interactions (30). Moreover, such interactions may not only occur between VWF molecules (intermolecular interactions), but also within the VWF molecule itself (intramolecular interactions). Two examples have been reported in this regard. First, Ulrichs et al. described an interaction between the amino-terminal D’-D3 domains with the adjacent A1 domain (31). This interaction was found to be of relevance with regard to the accessibility of the A1 domain for GpIbα. Indeed, removal of the D’-D3 region from the VWF molecule resulted in an augmented interaction with platelets. This increased binding could subsequently be reverted upon titration with the isolated D’-D3 fragment. In addition, an antibody directed against the D’-D3 domain not only inhibited the interaction with the A1 domain, but also increased the affinity for GpIbα (31). An alternative pathway to shield the A1 domain was described by Martin et al. (32). By using recombinant VWF fragments produced in *Escherichia coli*, it was found that the A2 domain efficiently inhibits binding of platelets to the VWF A1 domain. Considering their close proximity within the VWF molecule, the A2 domain is therefore an attractive candidate to act as a modulator of interactions between the A1 domain and platelets.

An intriguing issue relates to the notion that the VWF A1 domain is surrounded by sialylated O-linked glycans. Moreover, the D’-D3 and A2 domains harbour three and two N-linked glycans, respectively. For over two decades it has been known that modification of these carbohydrate structures may result in altered binding to platelets (33–40). It is possible that glycan structures themselves contribute to the interaction between VWF and platelets, explaining why their modification could affect this interaction. With the current knowledge that adjacent domains have the potential to modulate platelet accessibility within the A1 domain, there might also be an alternative explanation (which is not necessarily mutually exclusive). It seems well possible that by modifying carbohydrate structures, one also modulates the interaction between the A1 domain and its adjacent domains, thereby indirectly affecting the accessibility of the A1 domain for platelets.

**Presence of platelet-binding VWF under pathological conditions**

One of the remarkable findings in the studies by Schneider et al. is that the VWF molecule may rapidly react following changes in shear stress with regard to its conformation. This sense of reversibility indicates that there is equilibrium between VWF being in the closed, globular configuration (in which it is unable to interact with platelets) and VWF being in the open, stretched conformation (in which it is able to bind platelets). In support of this mechanism is the finding that VWF and platelets aggregate in a re-
versatile manner at shear rates exceeding 10,000 s⁻¹ (41). It seems obvious, that under normal conditions the vast majority of the VWF molecules exist in the non-platelet binding form. However, a number of pathological conditions are known, which are characterised by the undesired formation of VWF-platelet aggregates (42). As indicated above, a deficiency of ADAMTS13 as found in TTP is associated with the occlusion of microvasculature by VWF-rich platelet thrombi. The presence of circulating platelet-aggregates is also observed for von Willebrand disease (VWD)-type 2B. This disorder is caused by gain-of-function mutations in the platelet-binding A1 domain of VWF, resulting in an enhanced affinity for GpIbα. In contrast to TTP-patients, however, VWD-type 2B patients display a mild to severe bleeding tendency, rather than a risk of thrombosis, despite the presence of circulating platelet aggregates (43).

Several years ago, we developed a tool to quantify levels of circulating VWF that is in its platelet-binding conformation. A recombinant llama-derived antibody was selected that showed preferred binding to active, platelet-binding VWF over quiescent VWF (44). This antibody is directed against the VWF A1 domain, and studies using isolated A1 domain variants suggest that this antibody is able to distinguish between the various conformations this domain can adopt. Indeed, VWF present in plasma of normal individuals showed little binding to this antibody. In contrast, enhanced binding was found for VWF present in plasma of VWD-type 2B and TTP patients. In quantitative terms, there was a two- to 14-fold increase in levels of active VWF in VWD-type 2B patients, and a two- to four-fold increase in patients with TTP (44).

Having this method established to detect circulating active VWF, we also investigated the presence of active VWF in other pathological conditions. For instance, we observed increased levels of active VWF in patients suffering from malignant hypertension, pregnant women with HELLP-syndrome or individuals infected with Plasmodium falciparum, the malaria-causing parasite (45–48). Relevant for this review is that we also detected circulating active VWF in a subset of patients with antiphospholipid syndrome (APS) (49). APS is an autoimmune disease characterised by a variety of clinical symptoms, like venous and/or arterial thrombosis, recurrent pregnancy loss in combination with the persistent presence of autoantibodies against a spectrum of (mostly phospholipids-binding) proteins, including β2-glycoprotein I (β2-GPI) (50). Antibodies against these proteins may be monitored via dedicated laboratory assays, the most commonly used variant of which is the phospholipid-based coagulation assay, lupus anticoagulants (LAC). The LAC test may be used to determine if the prolongation of the clotting time depends on the presence of antibodies against β2-GPI or other proteins, like prothrombin. It is important to make this distinction, because β2-GPI-dependent LAC is associated with a more than 20-fold increased risk for thrombotic complications compared to patients with a β2-GPI-independent LAC (51). When we were testing for the presence of active VWF, it appeared that these levels were increased two-fold in APS-patients with β2-GPI-dependent LAC, but not in those with a β2-GPI-independent LAC (49).

### β2-GPI as a natural inhibitor of active VWF

Having identified these slightly, but significantly increased levels of active VWF in plasma of APS-patients with β2-GPI-dependent LAC, the next step was to try to explain the underlying mechanism of these increased levels. Additional experimentation revealed that the presence of active VWF could not be explained by an acute activation of the endothelium, a reduced ADAMTS13 activity or even the presence of autoantibodies that convert VWF in its platelet-binding conformation. Ultimately, we challenged the option that β2-GPI itself could influence active VWF levels via direct interactions. Indeed, β2-GPI proved able to interact directly with VWF. However, the interaction was of remarkably low affinity, which would prevent complex formation in the circulation. Surprisingly, the affinity was enhanced several-fold upon conversion of VWF from its quiescent into its active conformation (49). In search of the β2-GPI interactive site within the VWF molecule, we discovered that β2-GPI binds to the VWF A1 domain, and actually interferes with VWF-GpIbα interactions. This effect was exemplified by the ability of β2-GPI to inhibit VWF-dependent platelet agglutination and adhesion. Importantly, this inhibitory effect could be neutralised by the addition of anti-β2-GPI antibodies, either monoclonal or isolated from APS-patients. This neutralising effect may explain why we could detect active VWF selectively in patients with a β2-GPI-dependent LAC.

Although identified as a serum protein in the early 1960s, its physiological function has remained unclear (52). In view of its phospholipids-binding properties, it was believed that the protein could exert an anticoagulant activity by displacing various coagulant proteins from anionic phospholipids sites (for review see [52]). Our research now indicates that β2-GPI acts as a natural inhibitor of VWF. This immediately leads to the question as to what is the physiological relevance of β2-GPI in the regulation of VWF-platelet interactions under normal conditions. What is striking is that in plasma the concentration of β2-GPI is approximately 150–200 μg/ml (3–5 μM), whereas the VWF concentration is approximately 10–13 μg/ml (35–50 nM, based on monomer concentrations). In other words, there is nearly a 100-fold molar excess of the inhibitor over its substrate. It seems conceivable therefore that β2-GPI should be regarded as a weak inhibitor in view of the normal haemostatic process that occurs in the presence of such molar excess. On the other hand, it also indicates that there is apparently a delicate balance between VWF levels and β2-GPI levels. We tested this hypothesis by investigating the effect of changing the β2-GPI / VWF ratio on platelet aggregation. The reduction of available β2-GPI levels by 25% (via the addition of anti-β2-GPI antibodies) in plasma of normal individuals was associated with an increased response to ristocetin-induced platelet aggregation. When β2-GPI levels were increased by the addition of highly purified β2-GPI, a reverse effect was observed, in that higher ristocetin-levels were needed to achieve platelet aggregation (49). Thus, in spite of the large molar excess of β2-GPI over VWF, relatively small changes in β2-GPI levels may already affect its inhibitory capacity towards the VWF-GpIbα interaction.

The physiological relevance of β2-GPI-dependent inhibition of VWF was subsequently investigated by analysing a large cohort of
elderly men with myocardial infarction (53). This analysis revealed an inverse relationship between β2-GPI plasma levels and myocardial infarction in older men. Moreover, an increase in β2-GPI/VWF ratio was accompanied with a two- to three-fold reduced risk of myocardial infarction in men aged above 60 years. The observation that this relationship exists between β2-GPI and VWF with regard to the risk of myocardial infarction may provide an explanation for the lack of consensus with regard to VWF as a risk factor for arterial thrombosis (1, 2). In previous studies, β2-GPI was not taken into account as a player in the pathogenesis of myocardial infarction. Also differences in age distribution may contribute to the apparent opposite findings. Plasma levels of both β2-GPI and VWF are known to increase with age (54, 55), and it seems that VWF levels increase stronger than those of β2-GPI (53). This suggests that, the ratio β2-GPI / VWF will decrease with aging, thereby reducing the efficiency by which β2-GPI is able to interfere with premature VWF-platelet interactions.

Conclusion and perspectives

Recent investigations have revealed that a number of different pathways seem to be involved in preventing the formation of circulating VWF-platelet aggregates. At the level of VWF, this may include limited exposure to shear stress, the presence of Mg2+ ions and/or the inhibition of VWF-platelet interaction by proteins that are co-localised with VWF in the Weibel-Palade bodies or eventually proteins located in the endothelial cytoplasm. Second, proteolysis of VWF at the endothelial surface by ADAMTS13 relieves VWF from wall shear stress, and allows the transition from an elongated, platelet-binding configuration into a globular quiescent form. In this globular form, intra-molecular interactions between the A1 domain and its adjacent regions (i.e. the amino-terminal D’-D3 domains and the carboxyterminal A2 domain) are in place to reduce platelet accessibility. Finally, under conditions where circulating globular VWF adopts an active platelet-binding conformation, β2-GPI may act as a “first line of defense” to prevent undesired platelet aggregation.

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The identification of these various regulatory pathways now opens avenues to further investigate their role in relation to the clinical situation. For instance, both TTP and VWD-type 2B are characterised by the presence of circulating active VWF. However, the clinical consequences are rather different. TTP is characterised by microvascular thrombopathy, whereas VWD-type 2B is associated with an enhanced bleeding tendency. One important difference is that despite the enhanced platelet-binding capacity under both conditions, ultra-large VWF multimers are found in TTP, and a loss of high multimers is found in VWD-type 2B. This could contribute to the different phenotype of disorders, but of course the next question is: why is one disorder associated with a loss of high multimers but not the other? It has been assumed that the loss of high multimers in VWD-type 2B is due to increased platelet-absorption. But why is that not true for TTP? In order to solve this
issue, we have recently developed a mouse model for VWD-type 2B (56). Our findings demonstrate that excessive proteolysis by ADAMTS13 rather than platelet-absorption causes the loss of high multimers in VWD-type 2B. Thus, the possibility exists that excessive proteolysis by ADAMTS13 prevents thrombotic complications in VWD-type 2B.

References

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