

# Perspectives Series: Cell Adhesion in Vascular Biology

## von Willebrand Factor

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A perspective on von Willebrand factor (vWF)<sup>1</sup> within a series on cell adhesion in vascular biology offers the opportunity to review the current understanding of platelet function in hemostasis and thrombosis. Platelets contribute to maintaining the normal circulation of blood through the preservation of vascular integrity and the control of hemorrhage after injury. Thus, the formation of platelet thrombi is a needed defense mechanism, but may precipitate diseases such as myocardial infarction in the setting of atherosclerosis (1, 2). Acute thrombotic arterial occlusion is the leading cause of morbidity and mortality in industrial societies, underscoring the relevance of studies aimed at unraveling how platelets respond to vascular injury. Particularly important in this regard is vWF, along with the subendothelial matrix components and membrane receptors that interact with it, owing to a key role in supporting unique aspects of platelet function. Indeed, vWF-dependent adhesion mechanisms can be viewed as the evolutionary adaptation to the need of establishing firm contact between a circulating element and the vessel wall meeting any mechanical challenge created by blood flow conditions.

*Platelet function: A paradigm of adhesion mechanisms for circulating vascular cells*

Platelets survey the inner lining of the vessel wall without interacting with it under normal circumstances, but respond rapidly to alterations of endothelial cells by attaching firmly to the site of lesion, where exposure of subendothelial structures may have occurred. A first layer of platelets adheres to the reactive surface, subsequently growing by accrual of additional platelets through homotypic aggregation. Both processes depend on the binding of membrane receptors to immobilized or soluble ligands, and are modulated by stimulus-coupled biochemical and cytoskeletal responses. Platelet thrombus formation is the paradigm of adhesion for circulating vascular cells, that can interact efficiently with the vessel wall only by withstanding opposing forces created by blood flow. In fact, the tendency of

cells to move with the layer of fluid adjacent to the reactive surface creates unique biomechanical requirements for the formation of adhesive bonds. In a vessel, the velocity of blood near the wall is lower than towards the center, a difference resulting in a shearing effect between contiguous layers of fluid moving at different speed. Thus, "shear" is the consequence of the relative parallel motion of fluid planes during flow; it is greatest near the wall and decreases progressively towards the center of the vessel (3). The local shear rate is expressed in cm/s per cm, or the equivalent inverse second ( $s^{-1}$ ). Fluid shear stress is force per unit area, the underlying cause of the shearing motion of blood. Shear rate is directly proportional to shear stress and inversely proportional to fluid viscosity. Assuming for blood a viscosity of 4 centipoise, the numerical value of shear stress (in dyn/cm<sup>2</sup>) corresponds to shear rate divided by 40.

The role of platelets requires that they become irreversibly attached at sites of vascular injury. The force opposing stable adhesion and aggregation is greater with increasing shear rate, i.e., in arteries more than in veins and, particularly, in arterioles. The highest wall shear rate in the normal circulation occurs in small arterioles of 10–50  $\mu$ m diameter, where levels have been estimated to vary between 500 and 5,000  $s^{-1}$  (4). Yet, these are the vessels where effective hemostasis is more strictly dependent on the ability of platelets to form thrombi. The relevance of adhesion mechanisms responsive to high flow conditions may be even greater in pathological conditions associated with the occurrence of acute arterial occlusion. Wall shear rates of 3,000–10,000  $s^{-1}$  have been measured at the top of plaques occluding the lumen of diseased coronary arteries by 50% (5), a degree of stenosis still considered of moderate clinical significance (1, 2), and considerably higher values may occur with more severe occlusion. These considerations highlight the importance of vWF as the essential adhesive substrate mediating platelet thrombus formation against high shear forces.

### *von Willebrand factor structure*

The structural organization of the vWF molecule has been elucidated (reviewed in reference 6). The glycoprotein is synthesized in endothelial cells and megakaryocytes as a precursor polypeptide of 2813 amino acids (pre-pro-vWF), including the 12-residue signal peptide, the 751-residue propeptide and the 2050-residue mature subunit. After cleavage of the signal peptide, complex intracellular processing leads first to dimerization of pro-vWF, initiated by the noncovalent association of propeptide moieties, then to covalent polymerization with numerous interchain disulfide bonds both at the amino- and carboxyl-terminal ends. Multimers are either stored in specific or-

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1. *Abbreviation used in this paper:* vWF, von Willebrand factor.

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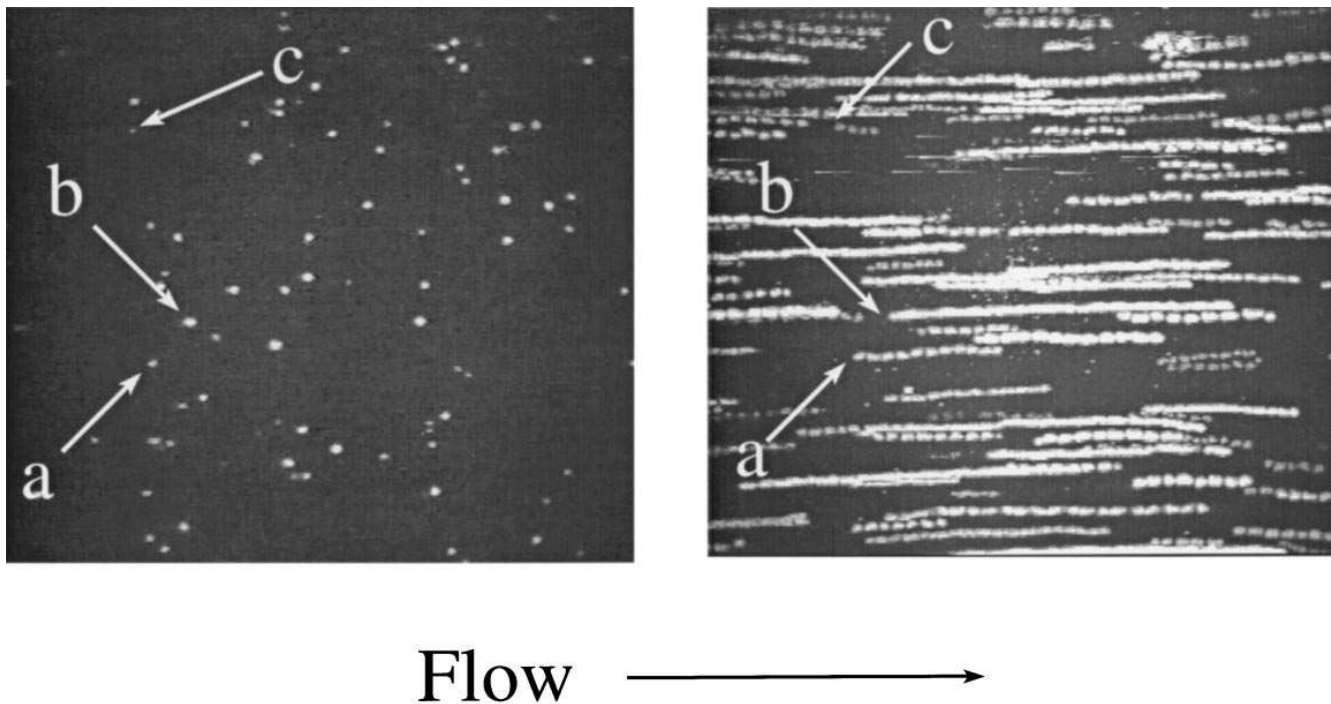
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ganelles for regulated release—Weibel-Palade bodies in endothelial cells and  $\alpha$ -granules in megakaryocytes (or platelets after thrombocytopoiesis)—or constitutively secreted. The latter pathway is operative only in endothelial cells, with bidirectional secretion both into the circulating blood and the sub-endothelial matrix. Megakaryocytes do not constitutively secrete vWF, but platelets release it during thrombogenesis. The 741-residue propeptide is normally cleaved before secretion of the multimers, and is found in the circulation where it may act as an independent modulator of cell adhesion to collagen. The degree of vWF polymerization is to some extent directly correlated to prothrombotic activity. The largest multimers are found in the subendothelium and in platelet  $\alpha$ -granules, whereas in blood (where soluble vWF and platelet membrane receptors are exposed to one another) a specific proteolytic cleavage in the constitutive subunit reduces multimer size after secretion (7, 8). Yet, vWF remains one of the largest circulating proteins, with molecular mass that can exceed 10,000 kD. Preliminary information has become available on the enzyme responsible for cleaving circulating vWF (9), opening a potentially important field of research on thrombotic disorders linked to accumulation of large multimers in blood. As important as the polymeric nature of vWF is for establishing

multiple adhesive bonds with platelets, specific substrate and receptor specificities crucial for this function reside in distinct subunit domains.

The binding sites for all molecules known to interact with vWF have been located with good approximation at the level of primary sequence. The amino-terminal domain within the first 272 residues of the mature subunit forms a complex with factor VIII, essential to maintain adequate circulating levels of this cofactor involved in thrombin generation. All other functional sites in the vWF molecule support platelet adhesion and aggregation by binding to extracellular matrix components or to membrane receptors. Two vWF domains, A1 and A3, have been shown to interact with collagen in a variety of different experimental models, but more recent evidence indicates that only the latter may be required for this function (10). However, the A1 domain can also bind to heparin-like molecules (11)—thus, possibly, proteoglycans—and to sulfatides (12), playing in any case a potentially important role in the immobilization of soluble vWF onto complex extracellular matrices (13). Notwithstanding other possible functions, it is established that the A1 domain and flanking regions (residues 449–728 of the mature subunit) represent the binding site for GP Ib $\alpha$  in the platelet membrane GP Ib-IX-V complex (14). The site of



**Figure 1.** Low velocity surface translocation of platelets interacting with immobilized von Willebrand factor. These images are derived from a real time experiment recorded at the video rate of 30 frames per second. Whole blood containing as anticoagulant D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride (PPACK), an inhibitor of  $\alpha$ -thrombin, was perfused onto a surface coated with immobilized vWF at wall shear rate of  $1,500 \text{ s}^{-1}$ . The image on the left represents a single frame, corresponding to a time period of  $1/30$ th of a second, recorded 1 min after initiating blood perfusion. Each fluorescent (white) particle is a single platelet tethered to the surface; three individual platelets are identified by arrows (a, b, and c). The image on the right is a composite created by the superimposition of 30 successive frames, taken at one second intervals beginning from the one shown on the left, thus covering a time period of 30 s. Each of the three platelets identified on the left appears at the origin of a track extending for variable length in the direction of flow, demonstrating movement from the position originally occupied. The composite image shows that all platelets tethered to the surface translocate for variable distances, thus have different velocities. The speed of motion has been found to vary between 2 and  $20 \mu\text{m}$  per second when the wall shear rate is  $1,500 \text{ s}^{-1}$ , corresponding to  $< 2\%$  of the calculated free flow velocity of platelets moving with the surrounding flowing blood in close proximity to the surface but not interacting with it (17). Freely moving platelets are not visible at the flow rate used for this experiment. Blocking  $\alpha_{\text{IIb}}\beta_3$  has no effect on the surface translocation of platelets interacting with immobilized vWF, while blocking GP Ib $\alpha$  prevents any interaction (17).

interaction for  $\alpha_{IIb}\beta_3$ , the second platelet receptor binding to vWF, is the RGDS sequence located at residues 1744–1747 in the carboxyl-terminal C1 domain.

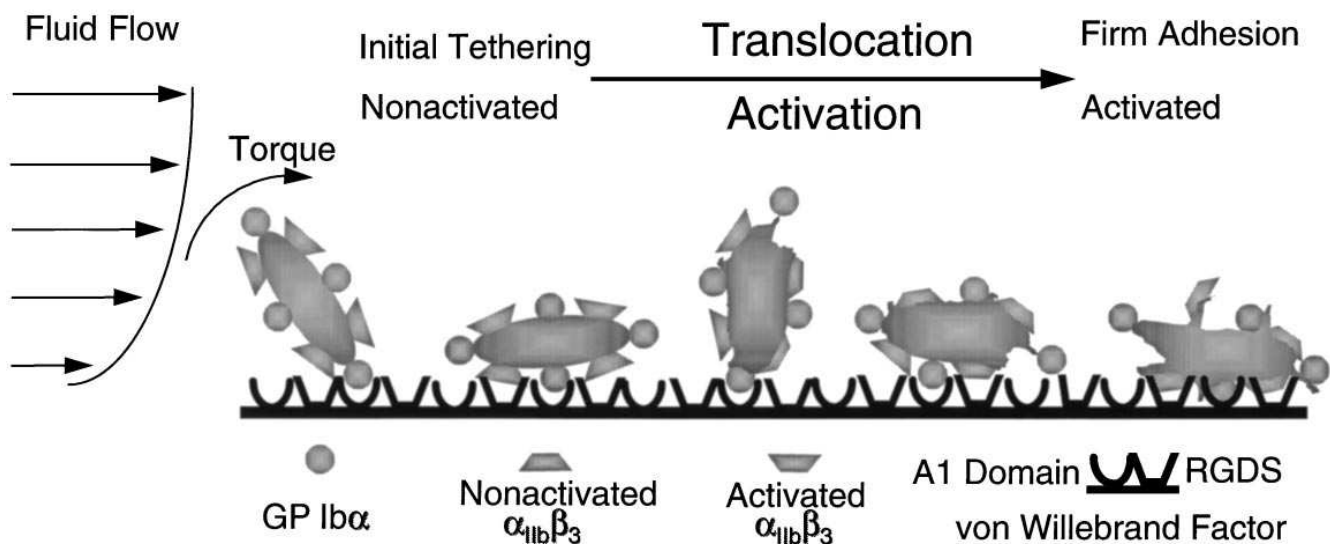
#### *von Willebrand factor function in thrombogenesis*

The function of vWF is to promote thrombus formation by mediating adhesion of platelets to the injured vessel wall and to one another. There is evidence that vWF in different anatomical locations participates in hemostatic processes. The circulating pool, however, is probably the most important in initiating platelet adhesion, since the distribution of subendothelial vWF is not homogeneous and the protein lacks in many vessels where platelet function is needed for hemostasis (15). Moreover, platelet vWF is not released until after activation, and may not be immediately available at a site of injury to promote adhesion, although its contribution to later phases of thrombus formation has been convincingly demonstrated (16). Plasma vWF is well suited to mediate early adhesion because it binds rapidly and tightly to collagen (and, possibly, to other matrix structures such as proteoglycans) whenever blood is exposed to injured tissues. Differential reconstitution of vWF in body compartments of pigs with severe von Willebrand disease provides good support for these concepts (16).

The unique mechanism supporting platelet adhesion to immobilized vWF under high flow conditions has been elucidated (17). The first step of this dynamic process is mediated

by the binding of platelet GP Ib $\alpha$  to the vWF A1 domain, an interaction characterized by a fast association rate that can tether platelets to exposed thrombogenic surfaces even when the velocity of flowing blood relative to the vessel wall is elevated. This event is favored by the multimeric nature of vWF, providing a high local density of active A1 domain sites and resulting in formation of multiple bonds. However, the vWF-GP Ib $\alpha$  interaction is also characterized by a fast dissociation rate and cannot provide bonds supporting irreversible adhesion, so that platelets tethered to the vessel wall in this manner move constantly in the direction of flow, albeit at a fraction of the free flow velocity (Fig. 1). During the slow translocation platelets become activated, and  $\alpha_{IIb}\beta_3$  can eventually mediate irreversible adhesion by binding to the RGDS sequence in the vWF C1 domain. The recognition specificity of  $\alpha_{IIb}\beta_3$  before activation, relevant for initial adhesion, is limited to interaction with immobilized fibrinogen or fibrin, consequently binding to vWF can only occur after platelet tethering to the surface mediated by GP Ib $\alpha$ . Moreover, the forward rate of binding interactions involving  $\alpha_{IIb}\beta_3$  appears to be relatively slow, explaining why this receptor is not sufficient to mediate initial platelet attachment to the vessel wall under high flow conditions, even though it is always required for firm adhesion (17). Thus, vWF is the indispensable adhesive substrate for thrombus formation in high shear environments because of the unique biomechanical properties of A1 domain binding to GP

## Dual Step Platelet Adhesion to von Willebrand Factor



**Figure 2.** Schematic representation of the dual step mechanism supporting platelet adhesion to immobilized von Willebrand factor. The first contact established between platelets and immobilized vWF is mediated by GP Ib $\alpha$ . Nonactivated  $\alpha_{IIb}\beta_3$  cannot pair with the RGDS sequence in vWF (38). The bond between the vWF A1 domain and GP Ib $\alpha$  must form rapidly, since platelets can be tethered to a vWF-coated surface regardless of flow conditions (17), and must also have high resistance to tensile stress, since adherent platelets can oppose the drag force created by flow even when the resulting wall shear rate is greater than  $30,000\text{ s}^{-1}$ , the highest tested (17). This bond, however, has an intrinsically high dissociation rate, thus a limited half-life, resulting in detachment where tension is greatest, and forward rotational movement (rolling) due to the torque imposed by the flowing fluid. New bonds are formed as different regions of the membrane of rolling platelets come in closer contact with the surface. Translocation continues until  $\alpha_{IIb}\beta_3$  becomes activated and binds to the RGDS sequence in the vWF C1 carboxyl terminal domain. The latter bond must have low dissociation rate and can mediate irreversible adhesion, dependent also on cytoskeletal reorganization. An important aspect of this mechanism is that, for tethering to be apparent, the surface density of vWF must be sufficiently high to support the formation of multiple interactions at any given time.

Ib $\alpha$ . Only this interaction can tether fast flowing platelets and markedly decrease their velocity relative to the vessel wall, allowing the occurrence of subsequent events (Fig. 2).

In experimental conditions where only purified vWF was exposed to platelets, and exogenous agonists were either not present or inhibited, demonstrable consequences of activation, i.e. thrombus formation, were seen only after several minutes and only at high shear rates (17). In the setting of vascular lesions, however, subendothelial components such as collagen, and soluble platelet agonists such as ADP and epinephrine, greatly enhance the efficiency of the hemostatic response by contributing synergistically to  $\alpha_{IIb}\beta_3$  activation. These mechanisms critically accelerate the attainment of irreversible platelet adhesion at sites of injury where plasma vWF becomes immobilized onto exposed collagen, and explain the functional relevance of  $\alpha_2\beta_1$  and other collagen receptors in hemostasis (18, 19). Indeed, irreversible platelet adhesion onto matrices containing collagen type I or III is essentially instantaneous even at the highest levels of shear stress, and thrombus formation ensues within seconds. This response is accelerated by the rapid activation of platelets, but it is still only possible owing to the unique function of GP Ib $\alpha$  and the vWF A1 domain (Fig. 2).

The relevant role of vWF in supporting initial platelet adhesion at sites of vascular injury is complemented by its function in mediating platelet-platelet cohesion. In fact, aggregation of platelets exposed to high shear stress is dependent on vWF binding to  $\alpha_{IIb}\beta_3$ , as well as GP Ib $\alpha$ , and fibrinogen cannot substitute for this function (20, 21). Studies with whole blood perfused over subendothelial surfaces support these conclusions (22). The reasons why vWF is required for aggregation under high shear are not fully understood, but are likely to be explained by specific biomechanical and structural properties of the molecule. There is evidence that shear forces may induce vWF to take the shape of extended filaments (23). Thus, the repeating subunit structure of the large multimers may offer an array of interaction sites capable of binding in a multivalent manner to receptors on the platelet membrane, increasing the strength of interaction and providing better linkage of platelets to one another. The divalent fibrinogen molecule, on the other hand, may provide sufficient adhesive strength to withstand opposing shear forces of lesser magnitude. Moreover, high shear stress induces vWF binding to platelets in the absence of any other chemical or physical modulator, a process dependent on interaction with both GP Ib $\alpha$  and  $\alpha_{IIb}\beta_3$  (24) that may promote preferential vWF localization on the platelet membrane. Shear-induced vWF binding is associated with platelet activation independent of other agonists (3), an additional factor favoring aggregation mediated by vWF in the initial stages of thrombus development.

#### *Perspective on future studies*

In spite of considerable progress in our understanding of the mechanisms involved in platelet thrombus formation, we still lack comprehension of the specific contributions provided by individual adhesive interactions to the process as a whole. This is due, at least in part, to the fact that the experimental models of platelet function traditionally used to mimic events occurring in flowing blood provide only a limited representation of the complexity present at a site of vascular injury, both with respect to the cells and molecules involved as well as the relevant hemodynamic conditions. Some answers to this problem will come from the use of mouse models allowing targeted manipu-

lation of individual components of the hemostatic system, coupled with studies of platelet response to vascular damage in intact organisms. Moreover, ongoing developments of confocal videomicroscopy will result in more accurate volumetric and morphometric analysis of thrombus formation during blood flow in ex vivo experiments. These approaches should provide some of the information necessary to design and use rationally new inhibitors of platelet adhesion and aggregation as anti-thrombotic drugs.

Animal models with targeted gene obliteration or mutagenesis should also help in obtaining more definitive information on whether vWF is involved in the development of atherosclerotic lesions, the process that develops over a long period of time and precedes acute arterial occlusion. High plasma levels of the protein have been found to be an independent risk factor for recurrent myocardial infarction and death (25). Because endothelial cells are the origin of all circulating vWF, increased plasma levels may reflect the extent of vascular damage. Experiments in animals have suggested a causative link between vWF and atherosclerosis, demonstrating that pigs with severe von Willebrand disease, who have markedly reduced levels of vWF, are protected from developing aortic plaques even when fed a cholesterol-rich diet (26). Furthermore, there is good evidence that decreased concentrations of circulating vWF limit the extent of occlusive thrombosis in stenosed and injured coronary arteries (27). The latter effect is not surprising in view of the essential role played by vWF in platelet thrombus formation under high shear stress. Less clear are the mechanisms to explain the possible participation of vWF in plaque development, although it seems reasonable to assume that influences on platelet adhesion and, consequently, generation of cytokines are involved. It also seems reasonable to anticipate that studies will be performed on genetic polymorphisms of vWF potentially linked to enhanced function, in order to evaluate their possible role as a risk factor for occlusive cardiovascular disorders.

Studies on von Willebrand disease (28), the most common genetic disorder in humans, will continue to focus on the definition of the molecular defects responsible for decreased vWF function, providing new information on the importance of single amino acid residues in supporting specific interactions. Progress has to be expected in elucidating the potential role of vWF in disorders such as thrombotic thrombocytopenic purpura and hemolytic uremic syndromes, that appear to be associated with the presence of unusually large vWF multimers in blood (29, 30). Preliminary evidence indicates that decreased plasma levels of a specific processing enzyme (9) are responsible for the accumulation of hyperactive forms of vWF, potentially explaining the diffuse thrombotic occlusion seen in the arterial microvasculature of these patients. Clarification of such issues has the potential to lead to new therapeutic modalities for these serious and often life-threatening diseases.

With respect to vWF function, a number of unanswered questions must be addressed with additional experimental work. There is already sufficient evidence that any vWF-dependent platelet activity involves two membrane receptors, GP Ib $\alpha$  and  $\alpha_{IIb}\beta_3$ , and initial information has been obtained on the synergistic properties of the corresponding bonds in mediating platelet adhesion to reactive surfaces (17). Yet, crucial details on the functional interplay between these two distinct membrane sites interacting with vWF are still missing, particularly with regard to shear-induced activation mediated

by GP Ib-IX-V and its role in promoting subsequent vWF binding to  $\alpha_{IIb}\beta_3$ . Moreover, the possibility that GP Ib $\alpha$ -vWF A1 domain bonds contribute directly to supporting platelet attachment to one another, in addition to any participation in activation, must still be evaluated, notwithstanding the current dogma that  $\alpha_{IIb}\beta_3$  is the only receptor mediating aggregation.

Equally needed is more definitive information on the mechanisms that regulate the initial recognition of vWF by GP Ib $\alpha$ . It is generally assumed that conformational changes are induced in the vWF A1 domain by interaction with collagen or other matrix components at sites of vascular injury, and perhaps by shear forces in GP Ib $\alpha$  as well as vWF, allowing ligand-receptor pairing normally prevented in the circulation. To date, there is only experimental evidence demonstrating that the overall shape of vWF multimers may be affected by shear forces (23), but nothing is known about more subtle conformational changes in the relevant interactive sites. In vitro, soluble vWF binds to GP Ib $\alpha$  only in the presence of exogenous modulators, but all those identified to date, such as ristocetin and botrocetin, are nonphysiologic substances. The mode of action of these modulators has been elucidated, at least in part. Ristocetin has been shown to dimerize in solution and the dimeric forms interact with both platelets and vWF, thus bridging GP Ib $\alpha$  with its ligand (31). Botrocetin, on the other hand, forms a stoichiometric bimolecular complex with soluble vWF and this complex, in turn, interacts with GP Ib $\alpha$  (32). In either case, irreversible vWF binding to platelets requires only GP Ib $\alpha$ , an apparent contradiction with the results obtained in the absence of these exogenous substances indicating that the dissociation rate of the interaction may be too high to allow measurement of equilibrium binding. Thus, as an alternative hypothesis to be tested, the initiation of vWF-dependent platelet responses may be controlled not by regulation of the A1 domain-GP Ib $\alpha$  interaction (which may always occur with rapid on and off rates) but by modulation of the activation needed for irreversible vWF binding to platelets through engagement of  $\alpha_{IIb}\beta_3$ . The observation that the largest plasma vWF multimers are relatively decreased in patients with reactive thrombocytopenia (33) agrees with the notion that vWF binding to GP Ib $\alpha$  may be ongoing in the normal circulation. In these cases, the elevated platelet count, increasing receptor concentration per unit volume, would only contribute to making the event more easily detectable, while the preferential removal of larger multimers can be explained by their known higher binding affinity. The hypothesis that the vWF-GP Ib $\alpha$  interaction need not be regulated by an off/on switch is compatible with the evidence that specific residues in the A1 domain may be responsible for maintaining functional conformations with different affinity for GP Ib $\alpha$ , presumably allowing transitions from one to another. The occurrence in patients with type IIB von Willebrand disease of mutations causing measurable binding of soluble vWF to GP Ib $\alpha$  in the absence of any exogenous modulator (28) further indicates that the function of the A1 domain can be regulated. To date, however, there is no information on the mechanisms that could induce such affinity changes during hemostasis in vivo. Detailed knowledge on the three-dimensional structure of important functional domains of vWF, including A1, is rapidly becoming available through x-ray crystallography, setting the stage for a rational approach to answering these questions.

Finally, more studies will undoubtedly be devoted to test the hypothesis that a drug preventing the binding of vWF to

GP Ib $\alpha$ —or, presumably, the binding of vWF to collagen—should provide an efficacious antithrombotic intervention. Pigs with von Willebrand disease have a markedly decreased incidence of occlusive thrombosis in stenosed and injured coronary arteries (34), even in the setting of atherosclerosis (27). Experiments with a monoclonal antibody blocking vWF binding to GP Ib $\alpha$  have shown efficacy in preventing occlusive thrombosis in a pig coronary artery thrombosis model (35). At present, the only candidate GP Ib $\alpha$  inhibitor for use in humans is a recombinant fragment representing essentially the A1 domain of vWF. Studies with this compound, VCL, have shown antithrombotic efficacy and enhancement of thrombolysis (36), as well as prevention of intimal thickening after balloon injury of vessels (37). Whether new drugs based on the concept of inhibiting the unique functions of vWF in thrombogenesis will be developed remains conjectural at present.

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