Structure of von Willebrand factor and its function in platelet adhesion and thrombus formation

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The adhesive protein von Willebrand factor mediates the initiation and progression of thrombus formation at sites of vascular injury. von Willebrand factor is synthesized in endothelial cells and megakaryocytes as a very large polymer composed of identical subunits. In the plasma, it appears as a series of multimers of regularly decreasing molecular mass, from several thousand to 500 kDa. The size of circulating von Willebrand factor multimers is controlled by proteolytic cleavage carried out by a specific protease. The biological functions of von Willebrand factor are exerted through specific domains that interact with extracellular matrix components and cell membrane receptors to promote the initial tethering and adhesion of platelets to subendothelial surfaces, as well as platelet aggregation. Moreover, von Willebrand factor binds the procoagulant co-enzyme, factor VIII, contributing to its stability and, indirectly, to its function in the generation of fibrin. This chapter presents a review of current knowledge on the structure, biosynthesis and functions of von Willebrand factor.

Key words: von Willebrand factor; platelet; adhesion; aggregation; thrombosis, haemostasis.

The purpose of this chapter is to review current knowledge on the structure and function of von Willebrand factor (VWF), with an emphasis on the aspects more directly relevant for platelet physiology and the mechanisms of arterial thrombosis. VWF is a glycoprotein present in plasma, the α granules of platelets, and subendothelial matrices, with two known functions: to promote platelet adhesion to thrombogenic surfaces and platelet aggregation, and to act as a carrier for the plasma procoagulant co-enzyme, factor VIII.

The role of VWF in the arrest of haemorrhage or in the onset of arterial thrombosis depends on the ability to support platelet–surface and platelet–platelet interactions by bridging membrane receptors to components of the extracellular matrix and to one another. Moreover, VWF performs an essential, albeit indirect, function in fibrin clot formation by associating with factor VIII, which is a necessary co-factor for the rapid generation of factor Xa at sites of injury. Factor VIII bound to VWF is protected from inactivation by proteases and has a longer lifetime in the blood, thus being more efficient in performing its function. Indeed, in the absence of VWF, the factor VIII level falls dramatically, and blood clotting is severely impaired. Diminished VWF activity is
the cause of a bleeding disorder known as von Willebrand disease. An abnormally enhanced activity of VWF, on the other hand, is presumably a pathogenetic factor in thrombotic disease.

**STRUCTURE OF VON WILLEBRAND FACTOR**

Electrophoretic analysis in large-pore gels containing sodium dodecyl sulphate and in conditions preserving intact disulphide bonds reveals that mature VWF molecules are polymers of varying size (Figure 1). The smallest circulating species is formed by two subunits of 2050 residues linked by disulphide bonds at the C terminal ends. This dimer is the protomer, or building block, of the multimeric series, larger polymers being formed by protomers linked at the N terminal ends. Thus, the progressive increment in molecular mass corresponds to the size of a dimer, approximately 540 kDa. The largest molecules may exceed 10 000 kDa.

**von Willebrand factor multimers**

Purified VWF visualized by electron microscopy appears either as filamentous structures with a diameter of 2–3 nm and a length of up to 1300 nm, close to the diameter of a platelet, or as loosely coiled molecules with an apparent diameter of 200–300 nm. Atomic force microscopy has shown that the extended forms are ‘uncoiled’ molecules that have acquired an extended-chain shape orientated in the direction of flow under the effect of shear forces. The threshold shear stress found to cause this change is in the order of 35 dyn/cm², a value within physiological levels in the arterial circulation.

The subunits in VWF multimers are the product of one gene and are initially identical, being modified after secretion into the blood stream. The resulting heterogeneity explains why the spacing of plasma VWF multimers separated in agarose gels is more complex than expected for a constant difference in molecular mass between adjacent species (Figure 1).

An electrophoretic analysis of plasma VWF with reduced disulphide bonds reveals a predominant band with an apparent molecular mass of 225 kDa (Figure 2A). This value reflects an aberrant mobility of the VWF subunit, since the true molecular mass calculated by chemical composition is approximately 270 kDa. In addition to the main species, reduced plasma VWF of normal individuals reproducibly contains two smaller bands of apparent molecular mass 176 and 140 kDa respectively, as well as a minor band of 189 kDa (Figure 2A), which together represent approximately 5–10% of the total subunit content. In contrast, VWF from platelet α granules is composed exclusively of 225 kDa subunits (Figure 2B). This suggests that the smaller species, which originate from proteolytic cleavage of the peptide bond between residues Tyr 842 and Met 843 of the native subunit, are generated after secretion from the endothelial cells into the circulation. The 140 kDa fragment corresponds to residues 1–842 and the 176 kDa fragment to residues 843–2050.

This largest VWF multimers contain multiple sites of interaction with platelets and vessel wall components, thus having enhanced thrombogenic potential. These functionally multivalent multimers support platelet adhesion to reactive surfaces or to other platelets more efficiently than do smaller ones. The degree of polymerization of mature VWF appears to vary with the anatomical location of the molecule. The largest multimers with the greatest thrombogenic potential are present in cellular storage.
sites – endothelial cells and platelets – and not in the plasma, where they appear transiently after regulated secretion\textsuperscript{12,13} and from which they are rapidly cleared.\textsuperscript{14}

With respect to physiological conditions at rest, therefore, some of the VWF molecules present in platelets or endothelial cells are appropriately defined as ‘unusually large’.\textsuperscript{15} These findings, as well as the presence of ‘unusually large’ plasma VWF in patients with certain pathological conditions\textsuperscript{15}, suggest that the size of circulating VWF may be subject to controlled regulation, possibly to prevent the occurrence of thrombosis. Cleavage of the subunit appears to be the mechanism responsible for reducing the size of plasma VWF.

Figure 1. Multimeric structure of plasma von Willebrand factor (VWF). Multimers were separated by electrophoresis in sodium dodecyl sulphate (SDS)–agarose gels and then visualized with a monospecific anti-VWF antibody labelled with \textsuperscript{125}I. Purified proteins used as markers of molecular mass were analysed in identical conditions and visualized by staining with Coomassie Blue: IgG, 166 kDa; IgM (the top band in the corresponding lane), 900 kDa; fibronectin (FN), 450 kDa; fibrinogen (Fib), 340 kDa. This analysis was performed under non-reducing conditions, and all native disulphide bonds in the molecules were left intact. Electrophoretic migration was from top to bottom, so molecules closer to the top of the gel have larger mass. Note that even the large IgM molecules migrated close to the dye front in this large-pore agarose gel, signifying that only molecules with a mass greater than 1000 kDa were effectively separated based on size. On the other hand, the largest VWF multimers could barely migrate into the gel. The position in the gel of some of the predominant species is indicated with thick bars on the left, giving a sense of the regular distribution of multimer sizes; numbers indicate how many subunits are present in each species, a value deducted from the apparent molecular mass. Minor species are indicated with thin bars. The heterogeneity in size is the consequence of the physiological post-translational cleavage of the subunit that occurs in plasma VWF multimers, as described in the text. Adapted from Dent et al (1991)\textsuperscript{4} and Ruggeri (1987)\textsuperscript{103}, with permission.

Two studies have identified a protease in blood that acts on VWF multimers and cleaves the subunit at the bond between Tyr 842 and Met 843.\textsuperscript{16,17} The estimated molecular mass of this enzyme is between 200 and 300 kDa, and its action is greatly facilitated in vitro by the partial denaturation of VWF. In vivo, a similar effect may result from the action of shear forces that change the three-dimensional structure of VWF and expose the peptide bond susceptible to cleavage. The potential relevance of this protease, apparently VWF specific, is illustrated by a report showing that the plasma of patients with chronic relapsing thrombotic thrombocytopenic purpura, a disease characterized by the diffuse occlusion of small vessels variably associated with the presence of unusually large plasma VWF multimers\textsuperscript{15}, contains decreased VWF
cleaving activity. This observation demonstrates that the controlled regulation of circulating VWF multimer size is an important physiological event, which may contribute to the pathogenesis of thrombosis if altered.

**Structural and functional domains of the von Willebrand factor subunit**

The mature VWF subunit contains several discrete functional sites responsible for biological activities that involve interactions with other molecules. These domains exist in each subunit with conformation and function probably independent of multimer assembly, as indicated by the fact that isolated monomeric fragments generated by the proteolysis of larger native molecules, or expressed recombinantly, retain substrate recognition specificity.

The mature VWF subunit contains 2050 amino acids, of which 169 are cysteine residues clustered in domains located at the N and C terminal ends. The estimated carbohydrate content is approximately 18% of the total mass. The analysis of fragments generated by limited proteolysis has led to the identification of intermolecular disulphide bonds within two regions of the subunit. One, in the N terminal portion of the molecule, contains 30 cysteines between residues 283 and 695; the other, in the C terminal position, has 18 cysteines between residues 1908 and 2050. Only a limited number of these C terminal interchain disulphide bonds appear to be important for the
formation of subunit dimers. There are no detectable free sulphhydryl groups in VWF, all the cysteine residues thus being paired by inter- or intra-molecular disulphide bonds.

The domain that interacts with factor VIII

It is not known where the initial interaction between VWF and factor VIII takes place. The respective sites of synthesis are thought to be different, so the complex may form not inside a cell but after the secretion of the two molecules in a specific environment. The region of VWF that binds factor VIII lies between amino acids 1 and 272 (Figure 3), with a possible crucial role for residues 78–96. The N terminal domain of VWF contains intrachain disulphide bonds that appear to be essential for factor VIII binding, suggesting that they may be important in maintaining the appropriate spatial conformation of a functional site formed by discontinuous residues. Indeed, distinct single amino acid substitutions in mature VWF – Arg19Trp, Gly22Glu, Thr28Met, Arg53Trp, His54Gln and Arg91Gln – result in a decreased binding capacity for factor VIII, causing a variant form of von Willebrand disease, designated type 2N, that resembles mild haemophilia A but is clearly identified by the autosomal mode of inheritance as opposed to the X-linked inheritance typical of the latter. It is of note that factor VIII activated to FVIIIa during clotting is modified in a way that does not allow its continued association with VWF, the latter preventing the binding of FVIIIa to platelets.

von Willebrand factor domains that interact with extracellular matrix components: A1 and A3

Collagen is the main component of extracellular matrices capable of binding VWF. Two of the three type A domains in VWF, A1 and A3 (Figure 3), are responsible for this interaction, and their respective roles may vary depending on the type of
collagen involved. Domain A1, comprising residues 497–716, appears to be uniquely relevant for binding to collagen type VI, whereas domain A3, comprising residues 910–1111, is necessary for binding to both types I and III. Fluid dynamic conditions and mechanical forces may modulate these interactions, and a complex interplay of domains A1 and A3 may be necessary to effect VWF immobilization onto extracellular matrices containing various collagen types.

Moreover, it is important to note that VWF interactions with the vessel wall involve other structural components in addition to collagen, and their interrelationships may create a functional diversity that may be difficult to appreciate in model systems using purified molecules. In addition to the collagen-binding site, the A1 domain of VWF also contains apparently distinct sequences that support interactions with molecules capable of mediating immobilization onto exposed tissue matrices. A heparin-binding site is presumably located in the loop region between residues Cys 509 and Cys 695. A second heparin-binding sequence, albeit of lower affinity, exists in VWF within the first 272 residues of the mature subunit, in the same region that supports the formation of the complex with factor VIII. These heparin-binding sites may reflect the ability of VWF to interact with matrix proteoglycans that contain sulphated sugars.

The A1 domain and the interaction with glycoprotein Ibα.

The A1 domain of VWF contains the site of interaction with glycoprotein Ibα (Gplbα), a component of the platelet Gplb–IX–V receptor complex. Inhibition studies with synthetic peptides originally suggested that two discontinuous regions of the VWF subunit, comprising residues 474–488 (at the boundary between the D3 and A1 domains) and 694–708 (within the A1 domain loop), were probably involved in this function. It is now believed that those results were influenced by the large number of proline residues in the synthetic peptides, which alter the action of the modulator ristocetin used to induce VWF binding to platelets in vitro.

Other studies with synthetic peptides indicate that a different region of the A1 domain, corresponding to residues 514–542, may be involved in binding to Gplbα, but there is no direct proof that the sequence identified has a functional role in intact VWF. Alanine scanning mutagenesis experiments, on the other hand, have suggested that a number of residues in a discontinuous sequence location may contribute to form the site of interaction with Gplbα, an issue further clarified by the solution of the A1 domain crystal structure (see below).

Carbohydrate side chains may regulate the Gplbα binding function of VWF. In this respect, it may be relevant that 8 out of the 10 O-linked sugars in VWF flank the A1 domain loop, four on each side, a location in close proximity to functionally important residues. Glycosylation is not normally a necessary requisite for VWF binding to Gplbα, since *Escherichia coli*-expressed fragments totally devoid of carbohydrate exhibit a high affinity for this platelet receptor. Nevertheless, the orientation of some of the carbohydrate side chains may influence the interaction, thus explaining the enhanced binding of asialo VWF to Gplbα compared with the native molecule.

Additional regulatory elements may be revealed by the snake protein botrocetin, which promotes the interaction with the platelet receptor by forming a complex with soluble VWF. Botrocetin binds tightly to the VWF A1 domain, possibly through residues located in three discontinuous segments of the sequence, as suggested by experiments with synthetic peptides and alanine scanning mutagenesis. The site of botrocetin binding may correspond to regions in the A1 domain that control the association of VWF to Gplbα.
Crystal structure of domains A1 and A3

The crystal structure of the VWF A1 and A3 domains has been resolved. Both have a typical α/β fold homologous to that in the I domains of integrin subunits α₁<sub>M</sub> and α₁<sub>L</sub>, with a central hydrophobic parallel beta-sheet flanked by amphipathic alpha-helices on each side. The metal ion-dependent adhesion site (MIDAS) motif found in the I domains is, however, notably absent in domains A1 and A3. This may indicate that VWF and integrins interact with collagen via distinct mechanisms. Indeed, the A3 domain of VWF may appear to be homologous to the α<sub>2</sub>I domain of α₂β₁ with respect to collagen binding, but only the latter requires magnesium or manganese ions to function. Site-directed mutagenesis studies have confirmed that the MIDAS sequence is key for the interaction of the α<sub>2</sub>I domain with collagen, whereas the homologous residues have no apparent activity in the A3 domain, suggesting that the vestigial MIDAS motif in the latter is not directly relevant for function. In spite of the similar folding, therefore, there are no obvious shared features in the α<sub>2</sub>I domain and VWF A3 domain that could be suggestive of a common mechanism for binding to collagen. The location of the residues forming the contact surface between collagen and the VWF A3 domain remains to be elucidated.

The A1 domain has been crystallized in complex with the Fab fragment of the function blocking antibody NMC-4, the structure identified providing information not only on the VWF residues interacting with the antibody, but also on the possible location of the GpIb<sub>x</sub> binding site. NMC-4 binds to helix α₄, which is part of an extended surface that includes strand β₃ and helix α₃. The N-terminal portion of helix α₃ forms a depression in this surface, suggestive of a binding pocket bounded on one side by the N terminal portion of helix α₄ and on the other by the N terminal portion of strand β₃ (Figure 4). This may be the site where GpIb<sub>x</sub> binds, presumably through residues whose side chains point upwards from the groove, notably Glu 596 and Lys 599. Docking studies using a GpIb<sub>x</sub> peptide have confirmed this putative location for receptor interaction in the VWF A1 domain (Figure 5).

The Arg–Gly–Asp sequence in domain C1 and the interaction with the integrin receptors

The sequence Arg–Gly–Asp (RGD) was initially identified as being sufficient to express the cell-adhesive properties of fibronectin but was later found to exhibit a similar function in several other proteins involved in the interaction with integrin receptors. In VWF, the Arg–Gly–Asp sequence corresponding to residues 1744–1746 in the C terminal C1 domain of the subunit (see Figure 3) represents the essential, if not unique, α<sub>IIb</sub>β₃-binding site. Another β₃ integrin, α<sub>IIb</sub>β₃, can interact with the RGD sequence in VWF. Endothelial cells can adhere to immobilized VWF through the latter receptor, but the biological significance of the interaction remains to be proven.

Sites of interaction with other ligands

VWF binds to sulphated glycolipids, which are present on cellular membranes and may serve an accessory role in promoting interactions between VWF and platelets. The binding site for sulphatides has been localized within residues 512–673 of the A1 domain, possibly with a more direct involvement of residues 569–584. This location is near other key sites supporting binding to GpIb<sub>x</sub>, certain types of collagen, heparin and botrocetin (see Figure 3), but there is no evidence for structural or functional overlap. VWF can become cross-linked to the alpha-chain of fibrin, but there is no information on the residues involved in this process. The binding of VWF to fibrin may contribute to platelet deposition onto altered vascular surfaces.
FUNCTIONS OF VON WILLEBRAND FACTOR

VWF is essential for normal platelet adhesion and aggregation in vessels in which rapid blood flow results in elevated shear stress. These correspond to the segments of the vascular tree where efficient primary haemostasis is key to the arrest of bleeding, namely small arterioles and arterial capillaries in which median wall shear rate estimates are of the order of 1700 per second.8 For the same reason, VWF may be crucial for platelet thrombus formation in diseased arteries in which obstructive processes, such as atherosclerotic plaques, reduce the lumen diameter. For example, shear rates in excess of 5000 per second have been measured in coronary arteries with a 50% stenotic occlusion.82 The influence of shear-dependent phenomena may therefore be even greater in pathological conditions predisposing to the occurrence of acute arterial occlusion than it is in the course of normal haemostasis.

Blood circulation, shearing flow and platelet adhesion

Platelets are anucleated blood cells that perform their functions in an environment characterized by constant fluid motion. This has important consequences for the inter-
actions that allow the formation of platelet thrombi, opposing the disrupting potential of forces generated by the circulation of blood. The effects of different flow regimens on thrombus formation are well recognized. Thrombi that develop in arteries—characterized by high-velocity flow—tend to be rich in platelets (white thrombi), while those developing in veins—characterized by low-velocity flow—contain more red cells enmeshed in a fibrin clot but relatively few platelets (red thrombi).83

In a normal vessel, erythrocytes represent most of the circulating cellular mass. As a consequence of their lateral migration and erratic motion, they occupy the greatest part of the lumen, pushing white blood cells and platelets to the sides and creating a near-wall platelet excess.84–86 Blood flow is consequent to the pump action of the heart and is driven by pressure differences and counteracting boundary wall movements that result from the elasticity of the vessels.

The velocity profile of flowing blood, assuming the properties of a Newtonian fluid as a good approximation, is parabolic. Thus, the fluid velocity is maximal in the centre of the vessel and decreases towards the wall as a parabolic function of the radial distance from the central axis (Figure 6A). Consequently, the fluid can be imagined as being divided in a series of adjacent layers (laminae or planes) moving at a different velocity relative to one another (laminar flow). The velocity differential creates the shearing flow, namely the sliding motion between two adjacent planes moving at different speed. Shear stress is the tangential force per unit area of contact between the laminae. The unit of shear stress in the International System of Units (SI) is the

Figure 5. Residues involved in the interaction between the von Willebrand factor (VWF) A1 domain and the glycoprotein Ibα (GpIbα). The GpIbα sequence is presented in green, as described in the legend to Figure 4. The side chains of residues interacting with the A1 domain are depicted and numbered with their position in the native protein. A limited portion of the A1 domain structure is shown, with the alpha2-helices in red, the beta-strands in light green and the connecting loops in purple. This segment begins with a portion of the loop between strands β2 and β3 (G 561) and continues with strand β3, helix α2, helix α3, strand β4 and helix α4 as well as the intervening loops. The VWF A1 domain side chains are colored blue (the residues in strand β3) or purple (the residues in helix α4) and numbered according to the sequence of the mature protein.62 Note that the orientation of the GpIbα side chains is derived from a modelling procedure66, whereas that of the A1 domain side chains is based on X-ray crystallographic data. Adapted from Vasudevan et al (2000, Journal of Biological Chemistry 275: 12 763–12 768) with permission.
Pascal (Pa) corresponding to one Newton/m² (N/m²); in the CGS system, the corresponding unit is the dyn/cm² (1 Pa = 10^10 dyn/cm²).

Shear rate is the rate of change in velocity between laminae with respect to the distance from the axis of the tube (Figure 6A). It is expressed as the difference in velocity (distance over time) divided by distance, resulting in the unit s⁻¹. The relationship between shear stress and shear rate is linear in newtonian fluids, and the corresponding proportionality constant defines the viscosity of the fluid. Thus, shear stress is directly proportional to shear rate and fluid viscosity.

Platelets and the vessel wall can be viewed as two juxtaposed surfaces, one of which, the platelet membrane, is moving while the other is stationary. Molecules on the two surfaces cannot interact with one another as long as the separation is greater than 100 nm, whereas bonds can form when the distance is 10 nm or less. When a flowing platelet is at appropriate distance from a reactive surface, the binding of a membrane

\[ \dot{\gamma} = -\frac{dv_z}{dr} \]

Figure 6. (A) Blood flow in a cylindrical vessel can be visualized as a series of layers (laminae) moving with decreasing velocity from the centre towards the wall (depicted schematically by arrows of different length on the left). The corresponding velocity profile (the dotted line on the left) is more blunt than the parabolic profile expected with a homogeneous suspension (the solid line) owing to cell depletion in the boundary layer near the wall. The shear rate \( \dot{\gamma} \) is the rate of change of velocity with respect to distance measured perpendicularly \( r \) to the direction of flow \( z \). The negative sign indicates that the gradient is defined from the centre (where the velocity is maximal) to the wall (where the velocity is minimal). (B) Schematic representation of the adhesion mechanisms under flow conditions. Adherent cells surrounded by flowing fluid are subjected to a torque, i.e. a combination of forces that tend to produce a rotating motion resulting from the approximately spherical shape of cells and the presence of points of attachment to the surface. The bonds at the leading edge of the cell are compressed; those at the tailing edge are subjected to tensile stress and may yield, releasing areas of the cell membrane from contact with the surface. The intrinsic life time (dissociation rate) of any specific ligand–receptor pairing influences the duration of the interaction. Receptor and ligand density determine the number of tethering bonds effective at any given time and are crucial to enhance the overall adhesive strength.
receptor to an adhesive ligand can take place if the corresponding intrinsic association rate is faster than the relative velocity at which the molecules are moving with respect to each other. In other words, binding must be established within the time frame during which the two molecules are sufficiently close to interact, which is shorter as the velocity of the platelets relative to the surface increases. It follows that, at a high shear rate, only interactions with a fast forward rate can mediate adhesion.

When a platelet adheres to a reactive surface, the bonds holding it in place must result in an adhesive strength at least sufficient to balance the force applied by the moving fluid (Figure 6B). This force – drag – is proportional to the size of the arrested platelet and the velocity and viscosity of the streaming fluid. Consequently, the drag opposing stable adhesion and aggregation is of particular relevance in those districts of the vasculature in which the shear forces are greater, i.e. in arteries more than in veins, and particularly in arterioles.

The forces acting on the adherent platelet are transmitted to the bonds between the platelet itself and the reactive surface. The receptor–ligand pairs located in the platelet tail relative to the direction of flow will be 'stretched' by the drag, thus being subject to tension. These bonds under tension are the ones responsible for platelet arrest (Figure 6B). The stress can be separated into two different components, normal stress (perpendicular to the surface) and shear stress (parallel to the surface). The receptor–ligand pairs under tension are those which counteract the effects of the shear stress and are subjected to tensile stress. Resistance to tensile stress is, therefore, a crucial attribute of adhesive bonds.

VWF is essential to initiate platelet adhesion and thrombus formation at sites of vascular injury because of its ability to link components of the extracellular matrix with GpIbα on the platelet membrane through bonds that form rapidly and, at least transiently, resist high tensile stress.

**Role of von Willebrand factor in the initiation of platelet thrombus formation**

Platelets promptly interact with surfaces that present immobilized VWF, initiating a response in which two receptors, GpIbα and the integrin αIIbβ3, are sequentially integrated in synergistic functions that are absolutely required under high shear stress conditions to mediate stable platelet–surface and platelet–platelet contacts, i.e. adhesion and aggregation respectively. These processes, except for the initial transient tethering mediated by GpIbα, are strictly dependent on platelet activation, which can be induced by specific interactions with molecules present on thrombogenic surfaces, including VWF, as well as by soluble agonists at the site of injury.

**von Willebrand factor at the vessel wall**

In areas of vascular damage, VWF is a key substrate for initiating wound repair through platelet deposition and thrombus formation. Depending on the vascular segment involved in the process, the subendothelial matrix may contain endogenous VWF in a quantity sufficient to support maximal adhesion. In any case, regardless of the presence of endogenous subendothelial VWF, several components of the extracellular matrix can rapidly bind plasma VWF wherever injured tissues are exposed to circulating blood. In fact, this may be the predominant mechanism leading to the finding of immobilized VWF around bleeding wounds. Lesions through the entire thickness of the vessel wall expose deep layers of extracellular matrix synthesized by
cells other than endothelial cells, where intrinsic VWF is not present but collagens and other components capable of binding plasma VWF are abundantly represented.

Platelet adhesion and thrombus formation can undoubtedly occur even on exposed vascular walls that contain no endogenous VWF, as clearly demonstrated by experiments with pigs affected by von Willebrand disease. Animals with the congenital abnormality transplanted with normal bone marrow demonstrated endothelial cells retaining the genetic defect. In these animals, therefore, there was no VWF secretion into the blood stream or into the subendothelial matrix, whereas platelets derived from donor megakaryocytes had a normal content of platelet α granule VWF. Upon the correction of the plasma defect by an infusion of VWF concentrate, the chimaeric pigs exhibited normal plasma and platelet VWF but absent intrinsic subendothelial VWF, yet they possessed completely normal haemostasis.

There is thus good evidence to conclude that plasma VWF can support platelet adhesion after binding to extracellular matrix components in the vessel wall or surrounding tissues and can, by itself, ensure normal haemostatic function even in the absence of endogenous matrix VWF. Nevertheless, when subendothelial VWF is present, platelet adhesion is no longer dependent on the kinetics of soluble VWF binding to matrix components. Whether this can render thrombus formation more efficient is presently unknown.

Conformational changes in surface-bound von Willebrand factor and in the A1 domain

It is often stated that surface-bound VWF must undergo a conformational change to make the interaction with GpIbα possible and initiate platelet adhesion. The origin of this concept lies in the observation that there is no measurable binding of soluble plasma VWF to platelets in the circulation, whereas an adhesive interaction occurs when platelets are exposed to immobilized VWF.91 There is, indeed, good experimental evidence that VWF molecules may change shape depending on haemodynamic conditions, so that upon binding to the vessel wall under high shear stress, they may appear as elongated filaments rather than the loosely coiled structures seen under static or low shear stress conditions.7 Such an ‘uncoiling’ may expose the repeating functional sites present in multimeric VWF, allowing a more efficient support of adhesive interactions as a result of multivalency and, at least in a broad sense, regulating function.

Structural and functional studies have also demonstrated that the A1 domain may assume at least two different conformational states characterized by different binding properties for the platelet receptor GpIbα.92 Evidence to support such a conclusion is based on the crystal structure of a recombinant A1 domain fragment containing a single amino acid substitution previously identified in a patient affected by type 2B von Willebrand disease.93 The phenotype of this variant of the disease is characterized by enhanced VWF interaction with GpIbα94 and is caused by different mutations that occur within or in close proximity to the disulphide loop between Cys 509 and Cys 695 in the VWF A1 domain.2

It is noteworthy that most type 2B mutations are clustered in an area of the A1 domain located at a considerable distance from the proposed GpIbα binding pocket, which is thought to be centred around strand β3 and helices α3 and α4 (see Figure 4 above).62 The structural changes induced by the type 2B mutation Ile546Val, located in the loop between helix α1 and strand β2, demonstrate that an enhanced adhesive function is associated with structural changes that occur in the β2–β3 loop at a distance of 27 Å from residue 546 (Figure 7). A water molecule enters the cavity left by the deleted isoleucine methyl group, and the decreased hydrophobicity in the environment
forces the side chain of Met 541 into a new orientation. The positional shift is propagated along the direction of strand β2 and creates the space for a new water molecule that forms hydrogen bonds with Glu 557 and Tyr 565. Moreover, a new hydrogen bond between the repositioned Glu 557 and His 559 induces a drastic change in the orientation of the Asp 560–Gly 561 peptide plane (Figure 7). The type 2B mutation, therefore, leads to evident structural consequences in an area of the A1 domain that may control access to the putative receptor binding pocket formed by strand β3 and helices α3 and α4.

The structural and functional characteristics of the Ile546Val mutant indicate that distant areas of the globular A1 domain surface may modulate GpIbα binding, with an extended contact interface between ligand and receptor. This assumption is supported

Figure 7. A partial superposition of the structure of the wild-type and Ile546Val type 2B mutant A1 domain, the results being obtained by X-ray crystallography. The normal structure is depicted in blue, the mutant structure in red. Ca atom traces and relevant side chains are shown in the segments of sequence 541–546 (part of helix α1 and loop α1–β2), 555–574 (part of strand β2, loop β2–β3, strand β3 and loop β3–α2), and 596–600 (part of helix α3). Repulsive forces are represented by thick dotted lines, new hydrogen bonds by thin interrupted lines. Adapted from Celikel et al (2000, Nature Structural Biology 7: 881–884) with permission.
by the observation that platelet adhesion to the immobilized VWF A1 domain in a flow field with a high shear rate (Figure 8) is influenced by residues that define an area greater than 20 Å in length, extending from the \( \beta_2 - \beta_3 \) loop to the \( \beta_3 - \alpha_2 \) loop (see Figures 5 and 8). It should be noted that the residues required for normal function essentially correspond to those involved in the structural changes caused by the Ile546Val substitution.92

The consequences of type 2B mutations prove that the affinity of the VWF A1 domain for platelet GpIb\( \alpha \) can be positively regulated by conformational changes, but it cannot demonstrate that similar events occur in normal VWF at the onset of a response to vascular damage. This will remain a working hypothesis until pathophysiological conditions capable of inducing similar effects are identified. It is nevertheless now clear that structural transitions in the A1 domain can modulate VWF function and facilitate the initiation of platelet adhesion.

von Willebrand factor and platelet adhesion

The adhesion of platelets to immobilized VWF is a dynamic process initiated by GpIb\( \alpha \) binding to the A1 domain, a key interaction for the genesis of platelet thrombi under high flow conditions.91 The coupling of the VWF A1 domain to GpIb\( \alpha \) is characterized by a fast rate of bond formation and allows the tethering of platelets to exposed thrombogenic surfaces even when the velocity of blood flow relative to the vessel wall is elevated. Indeed, above a threshold value of wall shear rate – approximately 1000
per second – only this specific ligand–receptor pairing can initiate adhesion. The VWF-Gplbα interaction is, however, 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.

During this slow translocation, the prolonged and continuous contact between the platelets and the thrombogenic surface allows the occurrence of activation, in turn resulting in an irreversible arrest of individual platelets and subsequent thrombus formation, both mediated by αIibβ3 (Figure 9). Thus, the VWF binding site for αIibβ3 is involved in mediating initial platelet adhesion to exposed subendothelium, but only in synergistic function with the A1 domain and its Gplbα receptor. The mechanisms controlling platelet activation consequently have a fundamental role in regulating the initiation of thrombogenesis mediated by VWF since only activated αIibβ3 can lead to the irreversible adhesion necessary to allow subsequent thrombus growth.
von Willebrand factor and platelet aggregation

In the arterial circulation, where blood flow is fast, thrombus formation is initiated by the adhesion of platelets to a reactive substrate and by their activation. These two events occur rapidly and almost concurrently at sites of injury, leading to a local accumulation of additional platelets in a longer-lasting process of homotypic aggregation. During this phase of thrombus growth, adherent and activated platelets bind soluble adhesive proteins, mainly fibrinogen and VWF, which upon immobilization onto the cellular membrane become substrates for the adhesion of circulating platelets in successive layers (Figure 9). The activation of the newly recruited platelets continues the process until the thrombus mass grows sufficiently to arrest blood loss from an injured vessel or, in pathological conditions, until regulatory mechanisms interrupt the cycle or the vascular lumen is occluded.

Notwithstanding the importance of the initiating events, a thrombus in the arterial circulation is composed essentially of platelets linked to one another but not directly to subendothelial components, and its development is strictly dependent on the formation of interplatelet bonds. Consequently, soluble ligand binding to activated platelets is a regulatory step for thrombus growth. Shear forces directly related to the velocity of blood flow tend to oppose the attachment of circulating platelets, which are not activated, to those already deposited in a growing thrombus, which are activated (Figure 9). Thus, when the shear rate is elevated, VWF is required to counteract these forces, as for example during initial platelet adhesion at a wound site. In the venous circulation, where blood flow is relatively slow, the formation of a fibrin clot is presumably the main determinant of thrombus formation, and the role of VWF in platelet aggregation becomes less relevant, if not dispensable.

Fibrinogen bound to \( \alpha_{IIb}^\beta_3 \) is often considered to be the predominant link between aggregated platelets. This is likely to be the case at a wall shear rate of 100 per second or less, as in the venous circulation. When the wall shear rate reaches 300 per second, however, experimental evidence indicates that a selective inhibition of GpIb\( \alpha \) limits the height reached by the thrombi in spite of normal initial growth (Figure 10). Such an effect is not likely to be the consequence of impaired platelet–surface interactions because VWF is not required to initiate platelet adhesion to collagen fibrils at wall shear rates as high as 500 per second. In fact, the same result is observed when receptor blockade is induced after the first layers of platelets have started to accumulate, such that only aggregation can be influenced (Figure 10). These findings indicate that GpIb\( \alpha \) and its adhesive ligand, VWF, are necessary not only for adhesion, but also for platelet-to-platelet cohesion in rapidly flowing blood.

It is still debatable what relative contribution VWF and fibrinogen provide to platelet aggregation by binding to activated \( \alpha_{IIb}^\beta_3 \). There is clear experimental documentation that VWF can support aggregation even in the absence of fibrinogen, albeit perhaps less efficiently, as shown in patients with afibrinogenemia. By this account alone, the designation of \( \alpha_{IIb}^\beta_3 \) as the ‘fibrinogen receptor’ is misleading as it induces to the erroneous conclusion that fibrinogen is the only relevant ligand for \( \alpha_{IIb}^\beta_3 \). At high shear rates, such as those prevalent in arterioles, fibrinogen cannot by itself support aggregation, whereas VWF can. In fact, the initial rate of platelet aggregation is significantly faster when VWF is the only available ligand for \( \alpha_{IIb}^\beta_3 \) than when both VWF and fibrinogen are present, confirming that the two molecules compete for binding to activated \( \alpha_{IIb}^\beta_3 \). Aggregates formed only in the presence of VWF are, however, unstable.

These experimental observations, along with the knowledge that binding to \( \alpha_{IIb}^\beta_3 \) is necessary for VWF function, suggest that, in the course of thrombogenesis under...
Figure 10. (A) Structure of thrombi formed at different shear rates in the absence (control) or presence of monoclonal antibodies blocking glycoprotein Iba (GpIba) or \( \alpha_{\text{IIb}}\beta_3 \), visualized by confocal optical sections at 1 \( \mu \text{m} \) intervals in the z axis. Each plane shows an area of 52.345 \( \mu \text{m}^2 \). For clarity, the z scale is expanded and only sections at selected distances from the surface are presented. Confocal sections were obtained after 840 seconds of flow at either 100 or 300 \( \text{s}^{-1} \), or after 420 seconds at 1500 \( \text{s}^{-1} \). The inset in the middle panel displays the partial reproduction with less size reduction of a thrombus formed at wall shear rate of 300 \( \text{s}^{-1} \), demonstrating the resolution of single platelets in the confocal sections. The two insets in the bottom panel show with greater magnification part of the surfaces exposed to antibody-containing blood flowing with a wall shear rate of 1500 \( \text{s}^{-1} \). No thrombi were formed, but single platelets could easily be detected. (B) Stacks of z sections labelled A show thrombi formed after 100 seconds of perfusion with blood containing no antibody at a wall shear rate of 1500 \( \text{s}^{-1} \); those labelled B show the growth of thrombi after an additional 740 seconds of perfusion at 300 \( \text{s}^{-1} \) with blood containing either buffer or the indicated antibodies. Adapted from Ruggeri et al (1999, Blood 94:172–178) with permission.
the influence of rapidly flowing blood, VWF and fibrinogen bind concurrently to platelets but to different \( \alpha_{\text{IIb}}^3 \) molecules. In any case, regardless of the generation of thrombin or the action of local agonists that activate platelets, VWF is always required for the normal growth of platelet aggregates exposed to a high shear rate. Thrombin and fibrinogen may be particularly important in these processes because, regardless of flow characteristics, the generation of fibrin is likely to be crucial in conferring long-term stability on the aggregates.\(^{96,100}\)

The synergy of fibrinogen and VWF in support of platelet aggregation depends on the recognized ability of each of these molecules to establish bonds with distinct adhesive properties. The initial function of VWF may depend primarily on its rapid rate of association with GpIb\(\alpha\).\(^{91,95}\) This is particularly important in haemodynamic conditions characterized by high flow rates that increase the velocity differential between the adjacent platelets (see Figure 9 above). The interaction of multimeric VWF with both GpIb\(\alpha\) and activated \( \alpha_{\text{IIb}}^3 \)\(^{101,102}\) may favour the capture of circulating platelets onto the surface of a forming thrombus, then allowing permanent bridging mediated by fibrinogen.\(^{102}\)

The reason why blocking GpIb\(\alpha\) binding to VWF may affect aggregation even at wall shear rates that do not prevent the normal initiation of surface adhesion is that thrombus growth narrows the path available to flowing blood, causing local increases in the velocity of the circulation and thus in the shear rate (see Figure 10 above).\(^{95}\) Because the mechanisms that oppose fluid dynamic forces are similar in the case of adhesive interactions between two platelets and in that between platelets and vascular surfaces, VWF is required for continued aggregation when shear forces at the surface of a growing thrombus exceed a threshold value. These concepts explain the relevant role that VWF plays in arresting mucosal bleeding, which involves mainly arterioles, and in arterial thrombosis, since the shear rate is particularly elevated where atherosclerotic plaques restrict the lumen.\(^{82}\)

**CONCLUSION**

The identification of the main bonds that, with distinct functional properties, support platelet interactions at the vessel wall has considerably enhanced our knowledge of the mechanisms of platelet adhesion and aggregation in flowing blood. The results of past efforts indicate new directions for future research. Of key importance now are understanding how differences in the composition of extracellular matrices may influence the rate of thrombus formation, and how signalling networks support the amplification of the process linking initial adhesion to platelet aggregation. Progress in these areas will undoubtedly lead to significant advances in our ability to recognize the predisposition to thrombotic disease and to treat affected patients.

**REFERENCES**


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