Platelet and von Willebrand factor interactions at the vessel wall

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Summary
The process of platelet thrombus formation contributes to the haemostatic response that prevents excessive blood loss after tissue injury, but may become a life-threatening disease mechanism by causing the acute thrombotic occlusion of atherosclerotic arteries. The participation of platelets in the formation of thrombi is centered on their adhesive properties and the ability to respond to stimuli with rapid activation. Platelet adhesion and activation are multifaceted and modulated by different environmental conditions, suggesting that it should be possible to obtain a selective pharmacological inhibition of the pathways more relevant to atherothrombosis than to haemostasis. In particular, progress in understanding the structure and function of von Willebrand factor and the mechanisms that underlie its interactions with vascular surfaces and platelets can elucidate important differentiating aspects of normal haemostasis and pathological arterial thrombosis.

The initial adhesion and activation occur in a time scale of milliseconds to seconds and require the concerted interaction of matrix proteins with platelet receptors. Among the adhesive and activating substrates are vascular wall components, such as different types of collagen, as well as plasma derived and surface-immobilized proteins, e. g. von Willebrand factor (VWF) bound to collagen and other matrix constituents. The main platelet counterparts include

- collagen receptors,
- integrin \( \alpha_{IIb}\beta_{3} \)
- glycoprotein(GP) VI,
- VWF receptor, GP Ibα, and
- integrin \( \alpha_{IIb}\beta_{3} \) (GP Ib-IIIa), which binds to multiple substrates.

Activation is directly coupled to adhesion but is greatly enhanced by agonists secreted locally from platelets, such as ADP and thromboxane A2, and by the tissue factor-mediated generation of α-thrombin. Most of these agonists are ligands for G-protein-coupled receptors on the platelet surface that respond through complex signaling pathways with the activation of phospholipase C, the increase of cytosolic Ca\(^{2+}\) and the decrease of cyclic AMP levels.

The adherent and activated platelets become the substrate for the second phase of platelet plug formation, which develops in a time frame of minutes and involves the recruitment and activation of additional circulating platelets into the growing thrombus. This phase of homotypic aggregation depends on the formation of interplatelet bonds that involve primarily GP Ibα in the GP Ib-IX-V complex and activated \( \alpha_{IIb}\beta_{3} \) as receptors on the platelet surface, and plasma VWF and fibrinogen as the ligands that link platelets to one another.

Endothelial cells form the inner lining of blood vessels and, under normal circumstances, are resistant to the interaction with circulating platelets in blood. Damage to the endothelial cells, sometimes directly but more often after exposure of subendothelial matrix proteins, rapidly elicits a response that consists in the attachment of platelets to the site of injury coupled to their activation (79). These are the crucial initiating steps in the haemostatic and thrombotic processes and lead to the formation of a stable platelet monolayer that, albeit insufficient by itself to prevent blood loss, is the basis for the subsequent development of an efficient thrombus (Fig. 1).
The formed aggregates are then stabilized by additional interactions, such as the binding of CD40 ligand to CD40 receptors. CD40 ligand (CD40L), thromboxane A2 (TXA2) stabilize platelet aggregates (green) stabilized by fibrin (blue strands) that form a barrier to blood flow. CD40 ligand (CD40L) is involved in the pathogenesis of atherosclerosis, and may represent a link between platelet-surface contacts and the latter process (33). Thrombin also mediates the consolidation of the platelet thrombus through the formation of a fibrin clot. Overall, the haemostatic response to vascular injury is contingent on

- the extent of damage,
- the specific matrix proteins exposed, and
- the prevailing flow conditions.

Here, the main aspects of platelet interactions at the vessel wall are reviewed, with emphasis on the role of VWF, collagen and their platelet receptors.

### Platelet adhesion

Circulating platelets adhere to sites of vascular injury through specific receptors that, coupled to their immobilized ligands, oppose the haemodynamic forces of flowing blood that tend to prevent adhesion contacts. Depending on the velocity of blood flow, and the consequent wall shear rate, different receptor-ligand interactions may be adequate to support the initial platelet-surface contacts (Fig. 2). In the human circulation, a threshold may exist at approximately 800/s wall shear rate. Below this threshold collagen may be sufficient to promote adhesion but above it VWF becomes progressively more important (85).

This effect of flow on platelet adhesion can be explained as follows. Blood circulates with greater velocity at the centerline of a vessel than near the wall, and this difference creates a shearing effect between adjacent layers of fluid that is greatest at the wall. The shear rate (1/s) is directly proportional to the shear stress (N/m² or Pa) and inversely proportional to the viscosity of the fluid (Pa·s).

The drag that opposes platelet adhesion and aggregation increases with the shear stress; consequently, its effects on platelet thrombus formation are more relevant in arteries than in veins and, particularly, in arterioles. The highest shear rate values in the normal circulation (95) may range from 472 to 4712/s with a median value of 1700/s.

### Collagen receptors

Two major receptors for collagen are expressed on the platelet surface (6):

- Integrin αIIβ3 (49, 67, 82), which is also found on a wide range of other cells, and
- GP VI (58, 59), which appears to be confined to platelets and megakaryocytes.

The latter receptor is a member of the Ig superfamily and contributes in a major way to signaling and platelet activation (20). The functional role of GP VI and αIIβ3 is highlighted by the impaired collagen-platelet interaction and mild bleeding disorder in patients deficient in the expression of either protein.
Integrin α2β1 (GP Ia-IIa)

Integrin α2β1 (also known as GP Ia-IIa on platelets, and VLA-2 on lymphocytes) was the first major receptor recognized as mediating platelet adhesion to collagen (68). There are on average 2000-3000 copies on the platelet surface, although the expression levels can vary up to 10-fold in normal healthy individuals contributing to differences in the risk of cardiovascular disease (50, 62, 83). This large variation in α2β1 expression levels among normal individuals is linked to silent polymorphisms in the gene for α2 (47).

The α2 and β1 subunits are noncovalently bound and have apparent molecular weights of 165 kDa and 130 kDa, respectively. The collagen-binding site was localized to a domain in the α2 subunit called the VWF type A domain (VWFA; also known as the I-domain), a region comprising 200 amino acids with homology to the collagen-binding domain of VWF. The crystal structures of both the I-domain and its complex with collagen-related peptides were elucidated by X-ray crystallography (27-29), providing valuable insights into the role of a cation binding site in modulating the collagen binding site conformation. The recognition sequence on type I collagen for α2-VWFA was identified and contains an essential GER peptide sequence (46) which coordinates with a cation (Mg2+ or Mn2+) bound to the VWFA domain to form a metal-ion-dependent adhesion site (MIDAS) (29). The extracellular domain of α2 contains a 7-fold repeated segment which includes a EF hand motif with three cation binding sites, thought to optimize collagen binding to the VWFA domain (23).

Although α2β1 is considered essential for optimal adhesion of platelets to collagen, its role as a signaling receptor has been controversially discussed. A two site, two step model of platelet activation by collagen was proposed in which platelets first bind collagen through α2β1, and are subsequently activated by GP VI (61, 81). Under defined experimental conditions, platelet adhesion to monomeric collagen is mediated exclusively through α2β1, and is associated with platelet spreading and activation of αthb3, but not granule secretion or

Fig. 2 Schematic representation of the mechanisms of platelet adhesion and aggregation in flowing blood (reproduced with kind permission from American Society for Clinical Investigation: Ruggeri ZM. Old concepts and new developments in the study of platelet aggregation. J Clin Invest 2000; 105: 699-701, Fig. 2).

a) In a flow field with high shear rate (in human circulation >500/s to 1000/s), only GP Ibcα interaction with immobilized VWF multimers (for example bound to collagen) can initiate the tethering of circulating platelets to the vessel wall. This interaction supports initially transient bonds, and translocation of the tethered platelets may occur (depicted schematically from left to right). Activation of αthb3 occurs during the transient tethering or through the action of other receptors that bind collagen or other components of exposed thrombogenic surfaces. The final result is the stable adhesion of recruited platelets to the surface. At shear rates <500/s to 1000/s, the adhesive functions of VWF are no longer indispensable for the initial attachment to a thrombogenic surface, and collagen receptors (among others) can permit stable adhesive interactions to form rapidly.

b) After the first layer of adherent and activated platelets is formed on the thrombogenic surface, αthb3 binds soluble adhesive ligands from plasma (exemplified are fibrinogen and VWF), which provide the substrate for the additional recruitment of nonactivated platelets and lead to thrombus growth. Note that nonactivated αthb3 cannot bind soluble ligands. In this phase of thrombus growth (or platelet aggregation), the shear rate at the surface of the growing thrombus imposes the same constraint on the function of adhesive bonds as during the initial platelet adhesion to the thrombogenic surface. Thus, when the shear rate is elevated, the bridging effect of fibrinogen, which is required to stabilize platelet aggregation, only occurs after the initial tethering of platelets to one another through the interaction of VWF and GP Ibcα (78).

The four images (bottom) are single frames from a real time recording of perfusion studies and demonstrate how inhibiting VWF binding to collagen using a monoclonal antibody against the VWF-A3 domain abolishes thrombus formation when the wall shear rate is 1500/s but not 500/s (85).
thromboxane production. Binding of soluble collagen fragments to α2β1 is increased following platelet activation (43), lending support to the notion that α2β1 may exist in different functional states, and that its affinity for collagen is regulated by inside-out signaling.

GP VI

The detection of patients deficient in platelet GP VI, and with defective collagen-induced platelet responses, led to the identification of GP VI as a crucial collagen receptor (58-60). GP VI is a major transmembrane platelet glycoprotein (60-65 kDa) and is a member of the immunoglobulin superfamily (42) which forms a non-covalent association with the FcγR chain at the cell surface (35, 97) that is critical for collagen-induced signal transduction.

In mouse platelets, deletion of the FcγR gene is associated with the absence of surface-expressed GP VI (66) demonstrating that the expression and function of GP VI is dependent on its association with the FcγR chain. The cytoplasmic domain of the FcγR chain contains an immunoreceptor tyrosine-based activation motif (ITAM), which is believed to play a crucial role in collagen-induced signaling (34). The association with FcγR chain enables GP VI to activate protein tyrosine kinase mediated signaling pathways. Platelet activation by collagen leads to an increase in tyrosine phosphorylation of several proteins, including FcγR-chain, the protein tyrosine kinase p72SYK, SLP-76 (Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa) and phospholipase C-γ2 (PLC-γ2) (37, 69).

Signaling through GP VI is thought to be initiated through ITAM phosphorylation by Src family tyrosine kinases (including Lyn and Fyn) following receptor clustering (34). The kinase p72SYK then binds to the phosphorylated ITAM domains of the FcγR-chain through its Src homology (SH2) domains, and is subsequently phosphorylated. Activated p72SYK then phosphorylates PLC-γ2, recruited to the plasma membrane by phosphorylated SLP-76, leading to stimulation of PLC-γ2 (19, 37). Activation of PLC-γ2 results in the generation of the second messengers 1,2-diacylglycerol (DAG), which activates protein kinase C (PKC) isoforms, and inositol-1,4,5-triphosphate (IP3), which induces the release of stored calcium ions (12). Activated PKC isoforms regulate serine/threonine phosphorylation events required for full platelet activation and modulation of αIIbβ3 into a form competent to bind soluble fibrinogen (88).

Recently, definitive evidence on the role of GP VI in collagen-induced aggregation has been provided by studies in mice with a deletion of the corresponding gene (45). When mouse blood containing GP VI−/− platelets was perfused over fibrillar collagen type I at a wall shear rate corresponding to that in the human microarteriolar circulation, initial contact and adhesion were essentially normal, but stable adhesion was somewhat decreased and thrombus formation was totally abolished (Fig. 3). Essentially the same results were seen in FcγRγ−/− platelets, which are also deficient in GP VI owing to the necessary association between the two proteins for a normal transport to the membrane (97).

It has been proposed that GP VI may be an important contributor to arterial thrombus formation. In a mouse model of injured carotid artery, for example, experiments with intravital fluorescence microscopy showed that platelets rendered deficient in GP VI by antibody treatment failed to adhere normally and aggregated on the damaged vessel wall (53). Accordingly, mice treated with an anti-GP VI monoclonal antibody were afforded protection from a lethal thromboembolism following infusion of a mixture of collagen and ephrinephrine (65). A more general statement on the importance of GP VI, relative to other key adhesion and activation receptors, in the development of atherothrombosis awaits further experimental work.

GP Ib-IX-V complex

Initially recognized as a specific VWF receptor on platelets, the GP Ib-IX-V complex is now known to posses a diverse repertoire of functions in haemostasis and thrombosis. For example, the GP Ib-IX-V complex mediates the interaction of nonactivated platelets with the vascular endothelium by binding to surface expressed endothelial P-selectin (77), and with activated leukocytes through its binding to integrin Mac-1 (89). In addition to its adhesive role, the GP Ib-IX-V complex has been shown to interact with thrombin (11, 51), high molecular weight kinogen (14) and coagulation factors XI (8) and XII (15). It also plays a key role in regulating the cytoskeleton of platelets (41).

Adhesive function

Under high shear flow conditions, the GP Ib-IX-V complex is essential to mediate the initial adhesion of platelets to VWF immobilized at sites of vascular injury (5, 48, 52, 102, 104). Ex vivo flow studies have established a key role for the platelet GP Ib-IX-V complex in mediating the capture and translocation or rolling of platelets on VWF (85, 86). Translocation of platelets on surface-bound VWF leads to platelet activation and the subsequent arrest through ligation of VWF with the platelet αIIbβ3 (88). The GP Ib-IX-V complex comprises four distinct transmembrane subunits: GP Ibα, GP Ibβ, GP IX and GP V (2, 7, 11, 102).

GP Ibα (ca. 135 kDa) is covalently bound to GP Ibβ (ca. 25 kDa) via a disulfide bond near the extracellular surface of the plasma membrane. GP Ibβ is noncovalently associated with GP IX (ca. 22 kDa) to form a 1 : 1 complex (24), whereas GP V (ca. 82 kDa) is non-covalently associated with GP Ib-IX in a 1 : 2 ratio (10, 16, 52). Thus, the four subunits of the GP Ib-IX-V complex are expressed on the platelet membrane surface with an apparent molar stoichiometry of 2 : 2 : 2 : 1 for GP Ibα : GP Ibβ : GP IX : GP V, respectively.

Approximately 25 000 copies of GP Ibα, GP Ibβ and GP IX, and approximately half this number for GP V are present on the surface of resting human platelets (10, 57). GP V levels are decreased when it is cleaved from the platelet surface by proteolysis during stimulation of platelets by α-thrombin (56). The binding site for VWF was localized to the N-terminal domain of GP Ibα (39, 101), and the solved crystal structure of
a complex between this domain and the VWF A1 domain indicated the contact interface between the two molecules (40).

The cytoplasmic face of the GP Ib-IX-V complex was shown to be associated with several proteins including filamin (actin binding protein, also known as ABP-280), which binds within the region Thr \(^{536}\).Leu \(^{554}\) of the GP Ib \(\alpha\) cytoplasmic domain (3). Through this interaction the GP Ib-IX-V complex is linked to a submembrane network of F-actin structures that comprises the platelet membrane skeleton (32). The cytoplasmic tail of GP Ib \(\alpha\) also contains the binding site for the dimeric signaling molecule 14-3-3 \(\gamma\) within the five C-terminal amino acids, Ser-Gly-His-Ser(P)-Leu (25).

Phosphorylation of Ser609 is essential for high affinity binding of 14-3-3 \(\gamma\) (13). In resting platelets, 14-3-3 \(\gamma\) provides a link between GP Ib-IX-V and phosphatidylinositol 3-kinase (PI 3-K) (63).

Another 14-3-3 \(\gamma\) binding site was located within the cytoplasmic domain of GP Ib \(\beta\) following the phosphorylation of Ser \(^{66}\) by cAMP-dependent protein kinase A (4, 17). However, binding of 14-3-3 \(\gamma\) to GP Ib \(\beta\) appears to be contingent on the association of 14-3-3 \(\gamma\) with the C-terminal domain of GP Ib \(\alpha\) (31). The cytoplasmic domain of GP Ib \(\alpha\) also interacts with calmodulin within residues 149-167 (6).

**Signal transmission through GP Ib-IX-V**

Indirect evidence suggests that one mechanism of VWF-dependent signaling involves the cross-linking of GP Ib \(\alpha\) subunits by multivalent VWF (44). For example, a monomeric proteolytic fragment of VWF containing a single A1 domain binds to the GP Ib-IX-V complex and inhibits binding of native multimeric VWF, but does not induce cytoskeletal rearrangement and activation of \(\alpha_{IIb}\beta_{3}\) (2, 84). The observations that the GP Ib-IX-V complex is associated with two ITAM-containing proteins, FcR \(\gamma\)-chain and FcγRIIA (30, 96), lends support to receptor cross-linking as at least one mechanism of signaling.

FcγRIIA and GP Ib \(\alpha\) are proximal to within 10 nm of each other in the platelet membrane (93), and both FcR \(\gamma\)-chain and FcγRIIA co-immunoprecipitate with the GP Ib-IX-V complex (30, 93). On cross-linking, FcR \(\gamma\)-chain and FcγRIIA are tyrosine phosphorylated within the cytoplasmic ITAM sequence by src family tyrosine kinases (103), thereby permitting the binding and auto-phosphorylation of the tyrosine kinase Syk. Activated Syk initiates signaling that leads to activation of phospholipase C\(\gamma\) and subsequent formation of inositol triphosphate (leading to Ca\(^{++}\) mobilization) and diacylglycerol (leading to activation of protein kinase C) (30, 96).

The cytoplasmic region of GP Ib \(\alpha\) is associated with two dimeric signaling proteins, actin binding protein and 14-3-3 \(\gamma\) (3, 21, 26). These associations provide potential links to several signaling molecules, including PI 3-K, focal adhesion kinase, src-related tyrosine kinases (Syk, Src, Fyn, Lyn and Yes), GTPase-activating protein, and tyrosine phosphatases (PTP-1B and SHPTP10) (7).

Several studies indicate that 14-3-3 \(\gamma\) plays a crucial role in regulating signaling through GP Ib-IX-V leading to activation of \(\alpha_{IIB}\beta_{3}\) (31, 38, 63). CHO cells co-expressing GP Ib-IX and \(\alpha_{IIB}\beta_{3}\) spread on a VWF coated surface and subsequently bound fibrinogen in an \(\alpha_{IIB}\beta_{3}\) activation-dependent manner (38). Both PI 3-K and protein kinase C play important roles in this process since their inhibition blocked cell...
spreading (38). Moreover, deletion of the C-terminal 18 amino acids of GP Ibα, containing the binding site for 14-3-3ζ, prevented cell spreading and fibrinogen binding to αIIbβ3.

PI 3-K forms a constitutive complex with both GP Ib-IX-V and 14-3-3ζ in resting platelets (63), and may therefore play a key role in 14-3-3ζ signaling. Although dissociation of 14-3-3ζ from the GP Ib-IX-V complex is seen following platelet activation (31, 63), it is unclear whether this is a direct effect of platelet activation, or a process that precedes activation.

The binding of the VWF-A1 domain to GP Ibα is associated with transient elevations of intracytoplasmic Ca2+ concentration ([Ca2+]i), that appear to originate from the mechanical stimulation of bonds under tensile stress and indicate platelet activation (54, 108). This mechanism of signaling may reflect receptor ligation and differ from the lateral clustering process discussed above. A first signal, designated a type α/β peak, depends on Ca2+ release from intracellular stores and precedes stationary platelet adhesion.

Once adhesion is established through the integrin αIIbβ3, a different type of [Ca2+]i elevation occurs, designated type γ, which depends on a transmembrane ion flux and is of greater amplitude and duration. This signal precedes the recruitment of additional platelets into aggregates. Degradation of released ADP or inhibition of phosphatidylinositol 3-kinase prevent type γ [Ca2+]i transients and block aggregation (36, 54, 107). Thus, an initial signal induced by stressed GP Ibα-VWF bonds leads to αIIbβ3 activation sufficient to support localised platelet adhesion. Then, additional signals from ADP receptors and possibly ligand-occupied αIIbβ3, with the contribution of a pathway involving PI 3-K, amplify platelet activation to the level required for aggregation.

In addition to binding VWF, GP Ibα is also a receptor for α-thrombin and this interaction may be involved in the activation necessary for continued platelet recruitment in a growing thrombus. Moreover, GP Ibα is required for the expression of procoagulant activities that facilitate the stabilization of platelet aggregates through fibrin formation. The resolved crystal structure of the complex between the amino terminal domain of GP Ibα and α-thrombin elucidated the contact interfaces between the two proteins and shed new light on the possible functional significance of these interactions (18).

Platelet GP Ibα possesses two binding sites for α-thrombin. One of these establishes extensive contacts with the functionally relevant anion-binding exosite I, and thus modulates fibrinogen clotting, but is made available for binding only after an independent site is occupied by the anion-binding exosite II of another thrombin molecule. The binding of distinct α-thrombin molecules to the two sites on GP Ibα creates the possibility for cross-linking of the receptor on the platelet membrane. Ramakrishnan et al. identified a thrombin receptor function for platelet GP Ib-IX that is unmasked by cleavage of GP V but is in itself independent of proteolytic activity (70). It is tempting to speculate that this function may result from the thrombin-mediated cross-linking of GP Ibα on the platelet surface. The recent structural evidence indicates, therefore, that at least two divergent consequences may result from α-thrombin binding to GP Ibα:

- Cross-linking of the receptor may contribute to platelet activation and aggregation, which are prothrombotic events, while
- the masking of exosite I may reduce the rate of fibrin formation and therefore limit the stability of thrombi.

GP Ibα may thus be an important player in the maintenance of a proper haemostatic balance after α-thrombin generation at sites of vascular injury. Moreover, the acquired structural knowledge suggests that VWF and α-thrombin may bind to GP Ibα concurrently. The model also indicates that intermolecular contacts may be established between the two ligands once bound to the receptor. These findings delineate a possible mechanism through which the rapid generation of α-thrombin at a wound site may promote not only platelet activation and fibrin formation, but also modulate the initial adhesion of platelets to immobilized VWF. Accordingly, α-thrombin bound to GP Ibα may stabilize adhesion by mediating a tighter association of VWF-A1 domain with the platelet surface through the establishment of additional contacts when the A1 domain is bound to the same GP Ibα receptor.

**Initiation of platelet thrombus formation and the role of VWF**

Von Willebrand factor is present within the subendothelium of human blood vessels (74) where it may contribute to mediating platelet adhesion by providing a direct link with the membrane of circulating platelets (80, 91, 98). Our knowledge on the distribution of subendothelial VWF in the vascular tree is far from definitive. A detailed immunolocalization study using fresh porcine specimens concluded that VWF antigen can be consistently detected in the endothelium of veins (exception: pulmonary vein) but not in arteries (exceptions: abdominal aorta, vasa vasorum of the thoracic aorta, pulmonary artery) (71).

Therefore, it is generally accepted, that VWF synthesis in the vascular tree is heterogeneous, a concept also supported by the demonstration that endothelial cells of different origin express varying amounts of VWF mRNA and protein even when maintained in culture (9, 106). In particular, it is still debatable whether VWF is localized in the subendothelium of all arterioles and arterial capillaries (94, 99), the vessels most directly involved in haemostasis. Experimental evidence suggests that type VI collagen filaments have affinity for VWF (22, 73), and immunolocalization studies showed that type VI collagen and VWF are morphologically associated in situ (72).

In addition to subendothelial matrix-associated VWF, the binding of plasmatic VWF to exposed perivascular tissue is a crucial initial step in the normal haemostatic response to vascular injury (80). This is the only mechanism to provide initial platelet-surface contact in high flow areas of the circulation where VWF is not endogenously present in the subendothelial matrix. Although soluble VWF does not bind to nonactivated platelets, when immobilized it...
forms a reactive surface capable of capturing platelets in the flowing blood (85, 86). Possible binding sites in the vascular wall for plasmatic VWF include collagen types I, III, and VI (90, 100). It has been proposed that fibrillar collagen types I and III are important binding sites for VWF in deeper layers of the vessel wall below the subendothelial structures, whereas microfibrillar collagen type VI may be a physiologically relevant binding site at the exposed subendothelium, following superficial vascular damage (72, 100).

The collagen-binding function of VWF is contained in two type A domains: A1 and A3. The latter is necessary and sufficient to support binding to fibril-forming collagens, such as type I or type III, while domain A1 is involved in collagen type VI binding (55). Fluid dynamic conditions and mechanical forces may modulate these interactions, and domains A1 and A3 may variably contribute to the immobilization of VWF onto complex extracellular matrices.

This concept possibly explains the apparently discrepant observations that an antibody blocking VWF-A3 domain binding to collagen prevented the formation of platelet-rich thrombi and prolonged the skin bleeding time (105), while mutations that markedly inhibit the same interaction, such as Ser968 to Thr (75), caused no or only moderate bleeding symptoms. Thus, the immobilization of VWF onto extracellular surfaces mediated by the domain A3 may be variably relevant depending on the nature of the vascular lesion. The collagen-binding region in the A3 domain has been tentatively deduced from the structural analysis of the binding site of a function-blocking monoclonal Fab fragment (76). The folding of domain A3 is very similar to that of A1, and residues essential for collagen binding may be located in strand β3 and loop β3-β4.

Attempts to elucidate the mechanisms involved in the initiation of the haemostatic process were facilitated by the use of ex vivo perfusion chambers to simulate relevant in vivo conditions. Using these techniques, it has been shown that the interaction of platelets in flowing blood with immobilized VWF results in an initial continuous surface translocation at all shear rates ranging from 50/s to >6000/s (86). This process is inhibited by blocking platelet GP Ibα function with a soluble recombinant fragment corresponding to the domain A1 of VWF (92). Continuation of blood flow at a wall shear rate of 1500/s leads to firm platelet adhesion and the formation of platelet aggregates attached to the surface.

Definitive adhesion of platelets is prevented when integrin αIIbβ3 function is blocked with a monoclonal antibody, resulting in the continuous displacement of platelets on the surface. Likewise, firm attachment, but not the initial capture and translocation, is also prevented in the presence of PGE1, which blocks platelet activation and the subsequent modulation of αIIbβ3 to form a competent binding receptor for the sequence Arg-Gly-Asp in the carboxyterminal domain of VWF.

It was assumed that the primary function of the interaction between GP Ibα and VWF is to initiate platelet adhesion under high shear conditions. In fact, platelets transported to a VWF surface are captured and their velocity slowed by a series of multiple interactions, involving the formation and breaking of GP Ibα/VWF-A1 domain bonds, regardless of the prevailing flow conditions. These results also demonstrate that the adhesion of flowing platelets to immobilized VWF is a two-stage dynamic process:

- The initial tethering of platelets leads to their surface translocation with a substantially reduced velocity compared with that of free-flowing platelets proximal to the surface.
- Platelet movement on the surface continues until platelets become activated and firm platelet attachment, mediated by activated αIIbβ3 ensues, leading ultimately to thrombus formation.

Distinct adhesion pathways are functionally integrated during the initiation of thrombus formation on composite substrates such as fibrillar type I collagen, which rapidly binds VWF when exposed to blood, and extracellular matrices (85). On type I collagen fibrils, initial platelet interaction leads rapidly to firm attachment at a wall shear rate of 1500/s (conditions that simulate the haemodynamic challenge to platelets that may prevail in arterioles where platelets are essential for haemostasis). The formation of thrombi ensues, whose number and growth rate are governed by the volumetric flow rate. Functional inhibition of platelet αIIbβ3 prevents thrombus growth without affecting the stable attachment of single platelets. This strategy permits the study of platelet-substrate interactions in the absence of thrombus formation.

In contrast, inhibition of plasma VWF-A3 domain binding to collagen completely eliminates all initial platelet surface interaction at 1500/s, but has no effect on thrombus formation at, or below 500/s. In likewise manner, blockade of GP Ibα abolishes deposition of platelets at 1500/s, but only partially at 500/s, while having no effect at 100/s. Blockade of αIIbβ3 results in a partial (50%) reduction of thrombus volume at 1500/s on surfaces coated with collagen at low, but not high density, indicating a significant role of αIIbβ3 in thrombus formation under the former conditions. In addition, αIIbβ3 plays a significant role in the initial attachment of platelets, preceding thrombus formation since blocking αIIbβ3 causes a reduction in surface coverage before the appearance of aggregates at 1500/s on collagen coated at low, but not high density (85). Thus the function of αIIbβ3 becomes increasingly more relevant for platelet adhesion and thrombus formation when platelets are subjected to increasing haemodynamic drag on surfaces with relatively sparse collagen fibrils.

Chelation of divalent cations in blood by EDTA permits tethering and platelet rolling in continuous contact with the surface, but prevents irreversible adhesion to type I collagen fibrils, indicating the dependence of integrin-ligand interactions in the latter event. The displacement characteristics of platelets on collagen at 1500/s are similar to those seen on a VWF-coated surface or on collagen saturated with purified VWF before the onset of blood flow. Thus, the binding of plasma VWF to type I collagen fibrils, like GP Ibα-mediated platelet tethering, is a cation-independent process.

The transition from initial tethering to stable platelet attachment requires the functional integration of both αIIbβ3 and αIIbβ1, as illustrated by the effects of blocking αIIbβ3 and αIIbβ1 function, either separately or
concurrently. Functional inhibition of $\alpha_{IIb}\beta_3$ with a monoclonal antibody prevents aggregate formation and permits the visualization of single platelets interacting with the surface. Under these conditions, platelets attach in a predominantly stable fashion on the collagen surface at 1500/s. However, when $\alpha_{IIb}\beta_3$ and $\alpha_\beta_1$ are blocked simultaneously, the majority of platelets translocate on the surface in the direction of blood flow in a manner similar to that seen for platelets interacting with VWF immobilized onto glass (86). The average velocity of platelets translocating on the collagen-VWF surface under these conditions is 5-6 $\mu$m/s; a value similar to that measured at the same shear rate of 1500/s on purified VWF bound to glass (86).

Thrombus formation initiated by platelet adhesion to extracellular matrix involves the synergistic function of at least four platelet receptors (85), namely GP Ib$\alpha$, $\alpha_\beta_3$, $\alpha_{IIb}\beta_3$, and $\alpha_\beta_1$. At a shear rate of 1500/s, GP Ib$\alpha$ function is essential for initiating platelet recruitment on subendothelial extracellular matrix, and stable attachment, but not continuous translocation of surface tethered platelets, requires divalent cations. Selective inhibition of $\alpha_{IIb}\beta_3$ leads to firm attachment of approximately 50% of platelets initially tethered to the matrix, a value similar to that seen with fibrillar type I collagen. However, when $\alpha_{IIb}\beta_3$ and $\alpha_\beta_1$ are blocked concurrently, the number of firmly attached platelets is higher than that seen under similar conditions with fibrillar type I collagen.

This value is reduced by blocking the integrin subunit $\alpha_\beta_3$, indicating a possible contribution of matrix or plasma fibronectin to the stabilization of platelet interaction with the subendothelium. Recently, evidence has been provided that fibronectin may also contribute to the formation and stabilization of platelet aggregates that form thrombi exposed to flowing blood, a role that becomes particularly evident in the absence of both VWF and fibrinogen (64). Platelet adhesion and thrombus formation on subendothelial matrix requires both endogenous and plasma-derived VWF, since blocking selectively either the A1 domain of subendothelial VWF or the A3 domain of soluble VWF in the perfused blood leads to a dramatic (80%) decrease in thrombus volume at 1500/s. These results indicate that both the amount of endogenous VWF and the availability of VWF-binding sites are limiting, such that additional binding of plasma VWF can complement the function of the endogenous protein, but neither is sufficient independently.

The traditional understanding of the mechanism that initiates platelet adhesion under high shear stress envisions the binding of GP Ib$\alpha$ to the domain A1 of VWF immobilized onto components of extracellular matrices, notably collagen. Indeed, it is commonly assumed that the association with collagen may regulate the GP Ib$\alpha$-binding function of VWF, although there is no definitive evidence to validate this hypothesis. Recently, it has been demonstrated that a process of reversible self-association may rapidly occur between molecules of VWF immobilized on a surface and those in solution in flowing blood (87).

This can form a layer of VWF multimers interposed between the initially exposed thrombogenic substrate and blood cells, such that the VWF molecules presenting A1 domain for platelet binding may not be directly and irreversibly linked to the surface (Fig. 4). The significance of such a dynamic exchange between surface-immobilized and soluble VWF multimers with respect to platelet adhesion under high shear stress is not yet fully understood, but apparently the direct association between VWF and collagen is not an absolute requirement for A1 domain expression of the GP Ib$\alpha$ binding function.

Fig. 4 Role of soluble VWF-A1 domain in mediating platelet interaction with surface-bound VWF (Adapted from: Savage B, Sixma J, Ruggeri ZM. Functional self-association of von Willebrand factor during platelet adhesion under flow. Proceedings of the National Academy of Sciences 2002; 99: 425-30, Fig. 3, and reprinted with permission). A washed blood cell suspension devoid of plasma proteins and containing EDTA (to block integrin function in adhesion and aggregation) and prostaglandin E1 (to block platelet activation) was perfused over immobilized collagen type I fibrils at the wall shear rate of 1500/s. a) Control experiment with normal multimeric VWF added to the cell suspension. The A3 domain mediates VWF binding to collagen, and the domain A1 interacts with platelet GP Ib$\alpha$. Tethered platelets are seen rolling on the surface, which is represented by an electron micrograph of collagen fibrils. b, c) Experiments performed after adding to the cell suspension recombinant VWF devoid of the domain A3 ($\Delta$A3-VWF), which cannot bind to collagen (b) or recombinant VWF devoid of the domain A1 ($\Delta$A1-VWF), which binds to collagen but cannot interact with platelet GP Ib$\alpha$ (c). In either case, no platelets are seen tethered to the surface. d) The collagen fibrils were precoated with $\Delta$A1-VWF multimers, which cannot initiate platelet tethering, and then exposed to the blood cell suspension containing $\Delta$A3-VWF. Although the latter cannot bind directly to collagen (see b), it could compensate for the lack of domain A1 in the surface-bound VWF and restore platelet tethering. The association of VWF multimers with one another can explain this result. The two-sided arrows between multimers indicate that the association is reversible. The schemes presented with the images depict the soluble and immobilized VWF used in the experiments with respect to the presence and function of domain A1 and A3. The images are single frames from a real time recording representing an area of 65 536 $\mu$m$^2$. The bars represent the number of platelets tethered to the surface under the different experimental conditions as described (mean ±SEM of two separate experiments).
Conclusions

The participation of platelets in the formation of thrombi that acutely occlude arteries, causing serious disease, is centered on their adhesive properties and the ability to respond to stimuli with rapid activation. Platelet adhesion and activation are multifaceted and responsive to different environmental conditions, suggesting the possibility of specific pharmacological targeting aimed at a selective inhibition of the pathways more relevant to the pathological aspects of atherothrombosis than to haemostasis. In particular, progress in understanding the structure and function of platelet receptors and their complex mode of interaction with different ligands can elucidate crucial aspects of normal haemostasis and pathophysiological arterial thrombosis.

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