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 thrombus 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 $\alpha_{II} \beta_3$,
- glycoprotein (GP) VI,
- VWF receptor, GP Ib$\alpha$, and
- integrin $\alpha_{IIb} \beta_3$ (GP IIb-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 $A_2$, and by the tissue factor-mediated generation of $\alpha$-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 Ibo 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).

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The formed aggregates are then stabilized by additional interactions, such as the binding of CD40 ligand to platelet receptors. CD40 ligand (CD40L), thromboxane A2 (TXA2).

Fig. 1 Balance between antithrombotic and prothrombotic molecules synthesized by endothelial cells (reproduced with kind permission from Nature Publishing Group: Ruggeri ZM. Platelets in atherothrombosis. Nature Med 2002; 8:1227–34, Fig. 2)

The functional role of GP VI and αIIbβ3 (1). CD40 ligand is also involved in the pathogenesis of atherosclerosis, and may represent a link between platelets and the latter process (33). 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.

**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).

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 αIIbβ3 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 αIIbβ3, but not granule secretion or activation of GP Ibα. 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 $\alpha_2\beta_1$ is increased following platelet activation (43), lending support to the notion that $\alpha_2\beta_1$ 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 FcRγ chain at the cell surface (35, 97) that is critical for collagen-induced signal transduction.

In mouse platelets, deletion of the FcRγ 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 FcRγ chain. The cytoplasmic domain of the FcRγ 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 FcRγ 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 FcRγ 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 FcRγ 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 (IP$_3$), 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 $\alpha_{IIb}\beta_3$ 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 FcRγ$^-/-$ 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 intravalit fluorescent 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 ephinephrine (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 possess a diverse repertoiere 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 kininogen (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 $\alpha_{IIb}\beta_3$ (86). The GP Ib-IX-V complex comprises four distinct transmembrane subunits: GP Iba, GP Ibβ, GP IX and GP V (2, 7, 11, 102).

GP Iba (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 noncovalently 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 2:2:1:2 for GP Iba:GP Ibβ:GP IX:GP V, respectively.

Approximately 25 000 copies of GP Iba, 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 Iba (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 Ibα 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 Ibα also contains the binding site for the dimeric signaling molecule 14-3-3ζ within the five C-terminal amino acids, Ser-Gly-His-Ser(P)-Leu (25). Phosphorylation of Ser609 is essential for high affinity binding of 14-3-3ζ (13). In resting platelets, 14-3-3ζ provides a link between GP Ib-IX-V and phosphatidylinositol 3-kinase (PI3-K) (63).

Another 14-3-3ζ binding site was located within the cytoplasmic domain of GP Ibβ following the phosphorylation of Ser^160 by cAMP-dependent protein kinase A (4, 17). However, binding of 14-3-3ζ to GP Ibβ appears to be contingent on the association of 14-3-3ζ with the C-terminal domain of GP Ibα (31). The cytoplasmic domain of GP Ibα 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 is the cross-linking of GP Ibα 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 αIIbbβ3 (2, 84). The observations that the GP Ib-IX-V complex is associated with two ITAM-containing proteins, FeR γ-chain and FcγRIIA (30, 96), lends support to receptor cross-linking as at least one mechanism of signaling.

FcγRIIA and GP Ibα are proximal to within 10 nm of each other in the platelet membrane (93), and both FeR γ-chain and FcγRIIA co-immunoprecipitate with the GP Ib-IX-V complex (30, 93). On cross-linking, FeR γ-chain and FcγRIIA are tyrosine phosphorylated within the cytoplasmic ITAM sequence by src family tyrosine kinases (103), thereby permitting the binding and autophosphorylation of the tyrosine kinase Syk. Activated Syk initiates signaling that leads to activation of phospholipase Cγ2 and subsequent formation of inositol triphosphate (leading to Ca²⁺ mobilization) and diacylglycerol (leading to activation of protein kinase C) (30, 96).

The cytoplasmic region of GP Ibα is associated with two dimeric signaling proteins, actin binding protein and 14-3-3ζ (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 SHPPTP10) (7).

Several studies indicate that 14-3-3ζ plays a crucial role in regulating signaling through GP Ib-IX-V leading to activation of αIIbbβ3 (31, 38, 63). CHO cells co-expressing GP Ib-IX and αIIbbβ3 spread on a VWF coated surface and subsequently bound fibrinogen in an αIIbbβ3 activation-dependent manner (38). Both PI 3-K and protein kinase C play important roles in this process since their inhibition blocked cell

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**Fig. 3** Role of GP VI in the formation of platelet thrombi on the surface of fibrillar type I collagen exposed to flowing blood (reproduced with kind permission from American Society of Hematology: Kato K, Kanaji T, Russell S et al. The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion. Blood 2003; 102: 1701-7, Fig. 5).

Blood was collected from anesthetized mice via a retroorbital puncture using heparin (40 U/ml) as anticoagulant. Apyrase was added to a final concentration of 40 U/ml as anticoagulant. Apyrase was added to a final concentration of 1.5 U/ml. Glass coverslips were coated with insoluble fibrillar type I collagen (2.5 mg/ml) and placed in a parallel plate flow chamber. Mouse blood was treated with mepacrine for platelet visualization and perfused through the chamber at 1500/s wall shear rate.

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**a)** Single frames taken from a continuous recording show the collagen-coated surface after 2.5 min of blood perfusion. In the frame to the left, thrombi formed by normal platelets are seen at a relatively low magnification (100-fold). The frame in the center and the one to the right show the surfaces exposed to GP VIWT and FcRγnull platelets, respectively, at a higher magnification (40-fold). In either case, a complete surface coverage by single platelets is apparent, with formation of small clusters particularly in the case of FcRγnull platelets, but absence of thrombus formation.

**b)** Post-perfusion thrombus volume was determined from serial z-sections. The results presented are mean values from three independent experiments.
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 Ca++ concentration [Ca++]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 Ca++ release from intracellular stores and precedes stationary platelet adhesion.

Once adhesion is established through the integrin αIIBβ3, a different type of [Ca++]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 [Ca++]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 localized 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 Ib 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 arteries and arterioles 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 lead-rich thrombi and prolonged the skin bleeding time (105), while mutations that lead to impaired PGE₂ production and inhibition of platelet aggregation (106). This concept possibly explains the apparent discrepancy between the effects of blocking VWF-A1 domain binding to collagen and the effects of blocking VWF-A3 domain binding to collagen. The former resulted in the prevention of collagen-induced platelet aggregation at low shear rates, whereas the latter resulted in the prevention of collagen-induced platelet aggregation at high shear rates.

Platelet movement on the surface continues until platelets become activated and form platelet attachment, mediated by activated α₂β₃, 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 arteries where platelets are essential for haemostasis). The formation of thrombus ensues, whose number and growth rate are governed by the volumetric flow rate. Functional inhibition of platelet α₂β₃ 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 like manner, blockage 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 α₂β₃ 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 α₂β₃ in thrombus formation under the former conditions. In addition, α₂β₃ plays a significant role in the initial attachment of platelets, preceding thrombus formation since blocking α₂β₃ 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 α₂β₃ 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 α₂β₃ and α₂β₃, as illustrated by the effects of blocking α₂β₃ and α₂β₃ 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 $\alpha_\beta_2$ 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 on 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 $\alpha_\beta_2$. 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 $\alpha_\beta_2$ 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 JJ, Ruggeri ZM. Functional selfassociation 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 E$_2$ (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 A1 domain 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).

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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 pathological arterial thrombosis.

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