Adhesion Mechanisms in Platelet Function

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Abstract—Platelet adhesion is an essential function in response to vascular injury and is generally viewed as the first step during which single platelets bind through specific membrane receptors to cellular and extracellular matrix constituents of the vessel wall and tissues. This response initiates thrombus formation that arrests hemorrhage and permits wound healing. Pathological conditions that cause vascular alterations and blood flow disturbances may turn this beneficial process into a disease mechanism that results in arterial occlusion, most frequently in atherosclerotic vessels of the heart and brain. Besides their relevant role in hemostasis and thrombosis, platelet adhesive properties are central to a variety of pathophysiological processes that extend from inflammation to immune-mediated host defense and pathogenic mechanisms as well as cancer metastasis. All of these activities depend on the ability of platelets to circulate in blood as sentinels of vascular integrity, adhere where alterations are detected, and signal the abnormality to other platelets and blood cells. In this respect, therefore, platelet adhesion to vascular wall structures, to one another (aggregation), or to other blood cells, represent different aspects of the same fundamental biological process. Detailed studies by many investigators over the past several years have been aimed to dissect the complexity of these functions, and the results obtained now permit an attempt to integrate all the available information into a picture that highlights the balanced diversity and synergy of distinct platelet adhesive interactions. (Circ Res. 2007;100:1673-1685.)

Key Words: adhesion molecules ■ platelets ■ vascular biology ■ extracellular matrix ■ collagen

Platelets in mammals are anucleated cells that originate from the cytoplasm of bone marrow megakaryocytes and circulate in blood as sentinels of vascular integrity. They show no interaction with the inner surface of normal vessels but adhere promptly where endothelial cells are altered or extracellular matrix substrates are exposed. This is a critical initial step in hemostasis and thrombosis, as well as in inflammatory and immunopathogenic responses. The functions of mammalian platelets are conserved throughout evolution and closely reflect those of nucleated thrombocytes in all other vertebrates. After adhering to vascular lesions, platelets can rapidly recruit to the site of injury additional platelets, which are necessary to achieve hemostasis, or different types of leukocytes, which set off host defense responses. Such selective recruitment is orchestrated by activation pathways stimulated by the initial adhesive interactions and by soluble agonists released or generated locally, which lead to the appearance on the platelet membrane of different adhesive molecules capable of attracting distinct circulating cells. Platelet adhesion in a broad sense is the...
Platelet Adhesion and Platelet Aggregation: Two Sides of the Same Coin

Adhesion and aggregation are often considered as distinct processes through which platelets establish individual contacts with extracellular surfaces or stick to one another, forming clumps, respectively. Yet, this distinction is based more on the interpretation of experimental studies than on cogent mechanistic reasons, because both adhesion and aggregation involve the transition of platelets from free flow in blood to arrest onto a surface, often mediated by the same adhesive ligand and receptor pairs. In the case of platelet adhesion, the surface is the extracellular matrix or the membrane of other cells of the vessel wall and surrounding tissues, and the adhesive substrates are endogenous matrix or cell membrane proteins and proteoglycans, along with locally bound selected plasma components. In the case of platelet aggregation, the surface is the membrane of other platelets that have already become immobilized at sites of thrombus formation and present membrane-bound adhesive substrates, either following activation-induced translocation from internal storage compartments or bound from plasma. An important consideration is that, by virtue of the mechanistic similarities, the same fluid dynamic constraints influence both platelet adhesion and aggregation.

Effects of Hydrodynamic Conditions on Platelet Adhesion and Aggregation

Platelets are essential for normal hemostasis and, in particular, for arresting bleeding from arterioles, where shear stress is elevated (see the online data supplement). In pathological conditions, platelets are a major contributor to arterial thrombosis, which typically occurs at sites of atherosclerosis with stenosis of the vessel lumen, where shear-stress values are considerably higher than in the normal circulation. For these reasons, and because the complex events that regulate platelet function are influenced by the flow of blood, the mechanisms that support platelet adhesion and aggregation under the constraints of elevated shear stress are of particular interest in the study of thrombus formation. Shear rate and shear stress have different effects on cellular adhesive interactions. The shear rate is directly related to flow velocity, including the velocity of cells in the fluid layer adjacent to the vessel wall, and limits the time of contact between membrane receptors and immobilized substrates on the vessel wall, thus the on-rate of the adhesive interaction. As a consequence, the efficiency of cell recruitment onto the surface decreases with increasing shear rate. Shear stress, in contrast, influences the lifetime of an adhesive bond once formed, thus the off-rate of the interaction, and the consequence is decreased efficiency caused by detachment of adherent cells with increasing shear stress. The point has been well documented for leukocyte adhesion.

Different pathways of platelet adhesion are variably affected by increasing shear force depending on the biomechanical properties of each receptor–ligand pair. Above a threshold shear rate of 500 to 800 sec⁻¹ in human blood and 2000 to 5000 sec⁻¹ in mouse blood, only the interaction between immobilized von Willebrand factor (VWF) A1 domain (VWF-A1) and membrane glycoprotein Ibα (GPIbα) has a sufficiently fast on-rate to initiate platelet adhesion. The higher threshold in the mouse is likely the consequence of a smaller platelet size. Thrombus development alters the hemodynamic conditions by restricting the lumen through which blood flows. To maintain the same volumetric flow rate (ie, the volume of blood transported per unit time) in spite of the restriction, the velocity of flow must increase, resulting in higher shear rate and stress. This explains why shear rates in excess of 20 000 to 40 000 sec⁻¹ develop at or just upstream of a severe stenosis in a human atherosclerotic coronary artery. For the same reason, the shear rate on the membrane of immobilized platelets exposed to flowing blood at the surface of an arterial thrombus is progressively higher as the protrusion into the vessel lumen increases. Thus, the fluid dynamic constraints that influence the adhesion of single platelets to the vessel wall affect also the recruitment of circulating platelets into a growing thrombus, as demonstrated by the required function of GPIbα and VWF-A1 to support platelet aggregation above a threshold shear rate (Figure 1). It is important to note that the threshold discussed here is not a minimum shear rate value to engage the function of immobilized VWF-A1, which can mediate platelet tethering even under venous slow flow conditions; rather, it is an upper limit for the function of most other platelet adhesive bonds in the absence of VWF.

Initial Platelet Adhesion to Thrombogenic Surfaces

Substrates and Receptors for Platelet Adhesion to the Vessel Wall

The hemostatic response to vascular injury is contingent on the nature of the lesion. Depending on the matrix proteins exposed to blood and the hemodynamic conditions, platelet adhesion requires the synergistic function of different platelet receptors, ultimately leading to platelet activation and aggregation. The extracellular matrix components that react with platelets include different types of collagen, VWF, fibronectin, and other adhesive proteins such as laminin, fibulin, and thrombospondin. Fibrinogen/fibrin are not synthesized by vascular wall cells but must be considered as potentially relevant thrombogenic substrates because they become immobilized onto extracellular matrix at sites of injury. One can assume that all tissue components capable of interacting with platelets can contribute to the initiation of thrombus formation when exposed to blood, even though only a few may have essential roles. Of note, experiments with purified proteins are useful to establish specific mechanisms of action but may not reflect functions...
within the complex supramolecular assembly of extracellular matrices. As a consequence, the relative contribution of several adhesive interactions to the process of platelet adhesion to vascular surfaces remains to be elucidated in detail. This is true in particular for insoluble proteins that exist only in the extracellular matrix, such as collagen, or for proteins that may exist in a soluble form in plasma, such as fibronectin, but differ in their structure and conformation when they assemble into complex matrices.24,25

Platelet Adhesion to VWF

Subendothelial Matrix VWF and Immobilized Plasma-Derived VWF

Among the substrates required for normal thrombus formation, VWF is unique for its role in initiating platelet adhesion and sustaining platelet aggregation under conditions of elevated shear stress.16,18 These functions are performed primarily through the tethering of GPIbα in the platelet membrane GPIb-IX-V receptor complex26 to the A1 domain of immobilized VWF exposed to flowing blood. As a constitutive component of the extracellular matrix of endothelial cells,27 in which it is associated with collagen type VI filaments,28–30 subendothelial VWF can directly support platelet adhesion.31–35 Nonetheless, hemostasis can be normal in the absence of endogenous endothelial VWF if plasma VWF is present (see the online data supplement). Consequently, the interaction of circulating VWF with exposed vascular and perivascular tissues is a key early event in thrombus formation.

The Transition From Soluble to Immobilized VWF

Plasma VWF can become immobilized onto subendothelial surfaces through the binding to extracellular matrix components and through self-association with other VWF multimers. The main substrate capable of binding VWF is collagen,36 particularly types I and III in deeper layers of the vessel wall and microfibrillar collagen type VI in the subendothelial matrix,30,37–39 with heparin, sulfatides, and fibrin providing additional interaction sites (see the online data supplement). Two of the 3 type A domains in VWF, A1 and A3, can mediate binding to collagens, and their respective roles may vary depending on the type of collagen involved and the fluid dynamic conditions.38,40 The ability to self-associate represents an additional mechanism for the transition from soluble to immobilized VWF, in which case circulating multimers interact in a reversible manner with matrix-bound and endogenous subendothelial VWF.41 This mechanism has been demonstrated by immobilizing a mutant VWF devoid of domain A1 (ΔA1-VWF), thus unable to promote platelet adhesion onto collagen and showing that GPIbα-mediated tethering was restored by the presence of soluble VWF in plasma (Figure 2). Very large VWF multimers locally released by stimulated endothelial cells42 may enhance the efficiency of the process, as these molecules form high-strength bonds with GPIbα.43 Self-association of VWF multimers can occur onto the platelet surface44 under conditions of hydrodynamic shear that favor the binding of soluble VWF.45 The self-association of VWF apparently involves multiple domains,46 and none has been identified as essential, including A1 and A3.41 In summary, the available evidence suggests that different types of injury may elicit distinct pathways for the local immobilization of soluble VWF. As a consequence, for example, VWF binding to collagen may not be essential to ensure normal hemostasis but may be a primary determinant of the pathological thrombogenic response caused by the rupture of collagen-rich atherosclerotic plaques. From an experimental point of view, it is difficult to recreate such a functional diversity using purified molecules, which can explain some of the inconsistencies found in the literature with respect to the mechanisms of VWF binding to vascular surfaces (see the online data supplement).

The Distinctive Functional Properties of Immobilized and Soluble VWF

Platelets have no measurable interaction with soluble VWF in the circulation but adhere promptly to exposed immobilized VWF. Such a tight regulation is necessary to prevent intra-vascular platelet aggregation and has led to the concept that surface-bound VWF must undergo a conformational change to make the interaction with GPIbα possible and initiate platelet adhesion. Indeed, VWF molecules may change shape depending on hemodynamic conditions, so that on 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.47 Such an “uncoiling” may expose the repeating functional sites present in multimeric VWF, allowing a more efficient support of
Membrane Receptors and the Mechanism of Platelet Tethering to VWF

Platelets have 2 main binding sites for VWF, $\text{GPIb}^\alpha$ and $\text{P-selectin}^\alpha$, which mediate the initial tethering of platelets to VWF-exposed surfaces. Platelet adhesion and accumulation depend on the interaction between VWF and $\text{GPIb}^\alpha$, which is a counter receptor for the GPIb-IX-V complex. The presence of additional structures signifying a serious lesion may be the required trigger for subsequent steps such as irreversible platelet adhesion and accumulation. The $\text{GPIb}^\alpha$-mediated
translocation velocity onto immobilized VWF is typically less than 2% of the free flow velocity of noninteracting platelets at the same distance from the luminal surface. This slow motion allows the establishment of additional bonds through receptors that belong mostly, but not necessarily, to the integrin superfamily. Such receptors, many of which depend on platelet activation to express function, typically have an intrinsically slower rate of bond formation but are capable of mediating stable interactions that lead to the definitive arrest of individual platelets and subsequent thrombus development. Notable in this regard is the role of the activated integrin $\alpha_{\text{IIb}}\beta_3$, which binds to the Arg-Gly-Asp sequence in VWF itself or to other adhesive substrates in a complex matrix, and of collagen and its receptors. Such a consideration highlights the true synergistic function of the VWF-collagen complex, which also leads to multiple activating signals coupled, in part, to the VWF-GPIb$\alpha$ interaction (see the online data supplement).

**An Integrated View of VWF-Mediated Platelet Adhesion and Aggregation**

The concept that the VWF-GPIb$\alpha$ interaction cannot support long-lasting adhesion must be modified in view of the recently demonstrated ability of nonactivated platelets to form aggregates that attach firmly to immobilized VWF under extremely high shear-stress conditions (Figure 3). Several unique features characterize this mechanism of platelet adhesion to extracellular surfaces and to one another, marking substantial differences with the process of single-platelet rolling. Perhaps the most relevant distinction is that GPIb$\alpha$-mediated, long-lasting adhesion and aggregation only occurs above a threshold shear rate of $\approx 10^4$ sec$^{-1}$, a feature that highlights its potential importance for pathological arterial thrombosis. A second key distinction is that platelet adhesion and aggregation at pathologically elevated shear rates depends on soluble as well as surface-bound VWF. Single-platelet adhesion and rolling, in contrast, requires only immobilized VWF, even though it is also enhanced by the presence of soluble VWF at the higher shear rates, likely as a result of VWF multimer self-association favored by shear-induced binding to platelets. A third and equally relevant feature is that GPIb$\alpha$-mediated and VWF-dependent firm platelet adhesion and aggregation occurs without any requirement for platelet activation and integrin function. Such a statement should not be taken to mean that activation has no influence on platelet thrombus formation at pathologically elevated shear rates, as it remains essential for the stability of aggregates and their attachment to the reactive surface. It is intuitive, however, that the ability of platelets to aggregate onto surfaces even before activation takes place greatly favors the establishment of growing thrombi in a high-shear-rate environment, in which elevated tensile stress limits the efficiency of adhesive bonds and rapid flow reduces the concentration of agonists required for activation. Under challenging hydrodynamic conditions, therefore, platelet interactions with adhesive surfaces and with one another appear to be synergistic. Of note, ADAMTS-13 can cleave circulating VWF multimers while they mediate activation-independent interplatelet cohesion under high shear stress, thus dispersing the aggregates.

**Platelet Adhesion to Collagen**

Different types of collagen (particularly I, III, and VI) are thought to be among the most active vessel wall components in the initiation of platelet adhesion and aggregation, but the relevance of their contribution to hemostasis and thrombosis relative to other extracellular matrix molecules remains to be defined. The conformation of different collagens may influence the mechanisms of interaction with platelets under flow and subsequent activation, which is a relevant factor in the interpretation of experimental results. Acid-insoluble fibers display a characteristic banded pattern attributable to the regular staggering of collagen monomers, whereas
pepsin-solubilized microfibrils lack this pattern and form spiral structures (Figure 4).74 Spiraled collagen formed by fibrils in helical assembly has been found in both normal and pathological vascular tissues, and may result from the degradation of fibers by matrix metalloproteinases, particularly matrix metalloproteinase 1.76 When immobilized, all of these substrates are thrombogenic but elicit platelet adhesion and aggregation through different mechanisms. This is best illustrated by the distinct consequences of blocking one of the platelet collagen receptors, the integrin α3β1, which impairs thrombus formation on spiral microfibrils but not on banded collagen fibers.74 In contrast, blocking the other receptor, GPVI, impairs thrombus formation on all types of collagen fibers.77,78 In fact, there appears to be a requirement for GPVI and α3β1 cooperation only when collagen fibers, and presumably their triple helical configuration, are not intact (Figure 4).74,77 As collagen is an insoluble matrix protein, preparations used in ex vivo experimental studies are often treated with proteases, such as pepsin, for solubilization, with the consequence of a functional behavior distinct from that of the native protein. For example, pepsin-treated acid-soluble collagen contains spiraled fibrils that can lead to overestimate the role of α3β1 in the interaction with platelets.

The Platelet Collagen Receptors

The platelet membrane is endowed with several collagen receptors, including the integrin α3β1,79–81 GPVI,82,83 GPIV (CD-36),84–86 and the 65-kDa protein (p65) reportedly specific for type I collagen.87 GPIV, which may be required for the interaction with collagen type V88 and is associated with Src kinases,89 is unlikely to make a major contribution to platelet activation because it is absent in approximately 5% of the Japanese population without hemostatic impairment. Likewise, the relevance of p65, which may be linked to nitric oxide generation,90 is unproven. Of the proposed receptors, only α3β1 and GPVI have a defined role in platelet–collagen interactions, but, in spite of extensive studies, their relative importance with respect to both adhesion and activation remains a topic for debate (Figure 4). Problems associated with the isolation of collagen from extracellular matrices, as discussed above, and possible but poorly understood differences between human ex vivo and mouse in vivo experimental systems explain the lingering uncertainties.36,71

The Integrin α3β1

The integrin α3β1 corresponds to the platelet membrane GPⅠa-ⅠⅧa, originally identified as the very-late-activation antigen-2 on T cells91 and class II extracellular matrix receptor on fibroblasts.80 The expression of α3β1 on normal platelets varies by as much as 1 order of magnitude,92 and may positively correlate to the rapidity of the initial phase of platelet adhesion to collagen.93 Several collagen sequences have been identified as targets for α3β1 binding,36 and the most relevant appears to be Gly-Phe-Hyp-Gly-Glu-Arg (where Hyp is hydroxyproline), which has been crystallized in complex with the receptor.94 Like other integrins, α3β1 requires activation and divalent cations to engage its ligands with high affinity, and although this may be a requisite for signaling (see the online data supplement), it may not be necessary for the initial contact. Thus, even in a low affinity state, α3β1 may be capable of mediating platelet adhesion to collagen preceding GPVI-induced activation,36 like nonactivated α3β1 mediates adhesion to immobilized fibrinogen.95 Abnormalities of ex vivo platelet interactions with collagen have been reported in α3-deficient mice, ranging from relatively mild, based on evaluating platelet adhesion under static conditions,96 to severe, based on evaluating platelet adhesion under flow conditions.97 In the latter study, with blood perfused over fibrillar collagen type I at shear rates between 400 and 1300 sec⁻¹, the isolated deficiency of α3β1 or GPVI-Fc receptor γ-chain (FcR-γ) complex caused an equivalent partial defect of platelet thrombus formation, whereas the combined deficiency caused complete inhibition.97 The conclusion was that the 2 receptors perform independent and critical roles in the interaction with collagen and operate synergistically for optimal function. Such a concept is in contrast to the alternative opinion that GPVI can mediate all aspects of collagen-dependent platelet adhesion and activation, even in the absence of α3β1.71 Although experimental conditions may tip the balance in favor of one of the other view, the 2 receptors are likely to contribute variably to platelet thrombus formation depending on the nature of the vascular lesion and the presence of yet unknown factors that can influence platelet and extracellular matrix components. In this regard, it is noteworthy that the congenital deficiency of either α3β181,98 or GPVI82 in humans results in a mild bleeding diathesis.
Experiments using in vivo mouse models have led to conflicting results on the role of α2β1 in platelet function. Some authors, who investigated the consequences of a mechanical injury in the mouse right common carotid artery, concluded that multiple integrin–ligand interactions synergize in promoting initial platelet adhesion (the study did not address thrombus formation) to the lesion, but without a significant contribution by α2β1. Rather, in agreement with previous ex vivo experiments using human blood and inhibitory antibodies,16 the results pointed to an important role of other platelet β1 integrins (α2β1 and α5β1; see below, under Platelet Adhesion to Fibronectin and under Platelet Adhesion to Laminin) in addition to the expected relevant contribution by α2β3.99 In contrast, other investigators who used a photochemical injury always in the mouse right common carotid artery, concluded that α2β1 plays a critical role in vascular thrombosis, although it is seemingly not involved in the formation of intravascular thrombi and pulmonary emboli following an injection of collagen.100 These authors observed that thrombus formation in the α2β1-deficient mice initiates normally but proceeds at a slower rate, taking about twice as long to occlude the vessel. The contrasting conclusions on the role of α2β1 in experimental arterial thrombosis cannot be reconciled at present with an objective explanation but likely reflect differences in the nature of the vascular lesion, including severity of the endothelial damage and exposure of extracellular matrix components. Thus, similar to ex vivo studies, different thrombogenic surfaces, whether because of the composition or relative proportion of their constituents, seem to elicit distinct pathways of platelet adhesion and activation in vivo.

**Glycoprotein VI**

This collagen receptor, a member of the immunoglobulin superfamily, is expressed uniquely on megakaryocytes and platelets. It is composed of 2 IgG domains, an O-linked carbohydrate-rich stalk-like region (analogous to the carbohydrate-rich region of GPIbα), a transmembrane, and an intracytoplasmic domain.101,102 Each GPVI molecule is non-covalently coupled to a disulfide-linked FcR-γ dimer,103,104 and coassembly of the 2 proteins is required for stable GPVI expression on the membrane (Figure 4).105 This receptor plays a major role in collagen-induced platelet activation (see the online data supplement). GPVI specifically recognizes the sequence Gly-Pro-Hyp, also known as collagen-related peptide.106 This peptide assumes a triple-helical conformation in solution and, when cross-linked for stability, is as efficient as fibrillar collagen.107 It appears that platelets activated by collagen through the GPVI/FcR-γ pathway play an important role in the process of neointimal hyperplasia after vascular injury.111 Finally, fibrillar collagen was found to enhance platelet-mediated thrombin generation through a pathway involving GPVI and supported by α2β1.112 This notwithstanding, other investigators who studied mice deficient in FcR-γ, thus unable to express GPVI, found that the extent of reduction in thrombogenic response caused by a defective GPVI pathway of interaction with collagen depends on the severity of the vascular injury. In fact, there was no major reduction in thrombus formation in a model of severe arterial injury and a 30% reduction in thrombus growth in a model of mild injury.113 Of note, inhibition of α-thrombin had a more profound antithrombotic effect in mice deficient in GPVI/FcR-γ than in normal controls, indicating that thrombin can greatly contribute to overcome the functional defect of platelets deficient in collagen-induced activation. In agreement with other experimental findings, these results confirm that the pathways of platelet adhesion/stimulation in injured arteries, likely to influence the severity of thrombotic com-
applications, are determined by the nature of the vascular lesion and of the substrates exposed to blood, including those leading to \( \alpha \)-thrombin generation.

Additional studies indicate that the contribution of GPVI to platelet thrombus formation depends on the nature of the surface to which blood is exposed and is influenced by still unknown factors. In ex vivo experiments, it has been found that the volume of platelet aggregates is normal when the blood of GPVI\(^{+/−} \) mice is perfused over extracellular matrix deposited by mouse skin fibroblasts.\(^{17} \) Such a finding, in marked contrast to the results observed on collagen surfaces,\(^{28} \) is in agreement with concept that the vessel wall may contain substrates onto which thrombus formation occurs independently of collagen-induced activation, at least as mediated by the known receptors. Moreover, the results of in vivo studies have shown that presently unknown variables influence the role of GPVI in hemostasis and thrombosis. Evaluation of the tail bleeding time\(^{78} \) and carotid artery occlusion following a ferric chloride–induced injury\(^{17} \) has concordantly indicated a dichotomous behavior of GPVI\(^{+/−} \) mice, because a group showed normal results and another a marked prolongation of the bleeding time and absent arterial thrombosis (Figure 5). Of note, the 2 phenotypes were inherited as distinct traits. The likely, but still unproven, explanation for these results is the existence of 1 or more modifier genes that, directly or indirectly, influence the thrombogenic potential of GPVI-deficient platelets. Ongoing studies appear to confirm this hypothesis. Thus, as a consequence of different conditions at a site of vascular injury, the relevance of the role played by GPVI in platelet adhesion and aggregation may vary. It is of interest to note that no exception is known to the required participation of GPIb\(\alpha \) in the formation of occluding arterial thrombi.\(^{17} \)

Platelet Adhesion to Fibronectin

Fibronectin is an essential adhesive substrate in many fundamental biological processes.\(^{24} \) Platelets possess 2 main receptors for this protein, \( \alpha_5\beta_1 \) and \( \alpha_{\text{IIb}}\beta_3 \),\(^{114} \) the latter of which requires activation to function.\(^{65} \) It has been known for some time that \( \alpha_5\beta_1 \) supports platelet adhesion to endothelial extracellular matrix,\(^{16} \) and more recently direct evidence has been presented that fibronectin may contribute to thrombus formation. A key observation has been that platelets from mice deficient in both VWF and fibrinogen can form thrombi at sites of vascular damage.\(^{115} \) Typically, there is no occlusion at the site of lesion, which can be explained by the required role of VWF in an area of increasing shear rate (see above, under Effects of Hydrodynamic Conditions on Platelet Adhesion and Aggregation), but platelet aggregates are unstable and embolize, causing downstream blockage of flow. In contrast, mice that lack \( \alpha_{\text{IIb}}\beta_3 \) are unable to form thrombi under the same conditions, suggesting that a \( \beta_3 \) ligand different from VWF and fibrinogen can contribute to platelet cohesion. A candidate for this function has been identified by demonstrating that mice with a conditional depletion of plasma fibronectin exhibit a delayed thrombus growth and decreased stability of platelet aggregates,\(^{116} \) suggesting that fibronectin can synergize with VWF and fibrinogen in supporting interplatelet cohesion through activated \( \alpha_{\text{IIb}}\beta_3 \). Soluble plasma fibronectin can assemble into fibrillar networks on the surface of fibroblasts, platelets\(^{117} \) and, possibly, other cells, thus providing a substrate that can contribute to the stable attachment of platelet aggregates.\(^{25} \) Fibronectin assembled into fibrillar structures may also support initial platelet adhesion, either directly or indirectly by association with collagen and/or VWF. In experimental conditions ex vivo, purified fibronectin is a rather inefficient substrate for platelet adhesion, and this may reflect the importance of supramolecular assembly with other ligands for the synergistic expression of adhesive function.

Platelet Adhesion to Fibrinogen/Fibrin

In addition to the role in platelet aggregation, which is discussed below, immobilized fibrinogen is a substrate for platelet arrest under flow conditions, selectively mediated by \( \alpha_{\text{IIb}}\beta_3 \) in the conformation present on nonactivated platelets.\(^{18} \) Fibrin, which is a cross-linked insoluble polymer of fibrinogen, retains the ability to support platelet adhesion, and in so
Platelet Adhesion to Thrombospondin

Thrombospondins are a family of adhesive proteins, of which thrombospondin-1 is contained in platelet α-granules. After activation-induced secretion, thrombospondin-1 binds to the platelet membrane and mediates adhesion. The significance of thrombospondin-1 in platelet thrombus formation is still unclear, although it is intriguing to note that this glycoprotein is abundant in atherosclerotic plaques. In experimental models, immobilized thrombospondin-1 has been shown to support stable platelet attachment up to a shear rate of 4000 sec$^{-1}$, a value typically associated with the participation of VWF in the process. Adhesion to thrombospondin-1, however, has been proven to be independent of VWF albeit mediated by GPIb with a minor contribution by GPIV (CD36) only when platelets were activated. The suggestion that thrombospondin-1 may mediate platelet adhesion under arterial flow conditions in lieu of VWF remains to be verified.

Thrombospondin-2 is a relevant constituent of extracellular matrices but is not present in platelets; nonetheless, its absence has been associated with a congenital hemostatic defect in mice. The extent to which this abnormality is directly associated with impaired adhesion is not clear, and alternative explanations are possible as megakaryocytes from mice deficient in thrombospondin-2 produce platelets that are not fully activated by agonist stimulation. This may exemplify a mechanism for an indirect effect on platelet thrombus formation caused by an adhesive molecule required during megakaryocyte maturation and/or thrombocytopenia.

Platelet Adhesion to Laminin

Different forms of laminin are highly expressed in the subendothelial extracellular matrix and, when exposed to blood, are potential substrates for platelet adhesion at sites of vascular injury. The subendothelial forms are laminins 8 (αβγ) and 10 (αβγ). Platelets also contain and secrete on activation laminins 8 and 10, as well as laminin 11 (αβγ). It has been reported that platelets can adhere to laminin but are not directly activated following the interaction. Other investigators, however, found that human laminin stimulates formation of filopodia and lamellipodia in human and mouse platelets through an adhesion/activation pathway that involves the integrin αβ, a well-known laminin receptor, and GPVI. Adhesion to laminin appears to depend on αβ with no required contribution by GPVI, whereas the latter is necessary for the formation of lamellipodia but not filopodia. These findings delineate a synergistic role for αβ and GPVI as laminin receptors, coupled to distinct activation pathways convergent on the function of the Syk tyrosine kinase. The relative contribution of adhesion to laminin in platelet thrombus formation remains to be delineated.

Platelet Adhesion and Thrombus Propagation: Platelet Aggregation

Soluble Adhesive Proteins and Their Platelet Receptors in Thrombus Propagation

Following the initial events that lead to adhesion and activation at sites of vascular injury, platelets bind soluble adhesive proteins and form a reactive surface for continuing platelet deposition. Subsequent thrombus growth is therefore strictly dependent on the formation of interplatelet bonds. Aggregation has typically been studied with agonist-stimulated platelets in suspension, without surface interactions, and under stirring conditions that create a turbulent flow with low shear rates. These studies have led to the assumption that fibrinogen binding to αβ is the only interaction relevant for platelet aggregation. This view has changed with the use of the cone-and-plate viscometer, where platelets in suspension can be exposed to defined levels of shear stress in a laminar flow field. High-shear stress by itself, without addition of exogenous agonists, can lead to platelet aggregation that is mediated by VWF and its 2 membrane receptors. Indeed, at elevated shear rates, typically in excess of 5000 sec$^{-1}$, VWF binds specifically to platelets in a process that involves sequentially GPIbα and αβ, GPIbα and αβ, GPIbα and αβ, have distinct but complementary roles in platelet aggregation depending on fluid dynamic conditions. The study of platelet aggregation in suspension fails to reproduce the correct spatial and temporal sequence of events that occur during thrombus growth in vivo, but there are alternative experimental approaches to measure in real time the 3D growth of platelet aggregates under defined hemodynamic conditions (Figure 1).

Ex vivo perfusion experiments indicate a synergistic role for fibrinogen and VWF in supporting platelet aggregation onto collagen fibrils. Without fibrinogen, thrombi mediated by VWF grow rapidly at high shear rate but are unstable; with both VWF and fibrinogen, thrombi grow more slowly but are stable. In mice selectively deficient in VWF, platelet adhesion at sites of experimental vascular lesion is delayed, but stable platelet aggregates eventually develop even though arterial occlusion is often impaired. In mice selectively deficient in fibrinogen, in contrast, platelet thrombi develop rapidly but detach from the surface and embolize causing vascular occlusion downstream of the lesion. Thus, in agreement with the ex vivo perfusion studies outline above, both VWF and fibrinogen are required to ensure stable aggregates. These results provide a plausible explanation for the altered hemostatic properties of platelets from patients with isolated congenital deficiency of either fibrinogen or VWF. Because neither protein by itself can sustain the development of stable thrombi under pathophysiologically relevant flow conditions, hemostasis cannot be normal unless both are present and functional.

Platelet Adhesion and Thrombus Stabilization

After platelet activation and aggregation have occurred in response to a vascular lesion, distinct adhesive mechanisms
become operative that consolidate the stability of the forming thrombus. The identification and characterization of the molecules involved in these processes have become the focus of increasing attention in recent years because of their seemingly important influence on hemostatic efficiency and, in pathological conditions, arterial thrombotic complications (see the online data supplement).

Conclusions

The last few years have witnessed major advances in our understanding of the mechanisms that support platelet thrombus formation in flowing blood. The results of ex vivo flow experiments and intravital microscopy studies have shed new light on the processes underlying hemostasis and thrombosis. Animal models with targeted gene deletions or mutations have greatly contributed to these advances and will certainly provide more insights in the future. Significant progress in genomics and proteomics has generated information relevant to elucidating the integrated processes that link platelet–substrate interactions and signaling pathways to thrombus growth and stability. Finally, the advent of improved drug development technologies is likely to permit the translation of this fundamental knowledge into more efficient therapeutic approaches to prevent excessive bleeding and thrombosis.

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Disclosures

None.

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Blood flow velocity, shear rate and shear stress

The velocity of flowing blood in vessels decreases progressively from the center to the wall and the relative motion between adjacent layers of fluid creates a shearing effect that, inversely to velocity, is greatest at the wall and decreases towards the center (supplementary Figure 1). Shear rate is the difference in flow velocity as a function of distance from the wall and is expressed in cm/s per cm or the equivalent inverse second (s⁻¹). Shear stress is the resulting force per unit area and is expressed in pascal (Pa), equivalent to one newton/m² (N/m²), or in dyne/cm² (1 Pa = 10 dyne/cm²). Shear rate is directly proportional to shear stress and inversely proportional to dynamic fluid viscosity, which is expressed in Pa·s or dyne/cm²·s, the latter equivalent to poise (P; 1 P = 0.1 Pa·s). In blood, the viscosity of which is ~ 3-4 millipascal (mPa)·s or 3-4 centipoise (cP), a shear rate of 25 s⁻¹ causes a shear stress of ~ 1 dyne/cm² (0.1 Pa). The highest wall shear rate in the normal human circulation, estimated to vary between 500 and 5,000 s⁻¹ (corresponding to a shear stress of ~ 20-200 dyne/cm²), occurs in small arterioles of 10-50 µm diameter.¹

Function of endogenous matrix VWF and immobilized plasma-derived VWF

The relative contribution of these two forms of VWF to platelet adhesion was elucidated by studies on the transplant of normal pig bone marrow into pigs affected by severe von Willebrand disease, characterized by a complete deficiency of VWF.² As expected, the procedure could not correct the endothelial cell defect, thus there continued to be no VWF secretion into blood or subendothelial matrix while the VWF content in platelet α-granules was normalized. This was not sufficient to restore a normal hemostasis, although some transplanted animals showed a partial amelioration of the prolonged bleeding time. The latter finding is in agreement with the concept that α-granule-derived VWF, released after activation, can contribute to thrombus growth³ but not to the initial platelet adhesion occurring before activation.⁴ In contrast, hemostasis was completely normal in the transplanted pigs after infusion of normal plasma VWF.² The prolonged bleeding time of pigs with severe von Willebrand disease was also normalized by transfusion of VWF concentrates alone,⁵ even though their platelet VWF
remained absent, however a considerably higher dose was required than in transplanted pigs with normal platelet VWF. These results prove that plasma VWF can initiate platelet adhesion after binding to the vessel wall in the absence of matrix VWF, and supports subsequent thrombus growth with the contribution of platelet-released VWF that, however, is not essential.

**The interaction of VWF with extracellular matrix components**

The VWF A1 domain (VWF-A1), comprising residues 497-716 of the mature subunit (add 763 to obtain the corresponding residue number in pre-pro-VWF), was initially shown to interact with collagen types I and III, but its main role may be binding to collagen type VI. The latter contains VWF type A domains in its non-collagenous regions that may become engaged in homotypic interactions with VWF-A1. The VWF A3 domain (VWF-A3), comprising residues 910-1111, also binds to collagen types I and III, and is apparently necessary and sufficient to mediate the interaction with fibrillar collagens. The VWF-A3 residues involved in collagen binding have been mapped, and a high affinity binding site for VWF has been identified in collagen type III. Fluid dynamic conditions and mechanical forces may modulate the VWF-collagen interaction, and the interplay of domains A1 and A3 may be necessary to support VWF immobilization onto extracellular matrices containing various collagen types. Of note, VWF multimer size directly correlates with the affinity for collagen binding.

Contrasting the information on the role played by VWF-A3 in the interaction with fibrillar collagens *in vitro*, supported by the demonstrated anti-thrombotic activity of a function-blocking anti-VWF A3 antibody, stands the evidence that mutations preventing collagen binding (such as Ser968Thr) are compatible with normal hemostasis *in vivo*. It appears, therefore, that different VWF domains can ensure the interaction with collagen in extracellular matrices, possibly depending on the nature of a lesion, and/or extracellular substrates other than collagen can support VWF in initiating platelet adhesion. In favor of the first hypothesis, evidence has been obtained that collagen type VI in the endothelial cell and fibroblast matrix is the main VWF binding site through an interaction mediated by the A1 domain. Moreover, particularly under flow conditions, VWF-A1 can substitute for VWF-A3 in supporting binding to collagen, in which the sites interacting with the two domains appear to be overlapping. Concerning the second hypothesis, it is known that VWF can interact with extracellular matrix components independently of collagen. The A1 domain contains a heparin-binding site that has been localized to the sequence Tyr565-Ala587. A second, lower affinity heparin-binding sequence
exists within the first 272 residues of the mature VWF subunit. These heparin-binding sites may reflect the ability to interact with matrix proteoglycans that contain sulfated carbohydrates. For example, the small proteoglycan decorin, which associates with several matrix components and contributes to matrix assembly, has been reported to bind VWF in an interaction mediated by the glycosaminoglycan chain and regulated by the degree of sulfation. In addition, VWF binds to sulfated glycosphingolipids (sulfatides) that are present on cellular membranes and may serve an accessory role in promoting localization on wounded tissues. The binding site for sulfatides has been localized within residues 512-673 of the A1 domain, possibly with a more direct involvement of residues 569-584 and/or 626-646. Sulfatides can inhibit platelet adhesion to VWF mediated by GP Ibα, suggesting an overlap of interacting sites. Because VWF is multimeric, sulfatides may contribute to its binding to surfaces and still allow platelet adhesion to different A1 domains in the same immobilized polymer. Similar considerations apply to heparin and its binding site in the A1 domain. Another pathway to VWF immobilization involves the interaction with components of a forming clot. Thus, the cross-linking of VWF to the α-chain of fibrin can contribute to platelet deposition onto altered vascular surfaces, and this may become a relevant adhesion mechanism in areas where acute or chronic inflammation causes fibrin deposition.

ADAMTS-13 and the regulation of VWF-mediated platelet adhesion and aggregation

VWF in plasma or released by altered endothelial cells and/or activated platelets at sites of vascular injury has a potent prothrombotic effect by promoting both platelet adhesion and aggregation, particularly under high shear stress conditions. The largest multimers of VWF, with the greatest prothrombotic function, are present inside cellular storage granules but are not normally found in the circulation. The reason for this is the efficient processing of all secreted VWF by the metalloprotease, ADAMTS-13, which cleaves one single peptide bond in the VWF subunit and in so doing reduces multimer size. Absence of ADAMTS-13 results in a thrombotic microangiopathy, suggesting that the physiologic function of the protease is to limit the activity of the most active VWF multimers to the sites where they are released from cells. Recently, the results of ex vivo perfusion experiments have added to this concept by showing that ADAMTS-13 can further cleave circulating VWF multimers while they mediate activation-independent interplatelet cohesion induced by elevated shear stress, resulting in a time-dependent dispersion of the aggregates. In contrast, the protease appeared to have no
effect, at least under the *ex vivo* conditions studied, when thrombus formation was induced by blood exposure to a collagen surface.

The latter finding stands in apparent contradiction with the results of *in vivo* studies in mouse thrombosis models, which have shown the ability of recombinant ADAMTS-13 to dissolve experimentally-induced thrombi in the arteriolar circulation leading to the conclusion that the protease could be used as an antithrombotic agent. While the effect of ADAMTS-13 on microarteriolar thrombi is in agreement with the phenotype caused by its deficiency, i.e. microarteriolar thrombosis, the situation may be different in larger arteries. In this case, the anti-thrombotic activity of ADAMTS-13 may depend on the extent to which adhesive molecules such as fibrinogen and fibronectin, rather than VWF, contribute to platelet aggregation. Thus, the anti-thrombotic activity of ADAMTS-13 may be selective for platelet aggregation under high shear stress conditions in which VWF is important for platelet cohesion. During hemostasis, ADAMTS-13 activity may be needed to avoid the propagation of platelet aggregates beyond the limits of a vascular wound, which typically involves the microarteriolar circulation with rapidly flowing blood. It remains to be demonstrated whether ADAMTS-13 may limit the potential role of VWF in mediating the occlusion of stenotic arteries where pathologically elevated shear rates develop. In this regard, it is intriguing to observe that a recent study found a positive correlation between ADAMTS-13 levels and the risk of myocardial infarction in men, a finding that is in apparent contrast with the suggestion that ADAMTS-13 may act as an anti-thrombotic agent. The mechanism through which increased ADAMTS-13 levels and/or activity might constitute a risk for arterial thrombosis remains to be understood.

**Activation of adherent platelets**

Outside-in signaling required for platelet activation is linked to the engagement of the primary adhesion receptors for both VWF (GP Ibα) and collagen (GP VI and the integrin α2β1). Other integrins, particularly αIIbβ3, also generate outside-in signals upon ligand binding, and their functions in cell activation have been reviewed elsewhere. Thrombogenic substrates interact with the first layer of adherent platelets, but thrombus growth depends on the progressive propagation of activating signals to the newly recruited platelets that are in contact with each other but not with the surface supporting the initial adhesion. These propagating signals, which are necessary for the binding of soluble adhesive proteins and aggregation, originate from the co-stimulation of platelet receptors by soluble agonists, such as ADP, α-thrombin and thromboxane...
A2, that become available at sites of vascular injury following the initial adhesion of platelets. Particularly in the arterial circulation, efficient platelet activation by soluble agonists is likely to occur only in the immediate vicinity of platelet-platelet adhesion contacts, where high local concentrations can counter the rapid clearance caused by blood flow.

**Activation through GP Ibα.** VWF binding to GP Ibα supports platelet tethering to thrombogenic substrates, but the extent to which it contributes to subsequent activation is less well defined. In fact, VWF in the vessel wall is associated with other potent platelet agonists, such as collagen, and it is difficult to ascertain whether VWF-induced GP Ibα signaling adds significantly to the overall activation response following initial adhesion. One problem is that, to date, it has not been possible to separate the adhesive and signaling functions of the receptor as the latter is dependent on the former, which in turn is essential for the initial tethering that precedes any other response when shear rates are elevated. It seems reasonable to assume that, if GP Ibα signaling has a role in platelet thrombus formation, this may be limited to conditions of high shear stress on surfaces with high density of immobilized VWF. Signaling may depend on the cross-linking of GP Ibα by multivalent VWF and involve FcR-γ, a signaling receptor subunit that contains an immuno-receptor tyrosine-based activation motif (ITAM) and is associated with the GP Ib-IX-V complex. On cross-linking, FcR-γ is phosphorylated within the cytoplasmic ITAM sequence by Src family tyrosine kinases, thereby permitting the binding and autophosphorylation of the tyrosine kinase Syk. Activated Syk initiates signaling that leads to activation of phospholipase C-γ2 (PLC-γ2) and subsequent formation of inositol triphosphate (leading to Ca2+ mobilization) and diacylglycerol (leading to activation of protein kinase C). It has been shown, however, that mouse platelets lacking FcR-γ undergo only slightly lower than normal Ca2+ oscillations after adhering to dimeric VWF-A1, suggesting that FcR-γ may contribute to but is not essential for activation induced by GP Ibα. Signals elicited by platelet adhesion to VWF-A1 under flow can activate αIIbβ3 independently of other receptors, with the contribution of the adaptor molecule, ADAP, for optimal efficiency.

The cytoplasmic tail of GP Ibα is associated with actin binding protein and 14-3-3ζ, which are linked to several intracellular signaling molecules including phosphatidylinositol (PI) 3-kinase, focal adhesion kinase, Src-related tyrosine kinases (Syk, Src, Fyn Lyn and Yes), GTPase-activating protein, and tyrosine phosphatases (PTP-1B and SHPTP10). Thus, 14-3-3ζ may be a relevant modulator of signaling through GP Ib-IX-V. Moreover, a membrane-permeable
inhibitor of 14-3-3ζ association with GP Ibα has been shown to inhibit VWF-mediated platelet adhesion under flow conditions, indicating a potential role of 14-3-3ζ in regulating the ligand binding function of the GP Ib-IX-V complex. This effect mimics the consequences of mutating the 14-3-3ζ binding site in the C-terminal domain of GP Ibα. Dissociation of 14-3-3ζ from the GP Ib-IX-V complex is seen following platelet activation, suggesting a potential pathway for the down regulation of the VWF-GP Ibα interaction after an initial adhesion.

The engagement of GP Ibα by surface-bound VWF elicits different kinds of transient cytoplasmic Ca\(^{2+}\) elevations (supplementary Figure 2).\(^{55,73}\) The first, designated a type α/β peak, is linked to the initial GP Ibα-mediated platelet tethering to VWF-A1 and precedes stable adhesion. These intracellular elevations are independent of extracellular calcium, indicating that signals downstream of GP Ibα may directly regulate Ca\(^{2+}\) release from internal stores. Type α/β Ca\(^{2+}\) peaks are partially inhibited by direct blockage of the P2Y\(_1\) ADP receptor but not by apyrase, which degrades ADP enzymatically.\(^{74}\) This indicates that GP Ibα binding to VWF-A1 induces release of ADP that, in turn, acts rapidly (to the extent that it cannot be blocked by apyrase) through P2Y\(_1\) to cause additional Ca\(^{2+}\) release from intracellular stores, reinforcing GP Ibα-mediated α/β peaks.\(^{74}\) Platelets that have established firm adhesion to VWF through αIIbβ3 exhibit sustained Ca\(^{2+}\) oscillations, designated γ peaks, which precede aggregation. Type γ peaks involve a transmembrane Ca\(^{2+}\) flux operating downstream of αIIbβ3 signaling. Thus, the initial GP Ibα interaction with the VWF A1 domain leads to a first level of αIIbβ3 activation sufficient for stable platelet adhesion to immobilized VWF but not for binding soluble VWF or fibrinogen, which is the step required for aggregation. Progression to thrombus formation requires further αIIbβ3 activation that is contingent upon signal amplification associated with type γ [Ca\(^{2+}\)], peaks (supplementary Figure 3). PI3-kinase inhibition or ADP removal by apyrase block γ peaks and prevent platelet aggregation.\(^{55,75,76}\) This ADP-dependent reinforcement of platelet activation is mediated by the P2Y\(_{12}\) receptor.\(^{74}\) Only a small proportion of platelets translocating on immobilized VWF exhibit type γ Ca\(^{2+}\) peaks,\(^{55,74}\) suggesting that a synergy between this and other activation pathways is necessary for platelet activation following adhesion. Such a functional arrangement may be important in the modulation of initial thrombus formation.

GP Ibα is the major α-thrombin binding site on the platelet surface, accounting for as much as 90% of the total protease bound.\(^{77}\) The functional significance of this interaction is still debated, in spite of the fact that α-thrombin binding to GP Ibα induces platelet adhesion, spreading, dense
granule secretion, and aggregation. Among the suggested possibilities is a role of GP Ib\(\alpha\) in positioning \(\alpha\)-thrombin for optimal cleavage of the protease-activated receptors, PAR-1 and PAR-4, which would then be responsible for the signaling. Crystallographic studies have shown the potential for GP Ib\(\alpha\) to bind two separate thrombin molecules by interacting with both exosite I and II of the protease. Such a mode of interaction may promote receptor cross-linking and aid in platelet activation. GP V, which is closely associated with GP Ib-IX, is a substrate for proteolysis by \(\alpha\)-thrombin, and the possible functional significance of its cleavage has emerged from the study of genetically modified mice. The platelets of GP V\(^{-/-}\) mice - which are normal in size, express normal amounts of the modified GP Ib-IX complex, and display normal function with regard to VWF binding - exhibit an enhanced response to thrombin. The finding has been explained by the observation that GP V\(^{-/-}\) platelets, but not those of wild type mice, are activated by proteolytically inactive thrombin, which cannot cleave the PARs but binds to GP Ib-IX with equal affinity as \(\alpha\)-thrombin. In contrast, proteolytically inactive thrombin could stimulate wild type platelets only if they had been pre-treated with a low dose of \(\alpha\)-thrombin, sufficient to cleave GP V but not to activate through the PARs. The conclusion based on these results is that \(\alpha\)-thrombin can initiate platelet signaling upon interacting with GP Ib\(\alpha\) independently of proteolytic activity, but proteolysis is required to release the inhibitory effect that GP V in the GP Ib-IX-V receptor complex displays on this signaling pathway.

Platelet activating signals transmitted through GP Ib\(\alpha\) appear to involve cGMP-dependent protein kinase (PKG), a conclusion proposed by a group of investigators who found that PKG knockout mice exhibit impaired platelet responses to VWF or low doses of \(\alpha\)-thrombin associated with a prolongation of the bleeding time. Moreover, the aggregation of human platelets induced by VWF or low concentrations of \(\alpha\)-thrombin was diminished in the presence of PKG inhibitors and increased by cGMP or by the cGMP-enhancing agent, sildenafil. Evidence has been presented that this activation pathway involves p38, a mitogen-activated protein kinase (MAPK), which in turn mediates the PKG-dependent phosphorylation of extracellular stimuli-responsive kinase (ERK). These findings are still considered controversial by some investigators, as they are in contrast to the currently accepted paradigm that PKG inhibits platelet activation. A potential confounding factor is that cGMP-dependent platelet responses following GP Ib\(\alpha\) ligation appear to be biphasic, with an initial transient stimulation that promotes platelet aggregation rapidly followed by an inhibitory state that limits the size of
The concept of an initial platelet stimulation mediated by PKG and induced by cGMP elevation has been expanded to include signaling pathways involving Gq- and Gi-coupled agonist receptors. A related and equally unexpected finding has been that nitric oxide (NO), also generally considered a potent inhibitor of platelet activation, appears to act as a key messenger in PKG-dependent platelet activation.

**Activation through the collagen receptors.** The two major collagen receptors on the platelet surface, α2β1 and GP VI, have both been implicated in generating signals that lead to platelet activation, although some investigators have concluded that GP VI plays the central if not exclusive role in the interaction with collagen. Like other integrins, α2β1 requires the transition from an “inactive” to an “active” state in order to bind to its ligand with high affinity, thus its function may be viewed as downstream of initial activating signals that proceed from the GP VI-collagen interaction or other stimuli. In this regard, evidence has been presented that activation of another integrin, αIIbβ3, is not only sufficient but also absolutely required for α2β1 activation on platelets. In any case, several experimental results indicate that α2β1 is a signaling receptor and contributes to collagen-mediated platelet responses. For example, antibodies and other reagents directed against α2β1 inhibit platelet activation and aggregation induced by collagen, but the effect can be overcome with increasing concentrations of the agonist. Similarly, the snake venom jararhagin, which cleaves the α2 subunit inactivating the integrin, inhibits collagen-dependent phosphorylation of Syk and other proteins at low but not high agonist concentrations. These findings support the conclusion that α2β1 may contribute to but is not essential for collagen-dependent platelet functions.

GP VI plays a major role in collagen-induced platelet activation, as illustrated by the lack of functional responses in human platelets that lack this receptor and confirmed by targeted ablation of the corresponding gene in the mouse. Each GP VI molecule is noncovalently coupled to a disulfide-linked FcR-γ dimer, in which each subunit contains an ITAM activation motif. Collagen induces platelet activation through the tyrosine phosphorylation of PLC-γ2, an event that is abolished in GP VI-deficient patients. The similarities in signaling between GP VI and the receptor for immune complexes, FcγRIIA, led to the demonstration that both stimulate Syk phosphorylation. Mice lacking either FcR-γ or Syk fail to aggregate or secrete dense granules in response to collagen. Major tools for studying GP VI receptor
signaling have been found in the snake venom toxin, convulxin\textsuperscript{115,116} and in collagen related peptides (CRP),\textsuperscript{117} both potent platelet agonists selective for GP VI.\textsuperscript{118,119}

**Platelet adhesion and thrombus stabilization**

Platelet aggregation in itself is not an irreversible process, which is an important characteristic allowing for the intervention of regulatory mechanisms capable of preventing an excessive, and potentially dangerous, propagation of thrombus growth. It is apparent that newly recruited platelets at the growing edge of a thrombus transmit feedback signals to aggregated platelets in deeper layers that are necessary to prevent disaggregation. The propagation of these signals throughout the thrombus is visible as recurrent cycles of intracytoplasmic Ca\textsuperscript{2+} elevations (supplementary Figure 2).\textsuperscript{120} Both ADP receptors, P2Y\textsubscript{1} and P2Y\textsubscript{12}, are involved in the process, which can be demonstrated experimentally by two-stage perfusion experiments in which normal blood is first exposed to a thrombogenic surface, such as collagen type I fibers, followed by blood containing specific inhibitors. In such a manner, it has been shown that a thrombus can begin to disperse even after several minutes of growth if ADP activation pathways are blocked, intracytoplasmic Ca\textsuperscript{2+} elevations are prevented or inhibitors of \(\alpha\mathrm{IIb}\beta3\) function are added.\textsuperscript{121} These findings indicate that platelet activation must be sustained in order to keep adhesive ligands bound to \(\alpha\mathrm{IIb}\beta3\) in a stable manner. It should be considered, however, that \textit{ex vivo} experiments might overemphasize platelet-dependent mechanisms of this kind because anticoagulants used to handle blood outside of vessels markedly suppress \(\alpha\)-thrombin generation and/or activity and reduce the deposition of fibrin that normally contributes to thrombus stability. Nevertheless, sustained platelet activation may be critical for maintaining the integrity of platelet aggregates, particularly in rapid arterial flow conditions and in the early stages of thrombus development.

Adhesive molecules bound to or expressed onto the membrane of activated platelet are crucial for the rapid growth of stable thrombi. One example of this function is the role that fibrinogen (likely after conversion to fibrin) plays in anchoring aggregated platelets to the site of vascular injury, thus preventing downstream embolization under the effects of flow.\textsuperscript{122} Several specific interactions have been identified that directly enhance the cohesion between platelets initially established through well-known adhesive ligands (VWF, fibrinogen, fibronectin) and receptors (GP Ib\(\alpha\) and \(\alpha\mathrm{IIb}\beta3\)). Two main mechanisms can be envisioned to operate in this regard, one resulting in the generation of signals that enhance or sustain platelet activation, and the other
based on homophilic or heterophilic interactions that add adhesive strength to platelet aggregates. The hormone leptin is an example of the first type of action, as it may reinforce the response of platelets to weak agonist stimulation thus contributing to the stability of platelet aggregation.\textsuperscript{123} The effect of leptin on thrombus growth has been confirmed with \textit{in vivo} experiments in obese mice, who lack leptin and exhibit delayed thrombotic occlusion with frequent embolization in injured arteries.\textsuperscript{124} Moreover, inhibition of endogenous leptin has been found to protect mice from arterial and venous thrombosis.\textsuperscript{125} In human obesity, therefore, in which leptin resistance leads to high circulating levels of the hormone,\textsuperscript{125} the effect of leptin on platelet activation may represent an additional prothrombotic factor.

Cellular pathways involved in inflammatory and immune responses also appear to have prothrombotic effects. CD40 ligand (CD40L), a member of the tumor necrosis factor family of ligands, has been detected on the surface of activated platelets where it interacts with \( \alpha{\text{IIb}}\beta{\text{3}} \) through an integrin recognition sequence (KGD). In the absence of CD40L, platelet aggregates formed in experimental \textit{in vivo} models of thrombosis are unstable, confirming the ability of this protein to support platelet-platelet cohesion under high shear stress conditions via an \( \alpha{\text{IIb}}\beta{\text{3}} \)-dependent mechanism.\textsuperscript{126}

Growth arrest-specific gene 6 product (Gas6) is a vitamin K-dependent protein homologous to the anticoagulant cofactor, protein S, whose absence or inactivation was reported to protect mice from fatal thromboembolism.\textsuperscript{127} Gas6 is present in plasma and in the \( \alpha \)-granules of mouse platelets, from which it is secreted upon activation, but may be absent in human platelets.\textsuperscript{128} Because of the latter observation, the relevance of Gas6 in human platelet physiology is being reevaluated as it may depend on the function of the plasma-derived protein only. On the other hand, all three known Gas6 receptors (Axl, Sky, and Mer) are present on mouse and human platelets, and their inactivation or stimulation results in the expected inhibition or enhancement of agonist-induced platelet activation responses.\textsuperscript{129} Thus, Gas6-dependent pathways may represent an amplification mechanism for platelet aggregate stability.

Eph kinases and ephrins are families of membrane bound molecules that interact with each other, exhibiting a major role in neuronal organization and as early markers for vascular commitment to arterial or venous development. Platelet-expressed Eph/ephrins include EphA4 and ephrinB1, which appear to contribute to “outside-in” signals originating from ligand-occupied \( \alpha{\text{IIb}}\beta{\text{3}} \). Inhibition of Eph/ephrin interactions results in a diminution of the volume of
thrombi formed on a collagen type I surface, presumably as a consequence of decreased aggregate cohesion.\textsuperscript{130}

A strategy has been proposed to identify molecules that are phosphorylated following the induction of platelet aggregation, as this could indicate a specific role in controlling thrombus stability.\textsuperscript{131} Two studies have recently verified the validity of this hypothesis. In one, a novel membrane protein has been identified, platelet endothelial aggregation receptor 1 (PEAR1), that appears to signal secondary to \(\alpha IIb \beta 3\)-mediated platelet-platelet contacts.\textsuperscript{131} Future studies will indicate the pathophysiological significance of this finding. In another study, evidence has been presented for the prothrombotic function of CD84 and SLAM (signaling lymphocyte activation molecule), both members of the same family of homophilic adhesion receptors. Immobilized CD84 can promote platelet microaggregation, and SLAM\(^{-/-}\) mice exhibit decreased agonist-induced platelet aggregation, a defect confirmed by intravital studies of thrombus formation in injured vessels.\textsuperscript{132} Because numerous cell signaling and adhesive pathways potentially involved in controlling platelet thrombus integrity have already been identified, an obvious challenge for the future will be the integration of this analytical knowledge into a comprehensive representation of the mechanisms of platelet response to vascular injury.

**Platelet adhesion in inflammation, atherogenesis and host defense mechanisms**

As sentinels of vascular integrity, platelets may be responsible for orchestrating the localization of other blood cells at sites where reactive processes are taking place. Such a function can be viewed as a generalization of the mechanism of platelet aggregation, whereby the platelets that react to an initial injury by adhering to the vessel wall become the surface onto which more platelets accumulate. In the case of inflammatory or immune-mediated processes, platelets may attach to endothelial cells perturbed by a variety of factors, including bacteria, viruses, their products as well as chemical mediators of cell responses induced by the presence of pathogenic stimuli. Platelets, by virtue of intrinsic properties including their number in blood, may be the first to detected alterations on the endothelial cell membrane, expressed through the presence of released molecules such as P-selectin\textsuperscript{133} and VWF.\textsuperscript{134} Once interacting with the endothelial surface, or subendothelial structures in case of a more severe damage, platelets may produce a varied response modulated by the nature and extent of the vessel wall perturbation, and thus selectively attract different types of leukocytes that, in turn, mediate specific defense mechanisms. In this regard, for example, it has been shown that granulocytes roll and adhere
onto activated platelets, and eventually transmigrate through the vessel wall, via a dual process mediated by P-selectin and β2 integrin ligands. Platelet and leukocyte adhesive interactions, therefore, may elicit reciprocal influences in the course of inflammatory, atherogenic and thrombotic processes. Lymphocyte-platelet interactions have been shown to be directly relevant to the development of immune-mediated mechanisms of viral clearance as well as organ damage in mouse models of viral hepatitis. In this case, in the absence of platelets or when platelets cannot become activated in response to stimuli, CD8+ antigen-specific T-lymphocytes fail to accumulate in the liver of mice transgenically expressing a hepatitis B antigen or infected by a specific adenovirus strain. Thus, both the removal of viruses and the severity of liver disease caused by lymphocyte-mediated killing and cytotoxicity are greatly reduced when platelet function is deficient. Such a finding establishes the concept that platelet adhesive properties are required for the targeting of lymphocytes to sites where their antigen-induced activities are required. The molecular mechanisms that mediate this platelet function are not yet understood in detail. They may involve the attachment of platelets to stimulated endothelial cells followed by lymphocyte adhesion to an activation-induced membrane-bound ligand; or, alternatively, platelets may interact with lymphocytes in the circulation and endow them with enhanced adhesive properties that allow localization at specific vascular sites. The latter process, for example, has been shown to support the homing of lymphocytes in high endothelial venules of lymphoid organs, a process related to but distinct from the peripheral targeting of antigen-specific CD8+ cytotoxic T-lymphocytes. The ability of platelets to promote the site-specific localization of leukocytes through selective adhesive interactions may be the paradigm of processes that involve other cell types and may be relevant in angiogenesis and tumor metastasis.

**The study of platelet adhesive functions by intravital microscopy**

Studying the mechanisms of thrombus formation in the vasculature of a living animal, typically performed with videomicroscopy techniques, provides a global view of the process, with the contribution of all pro- and anti-thrombotic factors responding to lesions of endothelial and subendothelial structures. Moreover, intravital experiments avoid the problem of using anticoagulants, as required for the *ex vivo* perfusion of blood specimens through flow chambers. One can thus monitor concurrently the aggregation of platelets and deposition of fibrin, and derive quantitative information that is temporally and spatially relevant to explain the process of
thrombus formation, in particular with respect to the events that initiate and consolidate the response to vascular injury. For example, intravital thrombosis models are providing a new perspective on the role of tissue factor,\(^{146,147}\) and have revealed the unexpected importance of coagulation factor XII in the formation of platelet thrombi. Factor XII (Hageman factor) contributes to the initiation of the contact phase of coagulation, which induces clotting on negatively charged surfaces but may be less crucial to secure normal hemostasis \textit{in vivo}. In fact, factor XII deficiency in humans is not associated with a bleeding phenotype, and affected patients have no apparent defect of coagulation or platelet function. It was, therefore, surprising to observe that mice with ablation of the factor XII gene exhibit a markedly decreased platelet response in an experimental model of arterial thrombosis,\(^{148}\) a finding reinforced by the observation that these mice are protected from pathological thrombosis caused by cerebral ischemia.\(^{149}\) The mechanism through which factor XII contributes to thrombus formation is not yet fully elucidated, but the relevance of its role is likely to be inversely related to the levels of exposed tissue factor, considered the main trigger of coagulation, which in turn may depend on the nature of the vascular lesion. Another important aspect of \textit{in vivo} thrombosis models is to provide information on the mechanisms relevant to the stability of platelet aggregates, which may be essential for the development of occluding arterial thrombi. Studies of this kind, for example, have shown that plasminogen activator inhibitor-1 and its cofactor, vitronectin, are required for the integrity of thrombi that develop in response to vascular injury.\(^{150}\)

In spite of the obvious relevance of the results provided by thrombosis model studied by intravital microscopy, it should be considered that no experimental method is without limitations, and conclusions from live animal experiments may not be directly applicable to human pathological conditions. This is particularly true with respect to vascular diseases, because experimental lesions in healthy vessels most likely elicit different thrombogenic responses as compared to those occurring in arteries altered by chronic degenerative processes such as atherosclerosis. In fact, experimental evidence indicates that intravital models of thrombosis are greatly influenced by the methods used to cause injury and the target vessel used, which may explain the variability of reported results.\(^{151}\) Understanding the mechanisms that modulate a thrombogenic response in relation to the nature of the initiating vascular lesion remains a challenge for both \textit{ex vivo} and \textit{in vivo} experimentation.
References


Supplementary Figure Legends

Supplementary Figure 1. Schematic representation of blood flow in a vessel. Normal endothelial cells are nonreactive for platelets, but exposed subendothelial structures induce rapid platelet adhesion and aggregation. Blood flow in a cylindrical vessel can be visualized as a series of fluid layers (laminae) moving at different velocity. The laminae near the center of the vessel have greater velocity than those near the wall (depicted by arrows of different length). The corresponding velocity profile (solid line) is more blunted than the parabolic profile expected with a homogeneous suspension (dotted line) because of cell depletion in the boundary layer near the wall. The shear rate is the rate of change of velocity with respect to distance measured perpendicularly to the direction of flow. The negative sign indicates that the gradient is defined from the center (where velocity is maximal) to the wall (where velocity is minimal). Adapted from reference 152 and reprinted with permission.

Supplementary Figure 2. Real time analysis of $[\text{Ca}^{++}]_i$ during platelet translocation and aggregate formation on immobilized VWF. Platelets loaded with fluo-3 AM (2x10^7/ml) were suspended with washed erythrocytes in homologous plasma and perfused over immobilized VWF for 3 min at the shear rate of 1500 s$^{-1}$. The sequence of images at the top shows an example of aggregate formation. At 0 s, platelet 1 appears in the optical field; at 10 s, it has moved in the direction of flow by approximately 20 µm; at 20 s, it has moved by an additional few µm; at 30 s, it is in the same position, and two new platelets (2 and 3) are attached in close proximity forming a small aggregate. The diagrams in the middle show $[\text{Ca}^{++}]_i$ and instant velocity of platelets 1, 2 and 3. The translocation of platelet 1 occurs mostly during a few seconds of relatively rapid movement, coincident with the appearance of transient $[\text{Ca}^{++}]_i$ peaks ($\alpha/\beta$); a higher and longer lasting increase in $[\text{Ca}^{++}]_i$ ($\gamma$) develops while the platelet is stationary. Cytosolic Ca$^{++}$ oscillations appear also when platelets 2 and 3 arrest on the surface, without a clear sequence from $\alpha/\beta$ to $\gamma$. The images at the bottom, captured between 60 and 63 s after the appearance of platelet 1 in the field, show the long lasting synchronous increase of $[\text{Ca}^{++}]_i$ in platelets forming a large aggregate. The 3D diagrams below each image show the measurement of $[\text{Ca}^{++}]_i$ in all the platelets in the field. Adapted from reference 55 and reprinted with permission.
Supplementary Figure 3. Schematic representation of the sequential signaling events induced by the interaction of platelets with immobilized VWF under high shear stress. On the left, a platelet is shown during the initial tethering to the A1 domain of immobilized VWF mediated by the GP Ib-IX-V complex. An α/β [Ca++]i elevation is elicited as a consequence of this interaction and leads to the release of ADP from intracellular storage granules. Src family kinases may be involved at this stage\textsuperscript{63,153} and cAMP/cGMP levels modulate this and other downstream responses.\textsuperscript{55} Subsequent events are shown in the platelet on the right. The released ADP binds to the G\textsubscript{q}-coupled P2Y\textsubscript{1} receptor, which leads to PLC activation and enhances Ca\textsuperscript{++} release from internal stores during α/β oscillations. At this stage, a first level of localized αIIbβ3 activation is reached that supports a more prolonged platelet adhesion mediated by the interaction with the RGD sequence in the VWF C1 domain. Initial PI 3-K activation may enhance this response. Subsequently, further PI 3-K activation and possibly the involvement of Src family kinases contribute to a more generalized αIIbβ3 activation that permits soluble ligand binding (exemplified here by fibrinogen and VWF) and supports the formation of platelet-platelet aggregates. This second level of αIIbβ3 activation is concurrent with or subsequent to a type γ [Ca++]i elevation dependent on a transmembrane ion flux. The second ADP receptor, P2Y\textsubscript{12}, supports the formation of larger platelet aggregates through mechanisms that occur after the measured Ca\textsuperscript{++} oscillations. The thromboxane A2 pathway inhibited by aspirin appears to have a very limited role in the successive stages of platelet adhesion, activation and aggregation induced by the interaction with immobilized VWF. Abbreviations: IP3, inositol-1,4,5-trisphosphate; Src, Src family tyrosine kinases; PLC, phospholipase C; PKC, protein kinase C; PI 3-K, phosphatidylinositol 3-kinase. Adapted from reference\textsuperscript{74} and reprinted with permission
Supplementary Figure 2
Supplementary Figure 3