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Platelets in hemostasis and thrombosis: role of integrins and their ligands

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Abstract

Platelet adhesion and aggregation at the site of vascular injury are two key events in hemostasis and thrombosis. The contribution of several platelet receptors and their ligands has been highlighted in these processes. In platelet adhesion, particularly at high shear stress, GP1b–von Willebrand factor (vWF) interaction may initiate this event, which is followed by firm platelet adhesion mediated by members of the integrin family, such as β1 (α2β1, α5β1) and β3 (αIIbβ3) integrins. In platelet aggregation, although GP1b–vWF, P selectin-sulfatides, and other molecules, may play some roles, the process is mainly mediated by β3 (αIIbβ3) integrin and its ligands, such as fibrinogen and vWF. Recent studies with perfusion chambers and intravital microscopy have revised the dogma established with the static (low shear stress) conditions. It is intriguing that platelet adhesion and aggregation do still occur in mice lacking both vWF and fibrinogen, suggesting that other unexpected molecule(s) may also be important in hemostasis and thrombosis.

1. Introduction

Platelets play a critical role in hemostasis and thrombosis. They may also contribute to several other physiologic and pathologic processes, such as inflammation, anti-microbial host defense, and tumor growth and metastasis [1]. Platelet adhesion and subsequent aggregation at the site of vascular injury are one of two key mechanisms required to stop bleeding, the other being mediated by the coagulation system, which generates polymerized fibrin. There are many interactions between these two mechanisms leading to clotting. For example, platelets accelerate the coagulation process by providing a membrane surface to enhance generation of thrombin, which then digests and converts fibrinogen to fibrin. On the other hand, thrombin generated via the coagulation process is a potent agonist for platelet activation, important for platelet adhesion and aggregation [2]. Platelet activation and platelet plug formation is important in normal cessation of bleeding and decrease in platelet count [3] or deficiencies of platelet adhesion and aggregation are associated with bleeding disorders [4,5]. In contrast, platelet adhesion and aggregation may result in thrombosis and vascular obstruction [6,7]. Unstable angina and myocardial infarction are typically the result of platelet adhesion and aggregation at the site of atherosclerotic lesions in coronary arteries and thrombosis in coronary or cerebral arteries is one of the major causes of morbidity and mortality worldwide.

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Therefore, the same processes, platelet adhesion and aggregation, play critical but contrasting roles (i.e., physiological and pathological).

2. Current theory of thrombosis: the processes of platelet adhesion and aggregation

Over recent years it has become clear that there are differences in the mechanisms of platelet adhesion at low and high shear conditions. In veins, the shear rate is low (20–200 s^{-1}), but high shear rates are found in arteries (300–800 s^{-1}), the microcirculation (500–1600 s^{-1}), or stenotic vessels (800-10,000 s^{-1}) [8]. It is known that the platelet membrane glycoprotein (GP) Ib-IX-V complex, together with its ligand, von Willebrand factor (vWF), is involved in initiating platelet adhesion to the vessel wall at high shear, such as the shear force in coronary arteries [5,6,9–11]. Stable adhesion is then mediated by several platelet integrin receptors and their ligands. These include the interaction of the α2β1 integrin (GPIaIIa) with collagen and the αIIbβ3 (GPIIbIIIa; CD41/CD61) integrin interaction with fibrinogen and vWF [6,10,12]. On the other hand, at low shear, such as in veins, the interactions of the platelet integrins αIIbβ3 with fibrinogen or fibrin, and those of the α2β1 integrin with collagen, may be able to directly initiate platelet adhesion [6,10,13]. Deficiencies of platelet adhesion receptors, or of their ligands, have been linked to bleeding diatheses, e.g. Bernard-Soulier syndrome, von Willebrand disease, afibrinogenemia [5,14,15]. However, our recent studies with vWF/fibrinogen double deficient (vWF/Fg-/-) mice demonstrated that platelet adhesion still occurred at high shear [16], suggesting that other interactions, independent of the GPIb–vWF interaction, occur between platelet receptors and ligands on the vessel wall [17,18], e.g. GPVI-collagen [19,20], GPV-collagen [21], and GPIb–thrombospondin-1 interaction [22].

Following platelet-vessel wall adhesion, platelets aggregate on the layer of adherent platelets to form a hemostatic plug. This requires platelet activation, stimulated by GPIb–vWF interaction and/or various platelet agonists, e.g. thrombin, collagen, complement C5b-9, ADP, epinephrine, thromboxane A_2 [19,23]. Platelet activation results in a conformational change in the αIIbβ3 (GPIIbIIIa) receptor on the platelet surface that is then able to bind its major ligand, plasma fibrinogen, resulting in outside-in signaling that furthers GPIIbIIIa receptor clustering and platelet cytoskeleton reorganization [24,25]. Fibrinogen bound to activated GPIIbIIIa cross-links adjacent activated platelets resulting in platelet aggregation and hemostatic plug or thrombus formation [26,27]. Understanding these mechanisms has lead to the use of novel anti-aggregating agents (e.g., the anti-αIIbβ3 antibody ReoPro and the αIIbβ3 binding venom peptide Integrilin) that block the GPIIbIIIa receptor [28,29]. Deficiency of αIIbβ3 receptor or fibrinogen, i.e. Glanzmann thrombasthenia and afibrinogenemia respectively, can be associated with severe bleeding.

Recently, using a monoclonal antibody to αIIbβ3 which specifically blocks vWF but not fibrinogen binding, it has been confirmed that vWF bound to activated GPIIbIIIa cross-links adjacent activated platelets [30], particularly at high shear. There is also evidence that other molecular pairings between platelet receptors and their ligands may be involved in aggregation. These include vWF–GPIb complex [31], CD36 and its ligand thrombospondin [32], P selectin–GPIb complex [33], and P selectin–sulfatides [34] interaction. Although P selectin can relocate from platelet α granules to the platelet surface after platelet activation [35] and P selectin ligand, PSGL-1, has been demonstrated in the platelet [36], there is no clear evidence that this molecular pair can support platelet aggregation. After potent agonist stimulation (e.g., by thrombin), released α granule proteins such as fibrinogen, vWF, thrombospondin [37], fibronectin, and possibly multimeric vitronectin [38], as well as other granular components [39,40], may also play an important role in platelet aggregation [41]. In addition, Matrix Metalloproteinases may also enhance platelet aggregation through outside-in signalling [42]. It is clear today that multiple platelet adhesion molecules are involved in platelet adhesion and aggregation. Among them, the integrin families and their ligands play pivotal roles.

3. Integrin receptors and their ligands

Integrins are a large family of cell surface receptors. At least 24 integrins have been found in different cell surfaces [43,44]. On platelets, β3 (αIIbβ3, αVβ3) and β1(α2β1, α5β1, α6β1, α8β1) integrins have been found. Their expression ranges from 2–4000 copies (α2β1, α5β1, αVβ3) to 50–120,000 copies (αIIbβ3) per cell [45]. αIIbβ3 is the predominant protein on the platelet surface, accounting for about 17% of the total platelet membrane protein [46].

All integrins contain an α subunit and a β subunit and there is homology in amino acid sequences and similarities of their structures [47]. Each subunit polypeptide contains a large extracellular domain, a single transmembrane domain, and an intracellular domain. A ligand-binding pocket is formed by the extracellular domains of both subunits [47]. Bi-directional signals (inside-out and outside-in) can be transmitted by the two subunits, which control integrin conformation and cell function [43,48,49]. For example, after ADP stimulation, the signal from ADP receptors [50,51] is transmitted to the cytoplasmic tail (i.e., intracellular domain) of αIIbβ3 and in turn transmitted to the extracellular domain (inside-out), driving a conformational change in the extracellular domain which is then ready to bind its ligand. After ligand binding, a signal is transmitted to the cell (outside-in), which controls cell functions such as morphology change, differentiation and proliferation, or apoptosis [25,47]. Both subunits contain multiple disulfide bonds and conformation and ligand binding of αIIbβ3 integrin may also be controlled through their disulfide bond exchanges [52–54], supporting earlier evidence that dithiothreitol can induce platelet aggregation [55]. Both subunits also contain divalent cation binding sites [56,57]. Three Ca^{2+} and one Mg^{2+} have been proposed in the β propeller domain of the α subunit; these divalent cations support one ligand binding site on this subunit [56]. One divalent cation binding site has been proposed in the I-like domain (vWF A domain-like domain) of the β subunit [57]; this domain contains an RGD peptide binding site in the up-face of a Rossman fold structure [57–59]. The RGD (arginine-glycine-aspartic acid) sequence in ligands (e.g., fibrinogen, vWF, fibronectin, vitronectin, prothrombin, etc.) is the recognition motif of the integrin family [60], and after ligand binding, some divalent cations may be replaced by an RGD portion of the ligands [58,61], but divalent cations are required to maintain the specific structure for ligand binding in most cases [62]. It is important to recognize that a key acidic residue (D, aspartic acid) in the RGD motif is essential for integrin recognition [44]. Some ligand binding sites, such as HHLGGAKQAGDV (H^{12}) in the carboxyl terminus of fibrinogen γ chain, KGD in Integrilin (snake venom αIIbβ3 antagonist) and in human CD40L, and QIDSPL in VCAM-1, do not contain the RGD sequence, but they do have a D in their binding sites [44].

Although the crystal structure of the extracellular portion of integrin (αVβ3) and its RGD binding form has been recently reported and other integrins may have similar structure [63,64], it is still not fully clear what biophysical events are happening at the interface between ligands and integrins. In the case of fibrinogen-αIIbβ3 recognition, three fibrinogen binding sites in αIIbβ3 have been localized, i.e. binding of the C-terminus of the chain of fibrinogen to the αIIb β propeller domain (residues 294–314), RGD sequence to β3 (residues 109–180), and the third site to β3 (residues 211–222, the conservative hydrophobic region in the integrin family) [65]. The last two sites are located in the upper face of the I-like domain. It is not as yet clear which binding site initiates fibrinogen-αIIbβ3 recognition in an activated platelet, but binding of the C-terminus of the chain to the αIIb may be able to support inactive platelet adhesion to immobilized fibrinogen [65], suggesting that this interaction may be easier than RGD-I-like domain interaction. In the in vitro environment, which may affect structures of both the I-like domain and the β propeller domain, the C-terminus of the γ chain-αIIb interaction may then become absolutely required and fibrinogen becomes the only ligand for platelet aggregation [66,67]. However, other ligand(s), from either plasma or platelet granules may still be able to support aggregation in more physiological conditions, as seen in our

recent study using an intravital microscopy in fibrinogen and vWF/fibrinogen double deficient mice [16].

Ligands of αIIbβ3 (GPIIbIIIa) include fibrinogen, vWF, fibronectin, vitronectin, thrombospondin, collagen, and PECAM-1 [44]. Recently,L1-Ig6 [68], prothrombin [69,70], Cyr61 and Fisp12 [71], serum amyloid A [72] and CD40L [73], have been shown to be possible ligands of αIIbβ3; these newly identified proteins may be able to mediate platelet adhesion to an immobilized surface, but there is no evidence indicating that they can support platelet aggregation. For aggregation, at least two integrin binding sites are required to cross-link two adjacent platelets and the ligand molecules must be in either the plasma or in the platelet where aggregation occurs. The concentration should be sufficiently high such as to support the low affinity interaction between integrins and their ligands, e.g. fibrinogen 3 mg/ml, vWF 5–10 μg/ml. Fibronectin appears to be a good candidate to fit these criteria (dimer contains two RGD motifs; 300 μg/ml in plasma), but there is considerable evidence that this molecule plays no, or even a negative, role in platelet aggregation [45,74]. Thrombospondin trimer in plasma contains three RGD motifs [75], but its concentration may be too low to support platelet aggregation [76]. Vitronectin concentration in plasma is high (450 μg/ml) but it contains only a single RGD sequence; it may in fact play a negative role in platelet aggregation through its occupancy of αIIbβ3 [77]. In addition to the plasma ligands above, fibrinogen, vWF, thrombospondin, fibronectin, vitronectin, and other proteins, may also be secreted from platelet α granules. These secreted proteins may also play an important role in platelet aggregation. At this time it appears that αIIbβ3 is required for platelet aggregation, since no platelet aggregation can be seen in either αIIb [78] deficient or β3 subunit deficient mice [79].

In addition to αIIbβ3, αVβ3 (20–40-fold less than αIIbβ3) is also expressed on the platelet surface [45]. In addition to the ligands mentioned above for αIIbβ3 (i.e., vWF, fibrinogen, fibronectin, vitronectin, thrombospondin), L1 adhesion molecules, collagen, osteopontin, and tenascin can also bind to αVβ3 [44], but interaction of these proteins and αVβ3 may be much less potent in platelet adhesion and aggregation.

β1 integrins (α2β1, α5β1, α6β1, α8β1) are another family integrins on the platelet surface. Ligands of α2β1 include collagens I–IV, laminin, vitronectin, tenascin [44], and the newly identified decorin [80]. Ligands of α5β1 include fibronectin, denatured collagen, and L1 adhesion molecule. Ligands of α6β1 include laminin and epiligrin and ligands of α8β1 include fibronectin, tenascin and vitronectin. These β1 integrin receptors may play an important role in platelet adhesion to injured vessel wall, but their roles in platelet aggregation are minor, as they are not able to independently support platelet aggregation in β3 integrin deficient platelets.

4. Future directions

The concept of platelet adhesion and aggregation at high shear has been significantly revised by recent studies using perfusion chambers [10,11,81] and intravital microscopy with gene targeted animals [16]. Although it is not clear whether platelet adhesion to injured vessel wall can still occur, as seen in vWF deficient mice, at high shear in type III vWD patients, it is clear that, in contrast to current theory established under static (low shear rate) conditions where fibrinogen is required for platelet aggregation, platelet aggregation does occur in flow conditions in both fibrinogen deficient mice [16] and afibrinogenemic humans [11].

It remains to be determined which molecules mediate platelet-vessel adhesion at high shear in mice lacking vWF. Although several molecular pairs are able to support platelet adhesion, including the β1 integrin family (α2β1-collagen, α5β1-fibronectin, α6β1-laminin, α8β1-fibronectin), the β3 integrin family (αIIb3-collagen, fibrinogen, fibronectin, thrombospondin, αVβ3-fibronectin, fibrinogen, collagen, thrombospondin), GPVI-collagen [19], GP V-collagen [21], and GP1b-thrombospondin, most of the above mediate adhesion after platelets are tethered by the GPIb-vWF interaction. It is unclear whether a single molecular pair is sufficient to support adhesion or whether the synergistic action of multiple pairs is

required to support this vWF-independent platelet adhesion. Identification of these GPIb–vWF-independent adhesion molecular pairs and their roles in human platelet adhesion and platelet activation will provide new insights into hemostasis and thrombosis.

Platelet aggregation independent of fibrinogen and vWF/fibrinogen in vivo is intriguing. Cross-linking two adjacent platelets for aggregation requires platelet surface receptors and their ligands. These receptor–ligand pairs include αIIbβ3 (GPIIbIIIa) and its ligands fibrinogen and vWF. Several other pairs, e.g. GPIb-IX-V and its ligand vWF, CD36 and its ligand thrombospondin, as well as P selectin and its ligands sulfatide and PSGL-1, also may be involved in this aggregation or in the stabilization of aggregates. However, our recent studies with the highly sensitive intravital microscope demonstrated that no platelet aggregation occurs in mice lacking β3 integrins [16]. This excludes the possibility of other platelet receptors independently mediating platelet aggregation in vivo, i.e. they are not sufficient in themselves to mediate aggregation, and the β3 integrin (αIIbβ3 or αVβ3) appears to be the essential receptor for this process. Interestingly, although a requirement of fibrinogen for platelet aggregation has been documented, and platelet aggregation cannot be induced in vitro if fibrinogen, or more precisely the QAGDV sequence in the carboxyl terminus of the γ chain, is not present [66,67], we found, using in vivo intravital microscopy, that very efficient platelet aggregation can be seen in mice lacking fibrinogen [16]. This is consistent with recent data from a perfusion chamber study of blood from afibrinogenemic patients [11]. Even more surprisingly, efficient platelet aggregation is also seen in mice lacking both vWF and fibrinogen [16], suggesting that other ligand(s) of β3 integrins can support robust platelet aggregation in vivo. It is currently unknown what this ligand is and where it comes from during platelet aggregation.

These 'new' platelet adhesion and aggregation related molecules may provide the basic hemostatic requirement for von Willebrand disease and afibrinogenemic patients. They may also be an enhancing factor in vWF and fibrinogen mediated adhesion and aggregation, or may modulate the control of thrombosis and hemostasis in normal human subjects, i.e. may competitively inhibit overreaction of fibrinogen or vWF mediated aggregation and enhance aggregation when either fibrinogen or vWF is insufficient. It may be asked why, if this new protein does exist, has it not been linked with a bleeding disorder? Explanations may be that genetic studies performed have been too limited or that the new protein(s) may, like fibronectin or other coagulant factors [82–85], be absolutely required for embryo development; thus, no deficient patient survives for study. Identifying and characterizing these proteins will enhance our understanding of these novel mechanisms of platelet adhesion and aggregation, contribute to a fuller understanding of thrombotic pathways, and may subsequently allow for development of new antithrombotic and anti-bleeding therapies.

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