Platelets in hemostasis and thrombosis: Novel mechanisms of fibrinogen–independent platelet aggregation and fibronectin–mediated protein wave of hemostasis

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Abstract
Platelets are small anucleate cells generated from megakaryocytes in the bone marrow. Although platelet generation, maturation, and clearance are still not fully understood, significant progress has been made in the last 1-2 decades. In blood circulation, platelets can quickly adhere and aggregate at sites of vascular injury, forming the platelet plug (i.e. the first wave of hemostasis). Activated platelets can also provide negatively charged phosphatidylserine-rich membrane surface that enhances cell-based thrombin generation, which facilitates blood coagulation (i.e. the second wave of hemostasis). Platelets therefore play central roles in hemostasis. However, the same process of hemostasis may also cause thrombosis and vessel occlusion, which are the most common mechanisms leading to heart attack and stroke following ruptured atherosclerotic lesions. In this review, we will introduce the classical mechanisms and newly discovered pathways of platelets in hemostasis and thrombosis, including fibrinogen-independent platelet aggregation and thrombosis, and the plasma fibronectin-mediated “protein wave” of hemostasis that precedes the classical first wave of hemostasis. Furthermore, we briefly discuss the roles of platelets in inflammation and atherosclerosis and the potential strategies to control atherothrombosis.

Keywords: platelets, thrombosis and hemostasis, integrin αIIbβ3, fibrinogen, fibronectin

Platelets: evolution, generation, and clearance
Platelets are small anucleate cells in the circulation, with a diameter of approximately 1-2 μm. They were first identified in 1874 by Osler¹; however, it was the Italian physician, Bizzozero, who in 1881 established the role of platelets in hemostasis and thrombosis in his seminal publications². By staining the granules of platelets (Wright stain), it was later demonstrated that...
these anucleate cells are generated from megakaryocytes in the bone marrow. In the following century, intensive investigation of the cellular and molecular mechanisms of platelets and megakaryocytes enabled the development of a number of therapeutic agents for the treatment and prevention of thrombotic disorders.

In non-mammalian vertebrates, hemostasis is mediated by nucleated cells called thrombocytes. Anucleated platelets evolved only in mammals, and to the best of our knowledge, there is no animal species that have been found thus far to have an intermediate state between thrombocytes and platelets. In addition, platelets are more effective than thrombocytes in forming occlusive thrombi under arterial shear stress. Since platelets have an average life span of only 8-10 days in humans and approximately 5 days in mice, they are constantly being produced from megakaryocytes in the bone marrow. During maturation, megakaryocytes undergo DNA replications without cell division (a process called endomitosis), leading to generation of polyploid megakaryocytes. The abundant genomic DNA in the polyploid megakaryocytes enhances their ability to synthesize proteins and package them into specific platelet granules.

The exact mechanism of platelet release from megakaryocytes is still under debate. In vitro studies demonstrated that platelet formation begins at one pole of megakaryocytes, then the whole cell is disintegrated, resulting in the generation of numerous proplatelets. However, a recent intravital microscopy study revealed that megakaryocytes extend long protrusions into bone marrow sinusoids and release proplatelets from the tip of the protrusions under shear stress, suggesting that platelet generation in vivo is drastically different from in vitro cell culture conditions. Proplatelets then undergo further division to generate mature platelets in vivo. In addition to bone marrow, new discoveries suggest that megakaryocytes can also mature in the lung and shed platelets into the pulmonary vasculature. Interestingly, a recent study suggested that platelets are capable of cell division and progeny generation even without a nucleus, although more evidence is required to confirm this finding.

The process and mechanism of platelet clearance is also not well understood, but it is assumed that this occurs in the reticuloendothelial system by macrophages. Aged platelets may express more phosphatidylserine (PS), which may attract macrophages for clearance. During red blood cell clearance, older cells may induce more autoantibody binding; however, whether this occurs in aged platelets and whether the Fc portion of the autoantibody interacts with Fc receptors on macrophages leading to phagocytosis still remains to be determined. A more recent study demonstrated that antibody opsonization can activate platelets, leading to platelet desialylation, a mechanism also involved in clearance of chilled platelets. These desialylated platelets can then be destroyed in the liver via hepatocyte Ashwell-Morell receptors. Whether this novel platelet clearance pathway plays a role in the clearance of aged platelets has yet to be investigated.

**Platelets in hemostasis and thrombosis**

Hemostasis is a critical physiological process to stop bleeding. Platelet accumulation at the site of injury is considered the first wave of hemostasis and the second wave of hemostasis is mediated by the blood coagulation pathway. Platelets play a central role in a series of sequential events during the platelet accumulation (i.e., platelet adhesion, activation, and aggregation) and are also actively involved in cell-based thrombin generation, which markedly amplifies the blood coagulation cascade. Thus, platelets contribute to both the first and the second waves of hemostasis.

**Platelet adhesion**

Platelet adhesion to the injured vessel wall can occur at both low and high shear conditions but are mediated through distinct mechanisms. Low shear rates (20-200/s) are observed in the venous system whilst higher shear rates are found in arteries (300-800/s) and stenotic vessels (800-10,000/s). Following vascular injury, subendothelial matrix proteins such as collagens are exposed to the blood components. Plasma von Willebrand Factor (VWF), originated from endothelial cells, megakaryocytes, and platelets, can then anchor onto the collagen. The VWF receptor on platelets (glycoprotein (GP) Ib/IX) interacts with the immobilized VWF, subsequently initiates platelet tethering to the site of injury. This binding is essential for platelet adhesion at high shear (e.g., coronary arteries), although the GP Ib-α-VWF interaction may also contribute to platelet adhesion at low shear. Following platelet tethering, GPVI and integrin α2β1 may interact with collagen and deliver activation signals to platelets. Stable adhesion is subsequently mediated by binding of several integrins to their ligands on the vessel wall (e.g., integrin αIIbβ3 to fibrinogen/fibrin and fibronectin, α5β1 to fibronectin or collagen, and α2β1 to collagen, etc.). At low shear (e.g., veins) the interactions between platelet integrins and their ligands (e.g., αIIbβ3 to fibrinogen/fibrin or fibronectin etc.) may directly initiate platelet adhesion.

In the last decade there have been significant advances in vivo models of platelet adhesion and thrombus formation using intravital microscopy.
VWF knockout (−/−) mice demonstrate decreased platelet adhesion[39,41], a phenotype that, interestingly, is not as severe as the GPIbα−/− mice, suggesting that GPIbα has additional hemostatic function[40]. Mice lacking GPVI present with prolonged bleeding times[39] and similarly, mice deficient in α2 or β1 integrins also have delayed thrombus formation, although these deficiencies are mild compared to GPIbβ−/− mice[41].

**Platelet activation and granule secretion**

The primary interactions between platelet surface receptors (e.g. GPIbα, integrins) and their ligands (e.g. VWF, collagen, fibrinogen/fibrin, fibronectin, etc.), can lead to platelet activation[30,52,53]. In addition, following vascular injury, the coagulation system is activated[31,54-55], which generates the most potent platelet activation factor, thrombin. Through cleavage of protease-activated receptors (PARs) and binding to GPIbα, thrombin activates platelets[56-59].

Platelet activation exposes PS on the membrane surface that drives the cell-based thrombin generation[34,35] and facilitates further platelet activation[53,60-61]. Activation signals induced by thrombin, collagen, or ligands of adhesion receptors with the addition of shear stress, can lead to platelet granule release. Platelet adhesion molecules, P-selectin[62], integrins, VWF, fibrinogen, fibronectin[63-64], vitronectin[55], multimerin[60], platelet factor 4, and approximately 300 other proteins are contained within the α-granules[56]. Dense granules release adenosine di-phosphate (ADP), which supports the second wave of platelet aggregation following integrin activation[65]. The release of Ca2+ from the endoplasmic reticulum and the dense granules via the Ca2+ sensor, stromal interaction molecule (STIM)1, and the Ca2+ channel, Orai, is also a significant contributor to platelet activation[66-69]. There are many positive feedback loops during platelet activation/granule release. Notably, ADP, likely via interaction with its receptors on platelets, initiates cell-based thrombin generation and further platelet activation/granule release[64]. These secretion events act as secondary messengers and, in combination with the generation of thromboxane (Tx) A2 and reactive oxygen species, amplify the activation process and integrin αIIbβ3 inside-out signaling, which in turn recruits more platelets for aggregation[70-71].

**Platelet aggregation: fibrinogen-dependent and –independent aggregation**

Following platelet activation, integrin αIIbβ3 binds fibrinogen and other ligands (i.e. fibrinogen-dependent and -independent pathways[31,39,64,75-76]), which leads to platelet aggregation. It is notable that following engagement of ligands, integrin αIIbβ3 can deliver outside-in signals, which further enhance platelet activation, cytoskeleton rearrangement, and granule secretion. These signal events facilitate hemostatic plug and thrombus formation.

For more than half a century, fibrinogen was considered required for platelet aggregation[64]. Through interaction with αIIbβ3 via its γ chain C-terminus, fibrinogen bridges adjacent activated platelets[22,71]. However, data from Ni et al. demonstrated that thrombus formation still occurred in fibrinogen−/− mice and in VWF and fibrinogen double knock-out (DKO) mice[59], indicating that fibrinogen was not indispensable for this process. Further studies demonstrated that DKO platelet aggregation occurred in vitro in platelet-rich plasma and gel-filtered platelets without anti-coagulant treatment (i.e. in a more physiological condition compared to anti-coagulated blood used in clinic and research). In contrast, integrin β3−/− mice exhibit no significant platelet aggregation, which indicates an essential role for αIIbβ3 in platelet aggregation and suggests the existence of other unidentified αIIbβ3 ligand(s)[60].

In VWF-fibrinogen DKO mice, fibrinogen−/− mice, and fibrinogen C-terminal γ chain mutant mice[72], as well as in afibrinogenemic patients[22,70], platelet fibronectin (an αIIbβ3 ligand) content was increased 3-5 fold due to enhanced internalization of plasma fibronectin (pFn) by integrin αIIbβ3. Conditional pFn−/− mice have impaired thrombus growth at arterial shear[70], implying that fibronectin may be a compensatory αIIbβ3 ligand that supports platelet aggregation. Unexpectedly, however, further depletion of pFn in VWF-fibrinogen DKO mice enhanced, instead of abolishing, platelet aggregation[51,75]. These results suggest that pFn can switch between supporting and inhibiting platelet aggregation, depending on the presence of fibrinogen/fibrin[72].

Another αIIbβ3 ligand, vitronectin, plays a dual role; aggregation is enhanced by granule-released vitronectin but is inhibited by plasma vitronectin[63]. These studies suggested that likely neither fibronectin nor vitronectin are the αIIbβ3 ligand that mediates fibrinogen-independent platelet aggregation. Cadherin 6 contains a canonical "RGD" (arginine-glycine-aspartic acid) integrin-binding motif and increases its expression on platelets after platelet activation[73]. Whilst cadherin 6 contributes to platelet aggregation, clearly other plasma and platelet proteins exist that can also mediate and facilitate fibrinogen-independent platelet aggregation[61]. However, what they are and how they contribute to thrombosis and hemostasis in different pathophysiological conditions requires further study.
**Platelet-mediated cell-based thrombin generation and blood coagulation**

In addition to their central roles in the platelet adhesion, activation, and aggregation (the first wave of hemostasis), platelets also contribute to coagulation pathway (the second wave of hemostasis). The blood coagulation cascade can be activated by either the extrinsic (tissue factor) or the intrinsic (contact activation) pathways in thrombosis. Thrombin, a vital product of the coagulation cascade, converts fibrinogen to fibrin, the end product of the coagulation cascade.

Besides these two classical coagulation pathways, the exposure of PS on platelets, following platelet activation, markedly potentiates thrombin generation by inducing a negatively charged surface that harbors the coagulation factors. Interestingly, in a study of platelet aggregation in fibrinogen and VWF DKO mice, Yang et al. found that ADP can induce thrombin generation that is required for platelet aggregation in the DKO mice. Recently, GPVI was also identified as a novel fibrin receptor involved in potentiating thrombin generation. Thrombin initiates robust downstream signaling, through PAR1, PAR4 and GPIbα, leading to platelet activation and further PS exposure, a positive feedback loop for thrombin generation and blood coagulation. Thus, there are many interactions between the first wave (platelet accumulation) and the second wave (blood coagulation) of hemostasis, which synergistically contribute to the arrest of bleeding.

**Platelets and the "protein wave" of hemostasis: new discoveries**

One of the most recent studies revealed a novel concept of a 'protein wave' of hemostasis, where pFn deposition on the injured vessel wall occurs prior to platelet accumulation (the first wave of hemostasis) and contributes to hemostasis. In mice lacking fibrinogen, further depletion of pFn markedly increased the mortality rate due to uncontrolled bleeding. Increased bleeding time was also observed in pFn conditional mice, treated with heparin and other anti-coagulants, suggesting that pFn is important for hemostasis in both genetic and drug-induced deficiencies of blood coagulation. We observed that the pFn deposition onto the injured vessel wall can occur independently of fibrinogen, VWF, β3 integrin, and platelets. It seems that the pFn-collagen interaction may play an important role in this process. pFn, likely via the covalent binding to fibrin, increases the diameter of fibrin fibers and enhances the mechanical strength of the clot and this mechanism likely contributes to the pFn deposition onto the injured vessel walls of normal individuals where fibrin exists. Interestingly, in the absence of fibrin (a product of fibrinogen), pFn switches its function from promoting to inhibiting platelet aggregation. As fibrin is mainly formed at the bottom of the hemostatic plug close to the vessel wall, pFn may support hemostasis at the base of the thrombi (likely through the formation of a pFn-fibrin complex) and switch to inhibiting excessive thrombus growth at the apical surface of thrombi. Through this mechanism, pFn serves to control bleeding, while preventing excessive thrombus growth and vessel occlusion. Further investigation of the interaction between platelets and circulating/deposited pFn may reveal novel therapeutic targets for thrombotic disorders, as well as usage of pFn for transfusion to control bleeding disorders, particularly those patients in association with anti-coagulant therapy. It would also be interesting to investigate whether the markedly increased platelet fibronectin content in fibrinogen−/− mice and afibrinogenemic patients can be released onto the injured vessel wall and contribute to the protein wave of hemostasis (Fig. 1).

**Conclusion and future directions**

The primary physiological function of platelets is to stop bleeding upon vascular injury. Platelets, via their contributions to the "protein wave" and to the classical first and second waves of hemostasis, play key roles in the arrest of bleeding. Thrombocytopenias caused by either genetic deficiencies or autoimmune and alloimmune responses lead to bleeding disorders. However, the same platelet accumulation and coagulation may lead to thrombosis. Thrombotic events occur at the site of a ruptured atherosclerotic plaque and can result in heart attack or stroke, the leading causes of mortality and morbidity worldwide.

In addition to thrombosis, the late stage of atherothrombosis, recent studies demonstrated that platelets are actively involved in the initiation of atherosclerosis. Platelets are sensitive to environmental changes, such as food products and advanced glycation end products in diabetes, which may affect atherosclerosis. Furthermore, as we demonstrated, platelets can respond to fibrinogen level changes. Through interaction with integrin αIIbβ3, platelets can use their residual mRNA to de novo synthesize P-selectin and other proteins, which may also affect inflammation and directly or indirectly affect atherosclerosis and the stability of atherosclerotic plaques.

In this review, we described the concept of fibrinogen/VWF-independent platelet aggregation, which was first noted in the early 2000s, and provided
insight into multiple and diverse interactions between platelets and their environment. Despite considerable efforts\textsuperscript{[62,76]}, the ‘x’ ligand(s) of integrin αIIbβ3 has yet to be uncovered. These concepts are an example of how diverse platelets can be and demonstrate the need for further investigation into platelet interactions. Furthermore, whilst platelets play a pivotal role in hemostasis and thrombosis, they are also versatile cells and are involved in multiple functions, including inflammation, immune responses, lymphatic vessel development, angiogenesis, tumor metastasis, as well as atherosclerosis\textsuperscript{[106-109]}. Further elucidations of platelet versatilities will provide insights into development of new methods to control not only thrombosis and hemostasis but also inflammation, cancer, and immunological disorders.

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**References**


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