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Chapter 2

The Traditional Role of Platelets in Hemostasis

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1. Introduction

Hemostatic balance is central to health maintenance. Hemostasis must be initiated rapidly to prevent excessive blood loss. However, it must be tightly controlled to prevent over exuberant thrombus formation with resultant pathologic occlusion of arterial or venous vascular beds. Platelets are central to this hemostatic balance via primary hemostasis, support of coagulation, and even anti-fibrinolytic effects. Quantitative and qualitative platelet disorders have classically focused on hemorrhagic and thrombotic diseases, the severity of which can range from mild to life-threatening. Recent advances have demonstrated that platelets have functions beyond their traditional hemostatic role such as supporting vascular integrity, angiogenesis, immune function, tumor metastases, etc. These "non-traditional" functions of platelet will be discussed in other chapters. In this chapter we present a brief review of the traditional roles of platelets in hemostasis and thrombosis.

2. Structure

Platelets have many unique structural features that facilitate their contributions to thrombus formation. The cell membrane of platelets consists of a phospholipid bilayer embedded with cholesterol, glycoproteins, and glycolipids. Platelet membranes are asymmetrically organized. Negatively charged phospholipids in resting platelets are preferentially present on the inner leaflet, most notably phosphatidylserine.[1] The platelet membrane is rich in a variety of glycoproteins (GPs) that bind agonists to activate platelets and that serve primarily adhesive functions (Table 1). Transmembrane glycoproteins may distribute preferentially to cholesterol-rich microdomains, called "lipid rafts."[2]

Platelet Surface Receptors for Adhesive Proteins			
GP designation	**Integrin designation**	**Other names**	**Primary ligands**
GPIa-IIa	$\alpha_2\beta_1$	VLA-2	Collagen
GPIb-V-IX	n/a	CD42	Von Willebrand Factor
GPIc-IIa	$\alpha_5\beta_1$	VLA-5	Fibronectin
GPIIb-IIIa	$\alpha_{IIb}\beta_3$	CD61 (β subunit), CD41	Fibrinogen (and several others)
GPIV	n/a	GPIIIB, CD36	Collagen

Table 1. Platelet Surface Adhesion Proteins

Resting platelets in circulation have a stable discoid shape that is maintained by a circumferential coil of microtubules and a membrane cytoskeleton composed of actin, spectrin and other proteins.[3] The platelet plasma membrane is contiguous with the open canalicular system (OCS), a complex series of connecting tunnels that are open to the extracellular space. In addition to supplying membrane surface area to the spreading platelet, the canalicular system provides a potential route for the release of granule contents critical for delivery of the numerous vasoactive elements present in platelet granules. This also serves as a storage site for glycoproteins that are receptors for adhesive molecules. While the OCS is contiguous with the extracellular space, the dense tubular system is a closed channel network analogous to the sarcoplasmic reticulum as a site where calcium can be sequestered. Release of calcium from this system is a critical step in platelet activation.

Platelets have three different types of granules (Table 2). Dense granules contain adenine nucleotides (e.g., ADP and ATP), calcium, bioactive amines (e.g., serotonin and histamine) and polyphosphates. α-granules are rich in larger adhesive proteins.

Dense granules
ADP
ATP
Calcium
Serotonin
α-granules
Platelet factor 4 (PF4)
von Willebrand Factor
Fibrinogen
Fibronectin
Factor V
Factor XI
Protein S
PAI-1

Table 2. Platelet Granule Contents important for hemostasis

3. Thrombopoiesis

In the healthy state, platelets have an average lifespan of 8-9 days. This requires an active production mechanism. Bone marrow megakaryocytes produce approximately 10^{11} platelets daily. Each individual magakaryocyte can produce between 1000 and 3000 platelets.[4] Most of the molecules present in the mature platelet are produced by the megakaryocyte, but some such as fibrinogen and immunogloulin, are endocytosed from the surrounding plasma milieu. Megakaryocytes produce platelets by extending long projections. Cytoplasm in the developing platelets largely resembles that of the megakaryocyte. However, certain contents, particularly granules, appear to be moved into the developing proplatelets by an active transport mechanism.[5]

Several cytokines effect the development of platelets. IL-3, GM-CSF, and stem cell factor all appear important in maintaining the health and proliferation of megakaryocytes. However, the key regulator of platelet formation is thrombopoietin (TPO). TPO is a 50-70 kDa protein that has homology to erythropoietin.[6] TPO interacts with its key receptor c-Mpl, leading to dimerization initiating a signal transduction cascade through JAK, STAT, and MAPK pathways. TPO is made in the liver and to a lesser extent the kidney.

4. Platelet-mediated hemostasis

During both normal in vivo hemostasis and pathologic thrombus formation, numerous physiologic responses occur simultaneously, such as vasoconstriction, platelet plug formation and coagulation. Platelet thrombus formation itself involves a set of unique molecular responses and signaling pathways that also occur simultaneously. From a discussion point of view, this complexity makes it convenient to arbitrarily compartmentalize these processes.

4.1. Tethering and firm attachment

Platelet plug formation is initiated by exposure to a break in the endothelial lining of blood vessels. This has two important sequelae. The first is the loss of a variety of inhibitors of platelet function. The intact endothelium produces nitric oxide and prostacyclin both of which are inhibitors of platelet function, and the loss of endothelium leads to the loss of CD39 which in its intact state breaks down adenosine diphosphate (ADP), an activator of platelets. Exposure of subendothelial elements also allows the initial recruitment of platelets from the circulation via interactions between adhesive glycoproteins on the platelet surface and subendothelial proteins. [7]

Von Willebrand Factor (VWF) is critical for platelet-mediated thrombus formation in vessels with high shear rates and high shear stress. VWF is a multimeric protein that ranges in molecular weight from 0.5 daltons (dimers) to greater than 20 million daltons (multimers).[8] The hemostatic efficacy of VWF is directly proportional to its size with the largest molecules being the most prothrombotic. Subendothelial VWF is derived from plasma VWF that binds

collagen after vessel injury and the abluminal secretion from endothelial cells. VWF circulates as a globular protein but undergoes conformational changes when exposed to high shear stress conditions. This unfolding exposes binding domains that allow the large von Willebrand multimers to form a bridge between subendothelial collagen and circulating platelets. The von Willebrand protein contains multiple functional domains including binding domains for both collagen and platelet GPIbα.

The initial binding of VWF to the platelets is mediated by interaction between the A1 domain of VWF with the GPIbα subunit of the GPIb-V-IX complex.[9] GPIbα has an N-terminal segment comprised of two β-loops flanking a leucine-rich repeat segment. GPIX is a small, single chain polypeptide. The exact contribution of this peptide to the function of the complex is not well understood. This bridging mediates a rapid but reversible platelet adhesion that allows for rolling of platelets along the damaged endothelium. Occupation of this complex by VWF also leads to platelet signaling responses, including rearrangement of the cytoskeleton, increase in intracellular calcium, and granule release. The reduced platelet velocity mediated by the VWF-GPIbα interaction, coupled with activation of integrin $\alpha_2\beta_1$ enables stable, irreversible interactions to form between collagen and platelet integrin $\alpha_2\beta_1$.

4.2. Activation

Once platelets are captured from the circulation, activation steps lead to numerous changes in the platelets. These include conformational changes, rapid calcium influx, degranulation, thromboxane production, etc. The changes are induced by numerous agonists interacting with specific receptors on the platelet plasma membrane.

With increasingly sophisticated technologies for assessing platelet function and thrombus formation in vivo and under flow condition, there is an enhanced appreciation of the heterogeneity of platelets in a developing thrombus. Thus, there appears to be diverse microenvironments such that regions near the vessel wall may contain degranulated and irreversibly activated platelets, while the luminal region may have minimally activated and reversible adhered platelets that may or may not undergo thrombus stabilization.[10, 14]

As noted above, exposure of subendothelial collagen begins the initial tethering process. Once platelets are engaged in rolling on this matrix they have the opportunity to interact with GPVI, which is the major platelet collagen signaling receptor.[15] GPVI is a type 1 transmembrane protein belonging to the Ig superfamily. It associates with an Fc receptor γ-chain which serves as the signal transducing unit. Engagement of repetitive motifs on collagen by multiple GPVI molecules leads to crosslinking of GPVI dimers and phosphorylation of the FcRγ chain immunoreceptor tyrosine-based activation motifs (ITAMs). This initiates a Syk-dependent signaling cascade finally resulting in activation of phospholipase Cγ2 (PLCγ2) and phosphoinositide-3 kinase (PI3K) that generates inositol-1,4,5-trisphosphate (IP3). IP3 induces calcium mobilization, degranulation and integrin $\alpha_{IIb}\beta_3$ activation. Activated $\alpha_{IIb}\beta_3$ binds fibrinogen and VWF, leading to platelet aggregation.

The exposure of subendothelial collagen also exposes extravascular tissue factor, initiating coagulation and thrombin generation. This cascade is enhanced by PS exposure on activated

platelet and endothelial cell membranes. Thrombin is a potent activator of platelets. Human platelets express two thrombin-activated G protein coupled receptors (GPCRs), PAR1 and PAR4.[16, 17] PAR activation occurs when a protease, such as thrombin, binds and cleaves the amino-terminus of the receptor. Binding of the new amino-terminus to the second extracellular loop of the PAR induces conformational changes in transmembrane domains enabling activation of G proteins.[18, 20] PAR1 and PAR4 activation lead to activation of Gαq, which activates PLCβ. PLCβ hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to generate diacylglycerol (DAG) and IP3, leading to PKC activation and increased calcium mobilization, respectively.[21] In platelets, these pathways work in concert to activate the integrin αIIbβ3 resulting in aggregation. PAR1 has a higher affinity for thrombin, and calcium transiently rises sharply after PAR1 activation followed by a relatively fast return to baseline levels. In contrast, PAR4 induces a more gradual and sustained rise in calcium and accounts for the majority of intracellular calcium flux.[22, 23] These platelet PAR1 and PAR4 kinetic signaling differences are reminiscent of the initiation and propagation phases of coagulation, where there is a burst of thrombin generation (quickly shut off by tissue factor pathway inhibitor [TFPI]) followed by a sustained and quantitatively greater production of thrombin by the intrinsic pathway.

Receptor	Agonist
GPVI	Collagen
PAR4, PAR1	Thrombin
$P2Y_{12}$, $P2Y_1$	ADP
α_{2a}	Epinephrine
TPα, TPβ	TXA2

Table 3. Important Platelet Receptors and Agonists

There are two important amplification pathways in platelet activation.[24] The first is through the release of ADP from dense granule secretion. ADP is a potent platelet agonist that, when added to in vitro platelets, leads to TXA_2 production, phosphorylation of a number of proteins, increased cytosolic Ca^{++}, shape change, aggregation, and secretion. This pathway is required for maximal platelet aggregation induced by other agonists. Platelets have two ADP receptors, $P2Y_1$ and $P2Y_{12}$, and both are GPCRs. $P2Y_{12}$ activates Gαi, which promotes aggregation by inhibiting cyclic AMP (cAMP) formation. $P2Y_{12}$ mediated activation of protein kinase A leads to VASP phorphorylation. $P2Y_{12}$ is inhibited by the thienopyridines, commonly used anti-platelet agents that have benefit in the management of ischemic vascular disease. $P2Y_1$ appears to be necessary, but not sufficient to induce full platelet aggregation. Platelets from $P2Y_1$ knockout mice cannot change shape or aggregate to ADP but cAMP is still decreased in those platelets due to its effect on $P2Y_{12}$. $P2Y_1$ activates Gαq with subsequent calcium mobilization.

The second feedback amplification pathway involves the metabolism of arachidonic acid (AA) to thromboxane A2 (TXA2).[24] A number of agonists stimulate the release of arachidonic acid from the stores in the plasma membrane, in particular phosphatidylcholine and phosphati-

dylethanolamine. Phospholipase A2 (PLA2) is the most important enzyme in the release of AA from those phospholipids. PLA2 can be activated by rising cytosolic calcium levels though there also appear to be calcium independent mechanisms. Released AA is then metabolized by cycloxegenase 1 (COX-1) to Prostaglandin G2 which subsequently is converted to Prostaglandin H2. Thromboxane synthase then produces TXA2. Aspirin irreversibly acetylates COX and also has benefit in preventing arterial ischemic syndromes. TXA2 diffuses out of platelets and binds to prostanoid GPCR family receptors, notably TPα and TPβ, which also activate platelets via Gq.

Epinephrine activates platelets through adrenergic α_{2a} GPCRs that couple with Gαi family members to inhibit adenylyl cyclase leading to decreased cAMP and increased intracellular calcium concentration. It appears that epinephrine synergizes with other agonists, particularly ADP. It is unclear if epinephrine can lead to full aggregation by itself in vitro, although there are reports of families with mild bleeding disorders due to defects in epinephrine-induced platelet aggregation.

4.3. Shape change

The most dramatic observable change to platelets as they undergo activation is the change from their discoid form to a spread form with many filopodia. Agonists, such as thrombin and TXA2, activate GPCRs coupled to Gα12/13, which signal through RhoA –ROCK and myosin to reorganize the actin cytoskeleton and produce shape change.[24] Platelets contain large amounts of actin in both the globular (G-actin) and multimeric filamentous (F-actin) forms. Following activation; the proportion of F-actin increases from 40-50% to 70-80%. In an organized process, actin filaments from the resting platelet are cleaved into smaller fragments. These then form the beginnings of new, longer actin filaments. This process is regulated, in part, by increase phosphatidylinositol-4,5-biphosphate (PIP_2). Simultaneous to the changes in actin, myosin is phosphorylated by myosin light chain kinase activated by the calcium-calmodulin complex. This leads to association with F-actin as well as binding the complex to the membrane via interaction with the GPIb-IX complex. In resting platelets, filamin acts to stabilize the actin framework underlying the membrane and limits the movement of the GPIb. Increasing cytoplasmic Ca^{++} concentrations activate calpain cleaving the actin binding protein leading to release from the GPIb complex. The outcome of this complex series of reactions is the centralization of actin into thick, fibrous masses associated with phosphorylated myosin filaments.

4.4. Degranulation

The above-mentioned agonists all induce platelet exocytosis of granules.[25] **S**oluble **N**-ethylmaleimide-sensitive factor **a**ttachment protein **re**ceptors or SNAREs mediate this delivery.[26] This includes t-SNAREs (target receptors), v-SNAREs (vesicle associated membrane receptors), and soluble components such as N-ethylmaleimide-sensitive fusion proteins (NSF) and NSF attachment protein. Reorganization of the cytoskeleton in conjunction with the SNARE machinery facilitates exocytosis of these granules, which contain a large variety of mediators important to the hemostatic and other roles of platelets.

Dense Granules. There are approximately three to eight dense granules per platelet. These are 20 to 30 nm in size and are electron dense due to the high calcium content. Dense granules also have high concentrations of serotonin, ADP, and ATP. ADP is an important platelet activator and this concentration of ADP in the dense granules and its delivery to developing thrombi by degranulation is an important amplification step in activating other platelets localized by adhesive molecules.

***α*-Granules**. There are 50 to 80 α–granules per platelet. They are much larger than dense granules at approximately 200 nm in diameter. Upon platelet activation, α granules fuse with the plasma membrane, releasing their cargo, substantively increasing the total platelet membrane surface area. α-granule membranes are rich in important adhesive integral membrane proteins, like GPIb-IX and $\alpha_{IIb}\beta_3$, which enhance adhesive properties. α-granule cargo includes adhesive proteins and coagulation factors like fibrinogen and VWF, representing an important amplification feature of platelet thrombus growth. Fibrinogen is present in concentrations greater than that of plasma. Notably, megakaryocytes do not appear to synthesize fibrinogen, and it is endocytosed via $\alpha_{IIb}\beta_3$. Patients lacking $\alpha_{IIb}\beta_3$ also lack α-granule fibrinogen. α-granule VWF has high molecular weight, which is the most efficient form for hemostasis. Platelets α-granules also contribute substantial amounts of coagulation Factor V and Factor XI, as well as thrombospondin-1 which is important for platelet activation via signaling through CD47. α-granules contain a number of antifibrinolytic molecules including α_2-antiplasmin and plasminogen activator inhibitor (PAI-1).

Proteomic studies indicate α-granules contain more than 300 different soluble proteins.[27] Many of the non-hemostatic and systemic effects of circulating platelets are mediated by these molecules, and include chemokines (e.g., PF4, β-TG, MCP-, RANTES and others), antimicrobial proteins (thymosin-β4 and thrombocidins), immune modulators (complement, factor H, IgG), growth factors (PDGF, TGF β and others) and pro-angiogenic (VGF, FGF) and anti-angiogenic (endostatin, angiostatin) factors.

4.5. Aggregation

Platelets contain ≈80,000 αIIbβ3 (GPIIb-IIIa) complexes, the most abundant plasma membrane GP.[28] In the resting platelet, αIIbβ3 exists primarily in a low affinity conformation that is not able to bind its major ligands, which are fibrinogen, VWF, fibronectin and thrombospondin-1. The final common pathway of platelet activation leads to integrin activation to a high affinity state.[29] This is referred to as inside-out signaling. The high affinity conformation binds fibrinogen (or other adhesive ligands), and the bound fibrinogen serves as a bridge to other platelets, resulting in an expanding platelet aggregate. The importance of $\alpha_{IIb}\beta_3$ in platelet function and normal hemostasis is underscored by the moderately severe bleeding seen in patients with Glanzmann thrombasthenia, an inherited disorder caused by absent or dysfunctional αIIbβ3.

αIIbβ3 is the prototypic member of the integrin family of heterodimeric integral membrane adhesion receptors. This receptor consists of 18 α subunits that associate noncovalently with 8 β subunits. αIIb is expressed only in megakaryocytes and platelets, and localizes to the plasma membrane, OCS, and α-granules. β3 has a broad tissue distribution. Platelets also

express the αvβ3 vitronectin receptor in low abundance. Crystalization of the extracellular domain of αvβ3 and the head domain of αIIbβ3 have provided detailed structural information about these integrins.[30, 32]

Talin is an abundant cytoskeletal protein that links integrins to the actin cytoskeleton. The agonist-induced rise in intracellular calcium results in binding of the talin head domain to the cytoplasmic domain of integrin β3. This interaction leads to an unclasping of the intracellular and transmembrane components of the α_{IIb} and β_3 molecules, causing spreading of the two proteins and exposure of the ligand binding site. The precise molecular details by which talin is enabled to bind β3 are unclear, but efficient integrin activation likely involves (1) the guanine nucleotide exchange factor CalDAG-GEFI, (2) activation of the small GTPase Rap1, (3) kindlin-3 binding to the β3 cytoplasmic tail, and (4) calpain cleavage of talin.

5. Role in coagulation

Platelets contribute substantially to thrombin generation, which further induces additional platelet activation. In addition, platelet thrombus stabilization requires local fibrin generation that depends on thrombin generation. When platelets are stimulated by strong agonists, the negatively charged phospholipids on the inner leaflet of the platelet plasma membrane are "flipped" to the outer leaflet. This reorganization may be mediated by the calcium activated scramblase TMEM16F.[33] Translocation of negatively charged phospholipids forms a stage upon which coagulation reactions occur. The formation of the "tenase" complex that converts Factor X to activated Factor X requires phospholipid. The development of the prothrombinase complex also requires negatively charged phospholipid as the surface upon which the complex assembles.

Activation of platelets by strong agonists also leads to the development and shedding of platelet microparticles. These have a high density of negatively charged phospholipids and are thus able to support the formation of the "tenase" and prothrombinase complex as noted above. They also contain coagulation Factor Va with which to support the formation of thrombin as well as supplying arachidonic acid which can contribute to further formation of TXA2.

Platelets α-granule release also provides coagulation factors V, XI and XIII. Factor V may be particularly important as platelet Factor V is modified in a manner rendering it more resistant to cleavage by activated protein C.

6. Platelets in pathologic thrombosis

Pathologic studies show that venous thrombi are platelet-poor, while arterial thrombi are platelet-rich. In addition, although anti-platelet therapy is known to have benefit in preventing recurrent venous thrombi, the benefits appear to be greater for myocardial infarction and

stroke. These pathologic and clinical observations are consistent with the known effect of shear stress on platelet thrombus formation. The effects of higher shear stress are clear for VWF. VWF adopts a folder globular structure under a low shear environment, obscuring the domains that mediate binding to platelets. In contrast, the mechanical effects induced by high shear unfolds VWF and exposes the GPIbα A1-binding domain of VWF. In addition, high shear rates are able to activate platelets directly.[34] In summary, platelets make a modest contribution to venous thrombosis and a more substantive contribution to arterial thrombosis. However, the fundamental molecular mechanisms of platelet thrombus formation appear to be similar in health and disease.

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