Platelet Interaction with the Vessel Wall

Philip G. de Groot, Rolf T. Urbanus, and Mark Roest

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Abstract Platelets have attracted a growing interest among basic scientists and clinicians, as they have been shown to play an important role in many physiological and pathophysiological conditions. Beyond hemostasis, platelets participate in wound healing, inflammation, infectious diseases, maintenance of the endothelial barrier function, angiogenesis, and tumor metastasis. Over the last 50 years enormous progress has been made in our understanding of the role of platelets in hemostasis. Platelets circulate in blood in a resting state, but they are able to react immediately upon a vessel wall injury by adhering to the exposed collagen, followed by platelet–platelet interaction to form a plug that effectively seals the injured vessel wall to prevent excessive blood loss. Comparable events will take place on a rupturing atherosclerotic plaque, which may result in a platelet-rich thrombus. This chapter will address the molecular basis of platelet adhesion and aggregation, the regulation of platelet function and the interaction of primary and secondary hemostasis.

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1 Introduction

Blood platelets originate from megakaryocytes present in the bone marrow (Thiery and Bessis 1956). Every hour about eight billion platelets are released into the circulation, where they circulate for about 10 days before they are removed by the spleen. Platelets are in fact relatively simple cellular fragments (White 2002). With a volume of about 7–9 fL they are small and they lack a nucleus, a clear endoplasmatic reticulum, and Golgi apparatus. The platelet interior is filled with different organelles (Fig. 1). The dense bodies (δ-granules) are the storage pool of low molecular weight components such as adenosine diphosphate (ADP), serotonin, and Ca^{2+}. The α-granules are the most numerous organelles present in platelets and they contain a large variety of proteins, including adhesive proteins, clotting factors, protease inhibitors, growth factors, cytokines, and chemokines. The third organelles are the lysosomes that are full of hydrolytic enzymes. The content of all these organelles can be released upon platelet activation. Platelets also contain large intracellular membrane complexes: the surface-connected open canalicular system and the dense tubular system. The open canalicular system increases the total surface area of the platelets enormously and provides a route for different components to penetrate deeply into the cell. The dense tubular system can be distinguished from the open canalicular system by its amorphous content when visualized with an electron microscope. The dense tubular system originates from the rough endoplasmatic reticulum and is a storage site of intracellular calcium.

Platelets play a pivotal role in normal physiology. Their primary function is to maintain an undisturbed blood flow, as evidenced by the bleeding tendency observed in patients with quantitative and qualitative platelet disorders and the therapeutic efficacy of antiplatelet drugs for thrombotic complications. Besides their role in hemostasis, platelets participate in many other metabolic processes, including wound healing (Nurden 2011), inflammation (Semple et al. 2011), infectious diseases (Yeaman 2010), maintenance of the endothelial barrier function (Nachman and Rafii 2008), angiogenesis (Sabrkhany et al. 2011), and tumor metastasis (Gay and Felding-Habermann 2011). Blood platelets play a major role, or are involved in diseases responsible for the large majority of deaths and disabilities worldwide.

Platelets are an essential component of normal hemostasis. In 1885, Lubnitzky was the first to notice that, in flowing blood, platelets are primary responders in the formation of a hemostatic plug and that fibrin formation occurs as a secondary effect (Lubnitzky 1885). These findings were largely ignored during the next 70 years. It took until the 1960s until it became clear that a platelet response is the first step in the arrest of bleeding and that they are the key players of what now is called primary hemostasis (French et al. 1964). It became obvious that studies on the adhesion and aggregation of platelets were essential not only to understand the

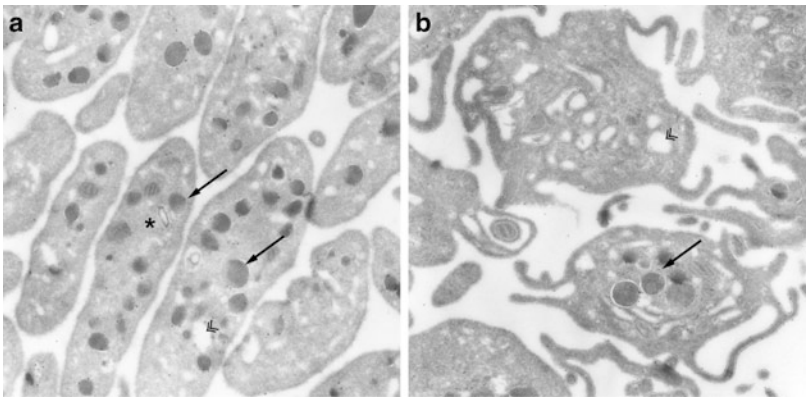

Fig. 1 The platelet interior. (**a**) Transmission electron micrograph (TEM) of resting platelets. Blood was collected in fixative, after which platelets were isolated. (**b**) TEM of thrombin-stimulated platelets. Washed platelets were stimulated with thrombin for 5 min prior to fixation. *Arrows* indicate α-granules, *asterisk* indicates a δ-granule, and the *double arrow* indicates the open canalicular system (OCS). Pictures courtesy of Dr. HFG Heijnen

pathophysiology of a bleeding tendency, but also for our understanding of the formation of arterial thrombi and thus for an optimal treatment of heart attacks and stroke. As a result, an explosive growth in publications on platelet function has been observed over the last 50 years and our knowledge on these little cells has increased concomitantly.

Platelets do not adhere to undamaged vessel walls. They circulate passively through the vascular tree, lined by an intact endothelium. Most platelets will never come into operation during their entire lifetime. Platelets circulate in our blood in a resting state until they are needed, but, if necessary, they can react immediately (Broos et al. 2011). They are circulating monitors of the integrity of the vessel wall and are designed to stop any leak. Platelets circulate in the blood close to the vessel wall on a continuous search for an injury. They have specific receptors on their surface that are able to recognize proteins that will be exposed to the blood after an injury has taken place. Platelets immediately respond when an injury is detected. They adhere, become activated, and start to interact with each other to form a platelet plug that covers the injury. The aggregated platelets also supply a surface to which coagulation factors can bind and fibrin can be formed to stabilize the plug. Platelet function is carefully regulated. Spontaneous or sustained platelet aggregation should be avoided at all times. An optimal platelet response is reached when blood loss is restrained without further damage caused by vascular occlusion. In normal physiology, the endothelium is critical in the control of the haemostatic response (van Hinsbergh 2011). This very efficient and strongly regulated process can be derailed when an atherosclerotic plaque is ruptured and a platelet thrombus is formed inside an intact blood vessel. Nonocclusive thrombi are far more frequently formed than occlusive thrombi (Davies et al. 1989). Mural thrombi are thought to be important contributors of the progression of an atherosclerotic plaque

(Ross and Glomset 1976), whereas an occlusive thrombus will result in the clinical manifestations of heart attack or stroke.

2 Platelet Adhesion

Platelet adhesion is the first step in the hemostatic response to a vascular injury. Platelet adhesion has several unique features. The adhesion of platelets occurs in flowing blood, which means that the adhesive process must occur rapidly and that the adhered platelet must withstand the exposed shear forces (Savage et al. 1996). This has lead to the evolution of adhesive receptors and ligands that are unique for platelet adhesion. Before the first interaction between a platelet and an injured vessel wall can take place, von Willebrand factor (vWF) present in plasma will adhere to the exposed collagens in the subendothelium (Fig. 2) (Sakariassen et al. 1979). The initial contact of the platelet can then take place via glycoprotein Ibα (GPIbα), one of the polypeptides of the platelet receptor complex GPIb–V–IX, with the immobilized vWF (Clemetson 2007). The interaction of vWF with GPIbα is transient and does not allow stable adhesion. This interaction initiates the tethering of platelets over the vessel wall. Platelets will roll over vWF in the direction of flow, driven by the shear forces exerted by the flowing blood (Savage et al. 1996). A continuous loss of GPIbα–vWF interactions at the downstream side of the platelet and the formation of new interactions at the upstream side mediate the rolling process. The rolling process slows down the platelets and enables interaction of platelets with vessel wall proteins via other receptors. The GPIbα–vWF interaction will also induce a weak intracellular signaling that will lead to integrin activation (Du 2007). The reduced velocity in combination with mild platelet activation allows subsequent interaction of additional receptors such as αIIbβ3 (glycoprotein IIb:IIIa; GPIIbIIIa), α2β1 and glycoprotein (GP)VI with vWF and collagen, respectively, resulting in firm adhesion (Savage et al. 1998). To further stabilize the interaction of platelets with subendothelium, platelets will spread (Fig. 3), thereby increasing the number of interactions with the surface. Spreading will help the platelets to withstand the shear forces exerted by the flowing blood. Platelet spreading is primarily mediated by the integrin αIIbβ3 (Weiss et al. 1991). Before stable adhesion and spreading is established, part of the platelets will detach and return to the circulation. The spread platelet provides a new surface for the next platelet to adhere to. The mechanism by which a circulating platelet attaches to already adhered platelets has many similarities to the adhesion of a platelet to the damaged vessel wall (Ruggeri 2000). The circulating platelet must attach to an adhered platelet when it is still subjected to shear forces. The adhered platelet will bind fibrinogen and vWF from the circulation via αIIbβ3 and GPIbα, respectively, creating an ideal surface for the next platelet to adhere.

Von Willebrand factor as adhesive surface is essential for optimal platelet adhesion at higher shear rates (Nieswandt et al. 2011). However, the subendothelium and connective tissue contain many other adhesive molecules, such as collagen types

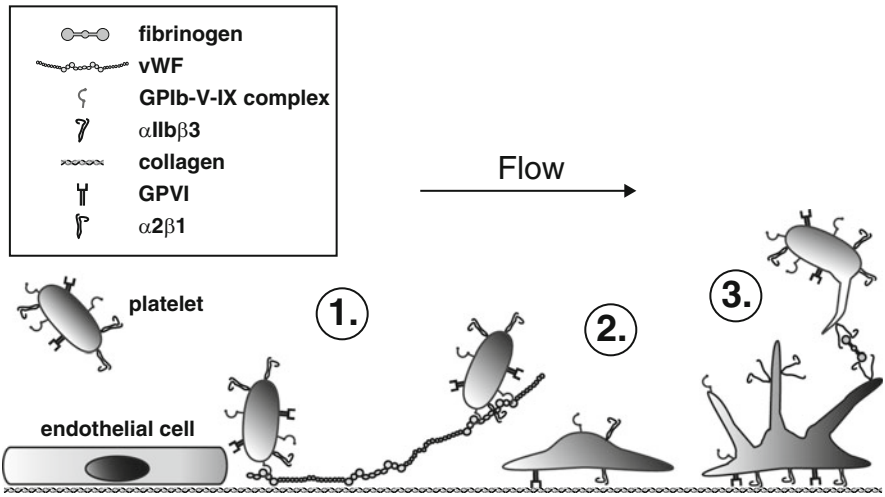

Fig. 2 Schematic overview of platelet adhesion. Platelets circulate in a resting state at high velocity. (1.) Upon vascular damage, the plasma protein Von Willebrand factor (vWF) will bind to the exposed subendothelial collagen via its A3 domain, after which it adopts its platelet-binding conformation. VWF then captures platelets from the circulation via the interaction of the vWF A1-domain with the GPIb–V–IX complex on the platelet. Since the interaction between vWF and GPIb–V–IX is transient, this will result in the slowing down and subsequent rolling of platelets over the injured vessel wall. (2.) The rolling platelet will then firmly adhere to the exposed collagen via the integrin α2β1. The interaction of GPVI with collagen will result in further platelet activation. (3.) Activated platelets will spread and undergo a shape change that dramatically increases their surface area. They will subsequently release the contents of the α- and δ-granules, which leads to the activation of the second layer of rolling platelets. Activated platelets express the integrin αIIbβ3 in its high-affinity fibrinogen-binding conformation, which will result in platelet aggregate formation

I, III, IV, V and VI, laminin, fibronectin and thrombospondin, for all of which platelets possess receptors (de Groot and Sixma 2002). All these adhesive proteins can support platelet adhesion at lower shear stresses. Which receptor(s) and extracellular protein(s) are involved in the different stages of adhesion depends on the vascular bed and nature of the injury. Local shear stress in combination with the composition of the exposed subendothelium, which depends on the site, type and severity of the injury, will probably determine the importance of the individual receptors. In the venous system, low shear rates allow the interaction of many different receptors with their ligands. In the arterial circulation, higher shear forces limit the participation of the majority of receptors and the major adhesive protein is thought to be vWF. VWF is present in the vessel wall, but its amount is often limited: the exposed collagen fibers present in the subendothelium rapidly bind plasma vWF (Lisman et al. 2006).

Platelets can adhere to laminin via α6β1 (Hindriks et al. 1992), to fibronectin via α5β1 (Beumer et al. 1995), to thrombospondin via GPIbα (Jurk et al. 2003), to fibrinogen via αIIbβ3 (Hantgan et al. 1992), and directly to collagen via α2β1 and GPVI (Pugh et al. 2010). The interaction of platelets with these different proteins

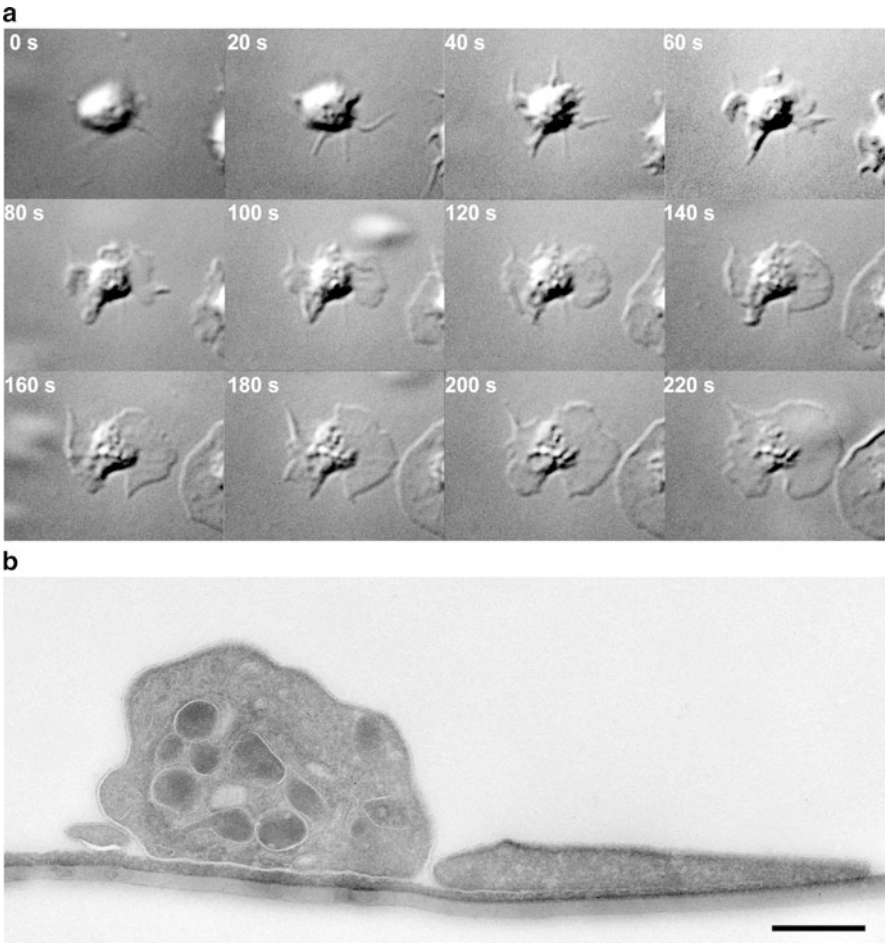

Fig. 3 Platelet spreading. (**a**) Washed platelets were perfused over a fibrinogen surface at a shear rate of 100 s^{-1}. Platelet spreading was visualized with differential interference microscopy. Snap shots were taken every 20 s. Pictures courtesy of Dr. V De Angelis. (**b**) TEM of surface-adhered platelets. Picture courtesy of Dr. HFG Heijnen

has been studied with isolated and purified proteins, but the contribution of the individual proteins in the much more complex subendothelium remains to be investigated. Some of these receptors have a very low surface density. However, there may be synergy of multiple substrate receptors. On the other hand, mutual interactions between proteins in the subendothelium might specifically shield off certain adhesive domains (Agbanyo et al. 1993). Studies with genetically modified mice have suggested significant roles for α5β1, α6β1, and αIIbβ3 in platelet adhesion (Gruner et al. 2003) indicating that platelet adhesion in vivo is a highly integrated process involving multiple interactions, none of which is essential by itself.

3 Von Willebrand Factor–GPIbα Interaction

It is apparent that vWF–GPIbα interactions are essential and unique for the adhesion of platelets at higher shear rates. VWF is present in plasma and subendothelium but there are additional sources of vWF in the Weibel-Palade bodies of endothelial cells and α-granules of platelets. The content of these organelles will be released when the hemostatic process is initiated and increase the local concentration. The plasma concentration of vWF is about 10 μg/mL, the concentration in platelets is about 280 ng/10^9 platelets (Sadler 2005). The normal range of plasma vWF is broad and strongly influenced by blood groups, with 25% lower levels in blood group O and high levels in individuals with AB loci (Sadler 2005). VWF is a large multimeric protein synthesized by endothelial cells and megakaryocytes. VWF present in the circulation cannot bind spontaneously to platelets, as the GPIbα binding site within vWF is cryptic. VWF must first bind to collagen via its A3-domain before it can interact with GPIbα via its A1-domain (Lankhof et al. 1996). The conformational changes that coincide with the binding to collagen and are necessary to expose the A1-domain are only partly understood. Other domains of vWF will probably shield the A1 domain (Ulrichts et al. 2006). Collagen is not the only binding site for vWF in the vessel wall, vWF can be immobilized via self-assembly (Savage et al. 2002). Circulating vWF can bind to vWF in the subendothelium via multiple domain interactions.

The overall shape of vWF in the circulation is dynamic. When vWF binds to collagen, shear stress will force vWF into an elongated conformation in the direction of flow, the ideal route for a platelet to roll on. This might explain why the hemostatic potency of vWF depends so strongly on its multimeric structure (Sixma et al. 1984). The length of the vWF molecules is accurately regulated. Individuals lacking the highest multimers of vWF, as in von Willebrand disease type 2a, have a severe bleeding tendency, while the presence of ultra-large multimers of vWF in the circulation will result in microangiopathy. VWF in the Weibel-Palade bodies of endothelial cells appears to be ultra-large. After secretion form the endothelial cells, the cleavage protease ADAMTS-13 will trim vWF to the length found in plasma (Dong 2005).

About 25,000 copies of the GPIb–V–IX complex are expressed on the surface of platelets. The complex consists of GPIbα, GPIbβ, GPV, and GPIX in a 2:4:1:2 ratio (Luo et al. 2007). The members of the complex belong to the leucine-rich repeat receptor family. GPIbα can bind many different ligands besides vWF, amongst others P-selectin, MAC-1, β2-glycoprotein I and the coagulation factors thrombin, VII, XI and XII (Andrews et al. 2003). The crystal structure of the complex between the extracellular part of GPIbα and the A1-domain of vWF showed GPIbα wrapped around one side of A1, providing two contact areas bridged by an area of solvated charge interaction (Huizinga et al. 2002). The bond between GPIbα and the A1-domain is termed a flex-bond, as it can exist in two states, a low-affinity and an extended, high-affinity state (Kim et al. 2010). One state is seen at low force; a second state begins to engage at a force of 10 pN and has greater force resistance

and an approximately 20-fold longer lifetime. Switches between the two states are induced by shear forces and determine the lifetime of an individual bond. This subtle interaction between shear stress and affinity of GPIbα for vWF allows the platelet to withstand a broad range of forces.

The essential role of vWF–GPIbα interaction is highlighted by the bleeding complications observed in patients with von Willebrand's disease and Bernard-Soulier Syndrome. Studies with mice lacking these proteins have confirmed the importance of this interaction (Denis et al. 1998; Ware et al. 2000). Furthermore, it has been shown that mice lacking these proteins are protected against thrombotic complications (Konstantinides et al. 2006). Deficiencies of ADAMTS-13 will result in thrombotic thrombocytopenic purpura, a syndrome characterized by the presence of ultra-large multimers of vWF, thrombocytopenia, hemolytic anemia, schistocytes, and organ failure (Chauhan et al. 2008).

4 Platelet Activation

The interaction between subendothelial collagen, vWF, and the GPIb–V–IX complex on platelets facilitates the initial capture of platelets from the circulation to the site of the injury. This interaction allows the direct binding of GPVI to collagen, which triggers immuno-receptor-based activation motif (ITAM)-regulated signaling pathways (Watson et al. 2010), leading to activation of adhered platelets (Fig. 4). Binding of GPVI to collagen furthermore triggers α2β1 inside-out activation (Chen et al. 2002). Activated α2β1 is important for firm adhesion of platelets to the collagen surface and further activation of the adhered platelets (Santoro 1986).

GPVI is a 62-kDa type I transmembrane receptor of the immunoglobulin superfamily of surface receptors, which is exclusively expressed in platelets and megakaryocytes. The signaling capacity of GPVI depends on its association with an Fc receptor (FcR) γ-chain homodimer. Each FcR γ-chain monomer contains a conserved ITAM-motif, which typically consists of two conserved YXXL motifs separated by 6–12 amino acids. Upon receptor cross-linking by the ligand, collagen, these two conserved ITAM tyrosine residues are phosphorylated by the Src family tyrosine kinases Fyn and Lyn. This phosphorylation then leads to recruitment and activation of the tyrosine kinase Syk, which regulates a complex downstream pathway that involves the adapter proteins LAT, Gads and SLP-76, the Tec family tyrosine kinases Btk and Tec, the GTP exchange factors Vav1 and Vav3, PI3-kinase isoforms and phospholipase C (PLC)γ2 [for detailed reviews, see (Watson et al. 2010; Jung and Moroi 2008)]. GPVI-deficient platelets aggregate normally on ADP and thrombin, but do not aggregate on collagen. GPVI-deficient patients have mildly prolonged bleeding times (Kato et al. 2003), which suggests that platelet activation by collagen can be by-passed by other activation processes. Inhibition of the GPVI pathway may reduce thrombosis risk (Nurden and Nurden 2011), which makes the GPVI-mediated signaling pathway an attractive target for novel antiplatelet therapy.

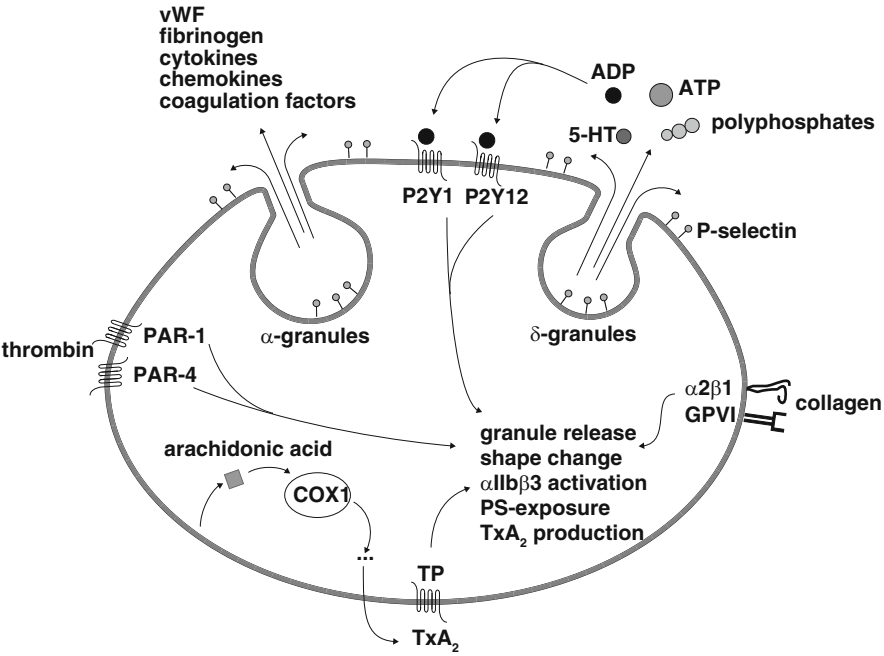

Fig. 4 Schematic overview of the major platelet activation pathways. Platelets can be activated by collagen via the integrin α2β1 and the platelet receptor GPVI, thrombin via the interaction with protease-activated receptor (PAR)-1 and -4 and ADP via the purinergic receptors P2Y1 and P2Y12. Interaction of each ligand with their respective receptors results in the transition of αIIbβ3 from a low- to a high-affinity conformation, platelet cytoskeletal rearrangement, the release of α- and δ-granule content and P-selectin expression. Dense granule ADP augments platelet activation. Upon platelet activation, arachidonic acid is liberated from the plasma membrane and converted to thromboxane A2 (TxA2) in a series of reactions that involves the enzyme cyclo-oxygenase (COX)-1. TxA2 furthers augments platelet activation

The integrin α2β1 requires an agonist-induced conformational change via inside-out signaling to bind to collagen. The underlying "inside-out" signaling events translate into talin binding to α2β1 (Nieswandt et al. 2009). Binding of α2β1 to collagen contributes to cellular activation both indirectly, by reinforcing GPVI–collagen interactions, and directly, by a series of intracellular signaling events, which are strikingly similar to GPVI-induced signaling and include Src, Syk, SLP-76, and PLCγ2. Thus, although structurally unrelated, the two major collagen receptors share important signaling molecules and act in a cooperative manner, reinforcing each other's activity. It is now generally accepted that they act synergistically in the process of α- and δ-granule release, activation of the integrins αIIbβ3 and α2β1, shape change and spreading, phosphatidylserine exposure, and matrix metalloproteinase activation.

Collagen-induced platelet activation triggers the formation of a baseline layer of aggregated platelets, but does not support additional layers at greater distance from

the subendothelium (Offermanns 2006). Second-wave mediators, such as ADP and thromboxane that are released from the baseline layer of activated platelets, are required for further thrombus growth. ADP is stored in the δ-granules of platelets. Upon platelet activation, ADP is rapidly released to cause auto-activation and to activate surrounding platelets. When ADP is secreted, it amplifies the response induced by other activators and it stabilizes platelet aggregates. Platelets contain two receptors for ADP: P2Y1 and P2Y12. Both receptors play a role in platelet aggregation and aggregate stabilization (Cattaneo 2011). Remarkably, although ADP stimulation of platelets has a strong effect on αIIbβ3 inside-out signaling, it has only minor impact on the release of α- and δ-granule content (Cattaneo et al. 2000).

P2Y1 is a G_q-coupled receptor that mediates the activation of PLCβ, which subsequently converts PIP2 into the second messengers DAG and IP3. IP3 opens calcium channels and DAG activates PKC and calDAG-GEFI (CD-GEFI). CD-GEFI contains binding sites for Ca^{2+} and diacylglycerol (DAG) and a guanine nucleotide exchange factor (GEF) domain catalyzing the activation of Rap1. Then the Rap1 effector molecule (RIAM) interacts with Talin-1 to unmask its integrin-binding site. The binding of Talin-1 to αIIbβ3 disrupts the salt bridges between the α and β subunits, which changes the integrin conformation from a low- to a high-affinity state. Kindlin-3 supports this process by binding to the β-tail. Individuals with mutations in Kindlin-3 show defects in αIIbβ3 activation and express a bleeding tendency.

P2Y12 is coupled to G_i-type G proteins, in particular G_{i2} (Offermanns 2006). Upon stimulation of the heterotrimeric receptor, the α-subunit is released from the βγ-complex. The βγ-complex is a second messenger that stimulates phosphoinositide 3-kinase (PI3K). The most important target protein of PI3K is Akt, which regulates platelet function by phosphorylating and inhibiting GSKβ. PI3K also activates the small GTPase Rap1b, which is a critical regulator of αIIbβ3 inside-out signaling. In addition, the liberated α-subunit of the G_{i2} protein inhibits adenylyl cyclase (AC) and thereby counteracts cyclic adenosine monophosphate (cAMP), which inhibits the release of Ca^{2+} from intracellular stores. This function is mainly important to overrule the inhibitory effects of prostacyclin (PGI_2) secreted by endothelial cells.

Patients with functional genetic modifications in P2Y12 show a variable bleeding diathesis, characterized by mucocutaneous bleedings, excessive postsurgical bleeding, and mild to moderate posttraumatic blood loss. These patients show impaired platelet reactivity to ADP even at high concentrations. P2Y12 is one of the most effective targets for platelet inhibition to prevent secondary cardiovascular disease events. Several different P2Y12 inhibitors have been widely used, including thienopyridines (ticlopidine, clopidogrel, and prasugrel), while direct P2Y12 inhibitors (tricagrelor) have entered the market (Storey 2011).

Analogous to ADP, thromboxane A2 can (auto-)activate platelets via a positive feedback loop during platelet activation. Thromboxane A2 is produced from arachidonic acid that is liberated from the plasma membrane by phospholipase A2, through conversion by cyclooxygenase-1 (COX1). Prevention of thromboxane

A2 formation in platelets by COX1 inhibitors, such as acetylsalicylic acid, belongs to the most commonly used medication in the prevention of secondary events of cardiovascular disease. Thromboxane A2 interacts with the thromboxane A2 receptor (TP) on platelets and triggers both G_q- and G_{13}-mediated signaling pathways (Stegner and Nieswandt 2011).

Another potent activator of platelets is thrombin, the final product of the coagulation system, which activates platelets via protease-activated receptor (PAR)-1 and PAR-4. The activation of PARs is a two-step process. First, the cryptic ligand is unmasked by proteolytic cleavage of the N-terminal domain of the receptor. Then, an intramolecular rearrangement allows the ligand and the receptor moieties to interact, which results in intracellular signaling. PAR-1 is activated at low thrombin concentrations, while PAR-4 mediates platelet activation at high thrombin concentrations (Leger et al. 2006). Both PAR-1 and PAR-4 couple to G_q, which mediates signaling through its interaction with PLCγ2 and the subsequent conversion of phosphatidylinositol bisphosphate (PIP2) into diacylglycerol and inositol-triphosphate (IP3). This opens calcium channels, and activates PKC and CD-GEFI, catalyzing the activation of Rap1 and Rap2 to regulate inside-out activation of αIIbβ3. In addition, PAR-1 and PAR-4 couple to G_{13} to activate guanidine nucleotide exchange factors (GEFs) for the small G-protein RhoA, which induces myosin light chain phosphorylation and myosin-dependent contraction. This pathway is of major importance for platelet shape change. Deficiency of G_{13} causes more dramatic effects for platelet adhesion than for integrin activation, aggregation or granule secretion. It has been suggested that PAR-1 and PAR-4 may also couple to G_i, although this interaction is still debatable.

5 Thrombus Stability

A growing thrombus typically consists of a dense inner core of fully activated platelets covered by a layer of loosely bound platelets. Platelet recruitment to this growing thrombus requires only minimal activation, since tethering platelets retain their discoid shape. Over time, some of these discoid platelets will become fully activated and release their granule content, whereas others will detach from the thrombus and embolize (Furie and Furie 2005; Nesbitt et al. 2009). The transition of αIIbβ3 from a low- to a high-affinity state that accompanies activation allows further platelet–platelet interactions to take place, a process dependent on the association of the integrin with its ligand and subsequent outside-in signaling. When close contacts between adjacent platelets are formed, several other surface molecules will interact with their counterparts and induce contact-dependent signaling, which regulates thrombus formation and stability.

One of the surface receptors reported to influence thrombus stability is CD40L (CD154), a receptor best known from its role in T-cell-mediated B-cell responses. Although platelets only express an estimated 600–1,000 copies of this receptor, it enhances the activation of αIIbβ3, the most abundant platelet receptor, by binding

to the extracellular portion of the β3 integrin via its KGD sequence (Andre et al. 2002). Interestingly, the absence of CD40L has no effect on hemostasis, but appears to protect against thrombosis in a murine thrombosis model. One of the most pronounced features in these mice is the increased embolization rate after thrombus induction, indicating thrombus instability. In humans, addition of soluble CD40L strongly augments thrombus formation under conditions of flow.

Activation of the integrin αIIbβ3 is also enhanced by the Eph kinases EphA4 and EphB1 and their ligand Ephrin B1. The formation of close contacts between adjacent platelets in a growing thrombus allows the interaction between the Eph kinases and Ephrin B1 to occur, which leads to intracellular signaling events. Interaction of the EphA4 or EphB1 kinases with Ephrin B1 results in the activation of Rap1, a small GTPase involved in αIIbβ3 engagement (Prevost et al. 2004). Moreover, EphA4 is constitutively associated with the β3 integrin, where it facilitates the phosphorylation of the β3 integrin cytoplasmic domain and its association with myosin (Prevost et al. 2005). Blockage of either the Eph kinases or Ephrin B1 results in decreased thrombus size and defects in clot retraction.

Semaphorin 4D is another platelet receptor involved in the regulation of stable thrombus formation. Engagement of semaphorin 4D by its ligands plexin-B1 and CD72, all of which are expressed by platelets, augment the response to collagen, but not to other stimuli. The effects of semaphorin 4D on the platelet response to collagen are self-limiting, as the protein is quickly shed from the membrane by the metalloprotease ADAM17. Similar to mice deficient in CD40L, mice deficient in semaphorin 4D show delayed arterial thrombus formation (Zhu et al. 2007). Interestingly, the function of another surface receptor involved in contact-dependent signaling, platelet and endothelial cell adhesion molecule (PECAM)-1, directly opposes the function of semaphorin 4D. PECAM-1 dimerization, either in trans or on the same platelet, specifically inhibits the collagen pathway of platelet activation, but has no influence on ADP or thrombin-induced activation (Newman and Newman 2003). Whereas binding of semaphorin 4D to CD72 results in the inactivation of SHP-1, which downregulates collagen signaling, PECAM-1 dimerization results in SHP-2 recruitment.

The vitamin K-dependent protein known as growth arrest-specific gene 6 (gas6) has also been implicated in the regulation of thrombus stability. Murine platelets have been shown to contain both gas6 and express its receptors Axl, Mer and Tyro3 on their surface, deficiency of which is associated with impaired arterial and venous thrombus formation (Angelillo-Scherrer et al. 2001). However, the presence of gas6 in human platelets remains controversial and the effects of gas6 on human platelets are questioned (Clauser et al. 2006). Nevertheless, there are reports that gas6 enhances the ADP-dependent activation of αIIbβ3 (Cosemans et al. 2010).

Most of the stimulatory regulators of contact-dependent signaling, i.e., CD40L, the Eph kinases and semaphorin 4D, are expressed in low levels on resting platelets, with increased surface expression upon platelet activation. Whether this additional pool of receptors originates from the open canalicular system or from α-granules is unclear. Another receptor involved in contact-dependent signaling that becomes surface-exposed upon platelet activation is endothelial cell-specific adhesion

molecule (ESAM). This receptor, a member of the CTX family of adhesion receptors, localizes to the junctions between adjacent platelets, where it negatively regulates stable platelet–platelet interactions. Similar to PECAM-1 knockouts, deficiency of ESAM is a gain-of-function mutation (Stalker et al. 2009), with ESAM-deficient mice exhibiting increased thrombus formation upon vascular challenge. The way in which ESAM regulates thrombus stability is currently unclear.

6 Platelets and the Endothelium

The vascular endothelium is the interface between blood and the subendothelial tissue and is critical for the regulation of the hemostatic response (van Hinsbergh 2011). Endothelial cells line the entire vasculature. Under normal conditions, circulating platelets do not adhere to intact endothelium. The endothelium releases substances that suppress unwanted platelet activation in an intact blood vessel. Damage of the endothelial cell layer and, to a lesser extent, endothelial cell dysfunction will promote a pro-thrombotic state.

The luminal surface of the endothelium is covered with a negatively charged glycocalyx. Resting platelets cannot bind to the surface of endothelial cells due to electrical repulsion by negatively charged heparan sulfates (Reitsma et al. 2011). This defense mechanism can be overcome by activated platelets. Binding of P-selectin or GPIbα on activated platelets to the protein PSGL-1 on the surface of the endothelium will result in rolling of platelets over the endothelium (Frenette et al. 1995). Platelet heparanase, which is released from activated platelets, can facilitate this interaction by degrading proteoglycans in the endothelial glycocalyx (Pries et al. 1997).

Endothelial cells contain prostacyclin synthase, an enzyme that converts endoperoxides into prostacyclin (Yuhki et al. 2010). Peroxides are synthesized from arachidonic acid by cyclo-oxygenases (COX) 1 and 2 in every cell, but the further processing of endoperoxides is cell type-specific. Prostacyclin binds to the prostanoid receptor IP, a seven transmembrane spanning G-protein-coupled receptor on the platelet membrane (Stitham et al. 2007). Binding of prostacyclin stimulates adenylyl cyclase activity. The resulting increase in platelet cyclic AMP leads to calcium re-uptake by the dense tubular system, thereby inhibiting stable platelet adhesion and platelet aggregation (Noe et al. 2010). Prostacyclin is one of the most potent inhibitors of platelet aggregation. Mice lacking the prostacyclin receptor IP show an increased thrombotic tendency, intimal hyperplasia, accelerated atherosclerosis, restenosis, and reperfusion injury (Yuhki et al. 2011). These observations have been supported in human population-based studies in which a significant increased coronary disease was observed in individuals with functional mutations in the prostacyclin receptor (Stitham et al. 2011). Furthermore, the world-wide withdrawal of selective COX-2 inhibitors due to an increased

incidence of myocardial infarction and thrombotic strokes in users points to the importance of COX-2-derived endothelial cell prostacyclin synthesis.

Nitric oxide (NO) is a second endothelial cell product that is involved in the maintenance of its antithrombotic properties (Palmer et al. 1987). Nitric oxide released from the endothelium diffuses through the platelet membrane and binds to the heme moiety of guanylyl cyclase. This will lead to increased levels of cyclic AMP and downregulation of platelet activation. The importance of NO as antithrombotic mediator was recently demonstrated in a mouse model of the antiphospholipid syndrome. The antiphospholipid syndrome is accompanied by recurrent thrombosis. Interaction of antiphospholipid antibodies with the LRP8 receptor on endothelial cells resulted in de-phosphorylation of NO synthase at its Ser1179 site, a reduced NO production and an increased tendency to thrombus formation (Ramesh et al. 2011). The antithrombotic effect of endothelial cells may be the consequence of a combined action of prostacyclin and nitric oxide. Synergistic antiaggregatory effects have been shown on platelets. Both prostacyclin and nitric oxide are not only involved in the prevention of platelet activation, they are also important regulators of the vascular tone.

In addition to the effects of prostacyclin and nitric oxide, endothelial cells can reduce platelet activation with a surface-bound ecto-ADPase (CD39) (Gayle et al. 1998). ADP released form platelets, which mediates an amplification loop for platelet activation, will be metabolized by CD39. The formed AMP will be further metabolized to adenosine, which inhibits platelet aggregation. CD39-deficient mice have an increased spontaneous thrombotic tendency, suggesting that CD39 plays an important role in the regulation of primary hemostasis. Besides these direct effects on platelet function, endothelial cells also influence platelet function indirectly via downregulation of thrombin formation via the expression of thrombomodulin (Esmon 2003), the synthesis of tissue factor pathway inhibitor (TFPI) (Broze et al. 1990), and the regulation of vWF mutimeric size via ADAMTS-13.

7 Interaction Between Primary and Secondary Hemostasis

The formation of a platelet plug to seal a defect in the vascular wall is only the first step in the prevention of blood loss. Without further consolidation, this platelet plug will dissolve within hours and merely delay bleeding. Platelet aggregates that are formed at a site of injury are therefore rapidly strengthened by the formation of an insoluble fibrin network. The importance of this process is illustrated by a test of platelet function, the bleeding time test, in individuals with reduced or deficient levels of coagulation factor (F)VIII (hemophilia A): despite initial staunching of bleeding from the skin lesion that is induced in this test due to the formation of a platelet plug, these individuals will re-bleed from the wound after several hours due to inadequate fibrin formation (Sixma and van den Berg 1984). Based on these and other observations, the process of platelet adhesion and plug formation is generally referred to as primary hemostasis, whereas the formation of a fibrin network that

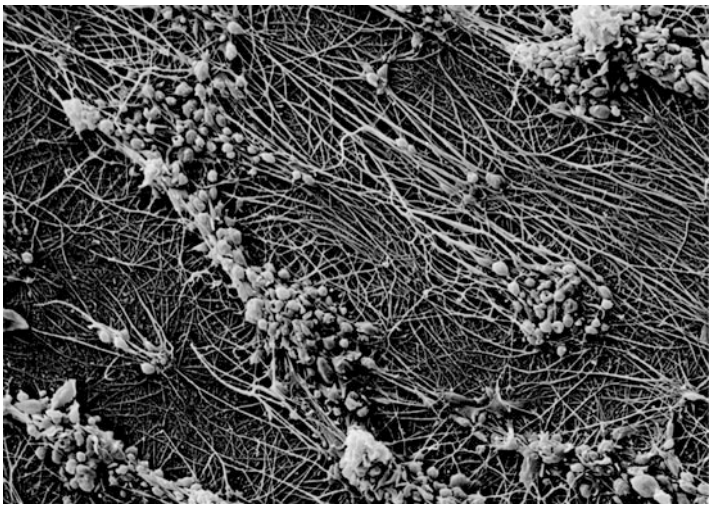

Fig. 5 Platelets and the coagulation system tightly interact during thrombus formation. Scanning electron micrograph of thrombi formed after perfusion of recalcified blood over endothelial cell matrix. Picture courtesy of Dr. HFG Heijnen

stabilizes this platelet aggregate is referred to as secondary hemostasis. We now know, however, that both primary hemostasis and secondary hemostasis tightly interact in the formation of a thrombus in vivo (Fig. 5).

Blood coagulation occurs when the soluble plasma protein fibrinogen is converted into insoluble fibrin fibers by the protease thrombin (Fig. 6). This enzyme is the final product of an intricate series of enzymatic reactions that starts with the exposure of blood to tissue factor (TF) (the extrinsic pathway of coagulation), or negatively charged surfaces such as glass (the intrinsic pathway of coagulation). We differentiate between these two pathways, because they largely depend on different enzymatic reactions. The intrinsic pathway of coagulation starts with activation of coagulation factor XII (FXII) on a negatively charged surface such as glass. This will lead to the activation of factor XI (FXI). Activated FXI (FXIa) will then activate factor IX, which forms a complex with its cofactor activated FVIII (FVIIIa) that is known as the intrinsic tenase complex and activates coagulation factor X (FX). The extrinsic pathway of coagulation starts with the exposure of TF to blood and the subsequent binding of circulating activated factor VII (FVIIa). This complex, known as the extrinsic tenase complex, can then activate FX. Both the intrinsic and the extrinsic pathway of coagulation culminate in the common pathway of coagulation, in which prothrombin is converted into thrombin by the prothrombinase complex, which consists of activated FX (FXa) and its cofactor activated factor V (FVa). Nevertheless, these differentiations largely apply to a diagnostic setting, because the initiation of coagulation by TF exposure is considered far more important than the activation of FXII by negatively charged surfaces in vivo. Moreover, the intrinsic and extrinsic pathway of coagulation partially

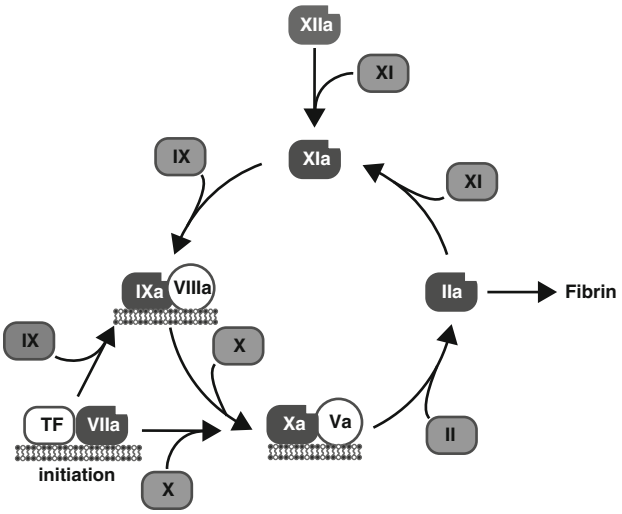

Fig. 6 The coagulation system. Coagulation is initiated by the exposure of flowing blood to extravascular TF, which rapidly binds circulating FVIIa. The TF–FVIIa complex activates FX, which—together with its cofactor FVa—converts prothrombin (FII) to thrombin (FIIa). Thrombin can then cleave fibrinogen into fibrin and coagulation ensues. Thrombin formation leads to more thrombin formation through a process called feedback activation, in which thrombin activates FXI. FXIa then activates FIX, which—together with its cofactor FVIIIa, (the intrinsic tenase complex) can then generate more FXa. Thrombin formation increases considerably through this process. When only small amounts of TF are exposed to blood, the TF–VIIa complex can also directly activate FIX. The intrinsic tenase complex then rapidly produces more FXa, which causes thrombin formation. FXII appears to be irrelevant for physiological hemostasis, but is reported to play a role in thrombosis

overlap, as the extrinsic tissue factor–factor VIIa complex can also activate FIX and the thrombin that is formed during the initial coagulation reaction causes feedback activation of FXI to generate more thrombin.

Platelets are considered crucial components of the coagulation reaction in vivo for several reasons. First of all, activated platelets are thought to provide the surface on which coagulation takes place. Most of the coagulation reactions take place on a surface containing the negatively charged phospholipid phosphatidylserine (PS). This greatly enhances the efficiency of the coagulation reaction, since it minimizes entropy, prevents the washout of activated coagulation factors by the flowing blood and ensures that coagulation only takes place at sites where it is needed. PS is normally present on the inner leaflet of the plasma membrane of every cell. PS exposure on the outer membrane is generally considered a marker for apoptosis and will result in rapid clearance. In platelets, a subpopulation expresses PS upon full activation, which is enough to support the coagulation reaction. The protein that mediates this PS exposure was recently identified as transmembrane protein 16F (TMEM16F) (Suzuki et al. 2010) in a patient with Scott Syndrome, a rare bleeding disorder characterized by defective PS exposure at sites of vascular injury.

A second reason why platelets are important regulators of coagulation is that platelet α-granules contain several coagulation proteins that are secreted upon activation, such as prothrombin and FV. Platelets contain approximately one-fifth of the entire FV pool in human circulation (Camire et al. 1998), which is both synthesized and taken up from blood plasma by the megakaryocyte. The importance of platelet FV for coagulation is also illustrated by the bleeding phenotype in severe FV-deficient individuals. Despite extremely low or undetectable levels of FV in the plasma of these individuals, the presence of minute amounts of FV in their platelets, which act as FV reservoirs, can ameliorate the severity of the bleeding phenotype associated with FV deficiency (Duckers et al. 2010).

Alpha-granules also contain several inhibitors of the coagulation reaction (Maynard et al. 2010), such as antithrombin and C1-inhibitor. Both of these molecules are serine protease inhibitors (SERPINS) and can inhibit several coagulation factors, amongst others FXIa, FIXa, FXa, and thrombin. Protein S is also reported to be in platelet α-granules. This protein acts as a cofactor for activated protein C, which degrades the cofactors FV and FVIII, thereby attenuating the coagulation reaction. Protein S also functions as a cofactor for tissue factor pathway inhibitor (TFPI) (Hackeng et al. 2006), an inhibitor of the extrinsic tenase complex, which has been reported to be present on the surface of activated platelets as well (Maroney et al. 2007).

The contact phase of the intrinsic pathway of coagulation, i.e. the activation of FXII by negatively charged surfaces, has been considered irrelevant for physiological hemostasis for a long time, because FXII deficiency is not associated with a bleeding phenotype. It has, however, gained renewed interest since the discovery that FXII plays a role in thrombus formation in a murine arterial thrombosis model (Renne et al. 2005). The mechanism via which FXII mediates this prothrombotic influence is currently under investigation. One of the first questions to address is how FXII is localized to the growing thrombus, as it lacks the ability to bind to negatively charged phospholipids to prevent being washed away by the flowing blood. One possibility is that it binds to platelet receptors. Indeed, FXII is reported to bind directly to platelets via the GPIbα subunit of the GPIb–V–IX complex (Bradford et al. 2000). Furthermore, it has been known for a long time that FXII can bind to negatively charged regions on exposed subendothelial collagen (Niewiarowski et al. 1964; Wilner et al. 1968). This brings us to the other issue that needs to be resolved: how does FXII get activated during thrombus formation? One possibility is the collagen, which has been shown to activate FXII (Wilner et al. 1968; Kawamoto and Kaibara 1990). Other potential activators of FXII in the setting of a growing thrombus are polyphosphates. Platelet δ-granules contain polyphosphates that are secreted upon activation (Ruiz et al. 2004). Recent experimental evidence suggests that polyphosphates can indeed activate FXII and mediate FXII-dependent thrombus formation (Muller et al. 2009). Moreover, polyphosphates have been shown to enhance both the activation of FV by thrombin and factor Xa activity, thereby influencing the propagation of coagulation (Smith et al. 2006). They have also been implicated as modulators of fibrin fiber thickness (Smith and Morrissey 2008). All of these processes have been attributed to different lengths of

polyphosphate polymers (Smith et al. 2010). Which of these effects of polyphosphates are most important in thrombus formation remains to be determined, although the polymer length of δ-granule polyphosphate seems to favor FV activation (Smith et al. 2010).

FXI has also been implicated as a major player in thrombosis, whereas its role in hemostasis seems to be of less importance. Deficiency of the molecule is associated with only a mild bleeding tendency, but appears to protect against excessive venous and arterial thrombus formation in in vivo models of thrombosis (Zhang et al. 2010; Wang et al. 2006; Rosen et al. 2002). Similar to FXII, FXI lacks phospholipid-binding capacity to support its localization to the growing thrombus in flowing blood. However, the enzyme can bind to two receptors on the platelet surface, GPIbα (Baglia et al. 2003) and LRP8 (White-Adams et al. 2009), which may retain FXIa on the platelet surface long enough for it to activate FIX. The role of FXI in thrombosis seems to be the amplification of thrombin formation via feedback activation on the growing platelet thrombus, but the exact mechanism of FXI-dependent thrombus formation is not fully understood. There are indications that the prothrombotic effect of FXI on thrombus formation is independent of both FXII and FVIIa (Tucker et al. 2009), which makes it hard to envision how FXI is activated.

Further strengthening of the thrombus by the fibrin network occurs when the clot contracts, a process driven by platelets and dependent on the interaction of the fibrinogen receptor αIIbβ3 with the actin cytoskeleton (Cohen et al. 1982). Interestingly, binding of αIIbβ3 to fibrin appears to involve a different epitope on the receptor than the epitope that binds fibrinogen, because some mutations that are associated with Glanzmann Thrombasthenia do not abolish the ability of platelets to mediate clot contraction, despite absent fibrinogen binding (Ward et al. 2000; Kiyoi et al. 2003).

8 Conclusions

A significant progress has been made over the last 50 years in our understanding of platelet function. We now have a detailed understanding of fundamental cellular processes and molecular interactions that determined platelet adhesion and activation. Thanks to structural biology, we have solved the structures of GPIbα and vWF and we start to understand the processes that underlie the flow-regulated interaction between these two unique proteins. We have unraveled the process of integrin activation at the atomic level. The next few years new information will be obtained from genomics, proteomics and system biology studies that will add new dimensions to our knowledge on platelet function in different parts of the vasculature, observations that may allow more rational drug design. As we are unable to identify the cause of a bleeding tendency in many patients at present, perhaps microarray-based gene expression analysis will allow a more in-depth analysis of bleeding disorders.

One of the major drawbacks in platelet research is the impossibility to genetically modify human platelets, making detailed studies on platelet function difficult. We completely rely on studies with genetically modified mice. We know that platelet function in mice can be different from platelet function in humans in many respects. The development of methods to produce platelets in significant amounts from cultured human megakaryocytes would significantly improve our possibilities to determine the importance of different signaling pathways for optimal platelet function.

Knowledge Gaps

- The differences in platelet responses in distinct parts of the vasculature.
- Regulation of secondary hemostasis by platelets.
- Approaches to modulate platelet function without risk of bleeding.
- Fine regulation of the release of granule contents.
- How do platelets participate in immunity and infections?

Key Messages

- The role of platelet in (primary) hemostasis.
- The regulation of platelet function.
- The roles of von Willebrand factor and glycoprotein Ibα in platelet adhesion.
- Signaling pathways involved in platelet activation.
- Factors determining the stability of a platelet thrombus.

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