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Regulation of platelet activation and coagulation and its role in vascular injury and arterial thrombosis

Maurizio Tomaiuolo, PhD [Research Investigator, Sr.],
Department of Medicine, Perelman School of Medicine, University of Pennsylvania, 821 BRB II/III, 421 Curie Blvd., Philadelphia, PA 19104, mtomai@mail.med.upenn.edu

Lawrence F. Brass, MD/PhD [Professor of Medicine and Pharmacology], and
Perelman School of Medicine, University of Pennsylvania, 815 BRB II/III, 421 Curie Blvd., Philadelphia, PA 19104, brass@mail.med.upenn.edu

Timothy J. Stalker, PhD [Research Assistant Professor]
Department of Medicine, Perelman School of Medicine, University of Pennsylvania, 809 BRB II/III, 421 Curie Blvd., Philadelphia, PA 19104, tstalker@mail.med.upenn.edu

Abstract

Hemostasis requires the tightly regulated interaction of the coagulation system, platelets, other blood cells and components of the vessel wall at a site of vascular injury. The dysregulation of this response may result in excessive bleeding if the response is impaired, and pathologic thrombosis with vessel occlusion and tissue ischemia if the response is overly robust. Extensive studies over several decades have elucidated the major molecular signaling pathways responsible for platelet activation and aggregation, and anti-thrombotic agents targeting several of these pathways are in widespread clinical use. This review will summarize more recent research examining mechanisms by which these multiple platelet signaling pathways are integrated in time and space at a site of vascular injury in vivo to produce an optimal hemostatic response.

Introduction

The hemostatic response to vascular injury is a complex process requiring regulated activation of coagulation proteins, platelets and components of the vascular wall to form a localized hemostatic plug that prevents bleeding. Many aspects of this process have been well characterized at the molecular and cellular level in vitro, and the major biochemical pathways responsible for coagulation and platelet activation have been reviewed extensively elsewhere[1–5]. This review will focus primarily on how the multiple components of the hemostatic system are integrated in time and space to generate an optimal response, including how fluid dynamics and the physical architecture of platelet plugs contribute to the formation of complex biochemical gradients at a site of vascular injury. While presented in

Correspondence to: Timothy J. Stalker.

the context of hemostasis, all of the players and processes discussed have an important role in pathologic thrombosis as well, as indicated by the clinical utility of multiple therapeutics directed against platelet and coagulation targets as anti-thrombotics. As we continue to gain a better understanding of how coagulation and various cellular signaling pathways are coordinated in time and space during hemostasis, we are more likely to uncover differences that may exist between hemostasis and thrombosis that could be targeted for safer anti-thrombotic treatments.

The hemostatic response to vascular injury

In a fairly simplified view, the hemostatic response can be considered as a sequence of cellular and molecular events delineated into the overlapping phases of initiation, extension and stabilization. Each of these phases involves pro-hemostatic molecular processes that result in the rapid plugging of a hole in the vessel wall to stem bleeding, balanced with anti-hemostatic processes that limit the response to the site of injury and prevent unwarranted vascular occlusion. The molecular players involved include adhesion molecules and their ligands, platelet surface receptors that initiate intracellular signaling pathways, and the coagulation cascade to generate thrombin and fibrin among others.

Initiation

Hemostasis is triggered by the exposure of blood to a breach in the vessel wall. During the initiation phase, circulating platelets are recruited to the injury site via adhesive interactions between von Willebrand factor (vWf) bound to collagen fibers in the vessel wall and the platelet GPIb-IX-V receptor complex. VWf is normally found circulating in the plasma in an inactive form, is secreted constitutively from endothelial cells as part of the extracellular matrix, and is also secreted from Weibel-Palade bodies of activated endothelial cells[6,7]. Following a breach in the vessel wall, circulating vWf is deposited on collagen fibers exposed at the injury site[8]. Unfolding of the protein as a result of shear forces exposes binding sites for platelet surface GPIb to rapidly recruit platelets from the circulation[6,7,9]. As the vWf-GPIb complex interaction is relatively weak, additional adhesive interactions mediated by integrin family adhesion molecules on the platelet surface are also required for firm platelet attachment at the site of injury. These include $\alpha_2\beta_1$ integrin binding to collagen and $\alpha_{IIb}\beta_3$ binding to vWf and other ligands. In order for platelet integrins to bind their ligands, they must undergo a conformational change from a resting to active state that requires platelet activation. Platelet activation during the initiation phase is likely mediated via multiple platelet signaling pathways, including activation of the GPVI collagen receptor, activation of platelet ATP and ADP receptors via release of these molecules from damaged cells and by signaling downstream of the GPIb-IX-V complex. In addition, escaping blood at the site of injury encounters tissue factor expressed by cells in the vessel wall and extravascular tissue initiating the generation of thrombin, which is a potent platelet activator.

Extension

Following initial platelet adhesion and activation, additional platelets are recruited from the circulation to form a platelet aggregate via platelet-platelet cohesion during the extension phase. This cohesion is mediated primarily by binding of the plasma protein fibrinogen to

$\alpha_{IIb}\beta_3$ integrin (aka GPIIbIIIa). Each fibrinogen molecule has two $\alpha_{IIb}\beta_3$ binding sites and can therefore mediate platelet-platelet interactions by binding to receptors on two adjacent platelets. Platelet recruitment and $\alpha_{IIb}\beta_3$ mediated cohesion require platelet activation by ADP released from platelet dense granules and thromboxane A_2 (TxA2) generated by platelets already adherent at the site of injury. Thrombin activity also continues to contribute to platelet activation. The importance of $\alpha_{IIb}\beta_3$ in mediating platelet aggregation is demonstrated by the lack of aggregation of platelets from Glanzmann's thrombasthenia patients, which results in a bleeding diathesis. Inhibition of platelet aggregation using $\alpha_{IIb}\beta_3$ antagonists is effective to prevent thrombosis in the setting of percutaneous coronary intervention[10,11].

Stabilization

Once formed, the nascent hemostatic plug must condense and become firmly anchored at the injury site to resist the force of flowing blood and prevent rebleeding. In addition to activating platelets, thrombin converts fibrinogen to fibrin forming a network of fibrin fibers that helps stabilize the platelet plug. Stabilization is also facilitated by consolidation of the platelet mass via actin-myosin mediated platelet retraction. Platelet activation is reinforced by positive feedback from soluble agonists (thrombin, ADP, TxA2), as well as contact-dependent signaling pathways that are initiated once platelets come in close proximity to one another such that receptor/ligand pairs on adjacent platelets become engaged. Again, $\alpha_{IIb}\beta_3$ integrin has an important role in this stage, now acting as a signaling molecule regulating platelet retractile processes[12].

An updated model of hierarchical hemostatic plug architecture

The above description of the hemostatic response provides a general sequence of events. It is consistent with, and in large part derived from, clinical experience regarding the importance of the various molecular players involved as defined by bleeding diatheses that result from either genetic or acquired deficiencies of specific molecular components. However, recent studies examining hemostasis and thrombosis in vivo show that this model is overly simplistic. Rather than a mass of uniformly activated platelets contained in a fibrin meshwork, hemostatic plugs formed in vivo develop a regional architecture where not all platelets are activated in the same way, and fibrin is distinctly localized (Figure 1). Platelets in different regions are morphologically as well as molecularly distinct, reflecting differences in activation state. The architecture of a hemostatic plug has been described as consisting of a core of highly activated, densely packed, degranulated platelets overlaid by a shell of less activated, loosely associated platelets (Figure 2A). This description is derived primarily from intravital imaging studies in the microcirculation[13], but evidence suggests a similar hierarchical organization of platelet activation in large vessels as well. The mechanisms responsible for heterogeneity of platelet activation include a complex interplay of platelet agonists and the physical microenvironment present within a platelet mass resulting in the development of gradients of soluble platelet agonists such as thrombin, ADP and TxA_2 (Figure 2B). Critical concentrations of these agonists are reached in different regions within the hemostatic plug, which means that individual platelets are exposed to different combinations of agonists that can vary over time as well as space. Importantly, as a direct consequence of the variable contribution of platelet agonists in time and space,

therapeutic approaches targeting specific platelet signaling pathways, such as $P2Y_{12}$ ADP receptor antagonists, aspirin and thrombin inhibitors have distinct effects on hemostatic plug architecture.

Regulation of platelet activation in vivo

Once platelets are captured at the damaged vessel wall, the primary drivers for platelet activation are collagen, thrombin, ADP, TxA_2 and, to a more limited extent, epinephrine (Figure 3). With the exception of collagen, each of these agonists signal through one or more members of the G protein coupled receptor (GPCR) superfamily. In general terms, activation of receptors results in an increase in the cytosolic Ca^{2+} concentration, followed by a series of downstream cell signaling events leading to multiple cellular responses that in aggregate are referred to as platelet activation (Figure 3). Although often depicted as such, platelet activation is not a binary process, but rather a graded sequence of events, some of which are reversible and some that are not. Early, reversible components of platelet activation include shape change from a discoid morphology to a variety of other shapes, $\alpha_{IIb}\beta_3$ integrin activation leading to platelet aggregation, and thromboxane A_2 formation. Later, irreversible activation events include dense and alpha granule secretion, phosphtidylserine (PS) exposure supporting coagulation factor complex assembly, and finally membrane blebbing and microparticle formation. The degree to which platelets are activated at a site of injury depends on the integration of the activating inputs each platelet experiences.

As described above and illustrated in Figure 2, platelet activation within an evolving hemostatic plug or thrombus is heterogeneous, resulting in the development of a platelet mass with a gradient of platelet activation extending from the injury site[13]. The recognition that platelet activation is not uniform during the hemostatic response leads to the question of how such a platelet activation gradient develops and whether there are implications for both the use of existing anti-platelet therapies and the development of new ones. One possible explanation is that platelets with different degrees of activation represent subpopulations of circulating platelets with distinct properties. While intriguing, there is at present little experimental evidence to support this hypothesis. Instead, the available evidence suggests that heterogeneity of platelet activation reflects non-uniformity in agonist distribution (Figure 2B). Here, we will discuss what is known about each of the major platelet agonist signaling pathways with regard to their contribution to the spatio-temporal regulation of platelet activation.

Thrombin

Thrombin is a key regulator of robust platelet activation in response to vascular injury. It activates platelets via two G protein coupled receptors on human platelets, PAR-1 and PAR-4. These receptors are coupled to Gq signaling pathways that lead to a rise in platelet cytosolic Ca^{2+} concentration, among other effects. Downstream signaling pathways culminate in activation of $\alpha_{IIb}\beta_3$ integrin and platelet aggregation, platelet granule secretion, generation of thromboxane A_2 and platelet retraction (Figure 3).

In mouse models, inhibition of thrombin generation/activity or genetic deletion of the platelet thrombin receptor (PAR-4 on mouse platelets) results in significantly impaired

platelet accumulation and a near complete lack of intracellular calcium mobilization[14,15] and P-selectin expression[13,16] as markers of platelet activation (Figure 4A–B). Thus, thrombin activity is critical for development of a stable core composed of fully activated platelets. However, the localization of thrombin activity in time and space is limited. The prothrombinase complex (factors Va and Xa) that generates thrombin is localized to procoagulant membranes of platelets and endothelial cells at or immediately adjacent to the site if injury, thus limiting the distribution of thrombin within a platelet mass[17–19]. Once generated, thrombin distribution is limited by its ability to diffuse away from the site of generation (discussed in more detail below) and by plasma-borne inhibitors that either directly inhibit its activity (e.g. antithrombin) or generation (e.g. tissue factor pathway inhibitor and activated protein C). This combination of factors limits thrombin activity to the core region of the hemostatic plug as demonstrated by studies using a fluorogenic thrombin sensor bound to the surface of platelets[20] and studies showing the localization of fibrin formation restricted to the core and extravascular space[13,16,21].

ADP

ADP is released from damaged cells at the site of injury as well as from dense granules of activated platelets. It acts on two platelet receptors, $P2Y_1$ and $P2Y_{12}$, to reinforce platelet activation in a paracrine or autocrine fashion (Figure 2)[22–27]. $P2Y_1$ is coupled to a Gq signaling pathway, although activation of Gq signaling by ADP is relatively weak compared to Gq signaling downstream of thrombin receptors, for example. $P2Y_{12}$ is coupled to a Gi signaling pathway[28,29]. Gi signaling results in inhibition of adenylyl cyclase and a decrease in cAMP levels in platelets. cAMP is an important inhibitor of platelet activation, normally acting to keep platelets in a quiescent state in the circulation via stimulation of adenylyl cyclase as a result of prostacyclin receptor activation. One effect of $P2Y_{12}$ signaling, therefore, is to turn off this inhibitory pathway to facilitate platelet activation. $P2Y_{12}$ signaling also contributes to $\alpha_{IIb}\beta_3$ activation and granule secretion. $P2Y_{12}$/Gi signaling is required for platelet activation in response to weak agonist stimulation, such as ADP activation of $P2Y_1$, and it potentiates platelet activation in response to submaximal concentrations of thrombin or thromboxane A_2. It is also required for maximal platelet activation downstream of the collagen receptor GPVI.

The importance of $P2Y_{12}$ in platelet activation in vivo is highlighted by the efficacy of $P2Y_{12}$ antagonists in inhibiting platelet activation and protecting against thrombotic events in humans[30]. This role for $P2Y_{12}$ signaling is recapitulated in animal models, where deletion of $P2Y_{12}$[23,31] and the introduction of $P2Y_{12}$ receptor antagonists have been shown to attenuate thrombus formation[32]. In general, these studies have ascribed a role for $P2Y_{12}$ in regulating thrombus stability[31,33,34]. Viewed from the perspective of spatio-temporal regulation of platelet activation, the effect of $P2Y_{12}$ signaling on thrombus stability is due to the importance of this signaling pathway in platelet recruitment and retention in the outer layers of a developing platelet plug, a region where thrombin activity rapidly declines. Inhibition of $P2Y_{12}$ activation greatly reduces platelet accumulation in the outer platelet shell (Figure 4C), while a gain of function mutation in Gi2α, the principal G protein coupled to $P2Y_{12}$ receptors, leads to an expansion of the shell[13]. In contrast, a $P2Y_{12}$ antagonist had no effect on robust platelet activation in the thrombus core, where thrombin activity is high

(Figure 4C)[13]. This latter finding may help to explain the relative safety of $P2Y_{12}$ antagonists used clinically.

Thromboxane A_2

Like ADP, thromboxane A_2 (TxA_2) generated and released by activated platelets acts to reinforce platelet activation in an autocrine and paracrine fashion. TxA_2 is generated via the aspirin sensitive cyclooxygenase-1 (COX-1) pathway in platelets. Upon release, it binds its receptors (TPα and β) on the platelet surface to activate a Gq signaling pathway (Figure 3). Like other Gq coupled receptors, downstream signaling includes a rise of cytosolic calcium concentration, activation of $\alpha_{IIb}\beta_3$ and granule release[29].

The importance TxA_2 in platelet activation in vivo is shown by a number of large clinical studies demonstrating the efficacy of aspirin treatment in the prevention of platelet-mediated cardiovascular events (i.e. myocardial infarction and stroke)[35]. Thrombus formation is also attenuated in TP-deficient mice[36]. The spatio-temporal distribution of TxA_2 within a growing hemostatic plug is not yet well defined. As a highly diffusible molecule, its localization will primarily be determined by its source (activated platelets) and its rapid metabolism in plasma to inactive metabolites. Initial studies indicate that like ADP, TxA_2 contributes primarily to platelet recruitment and retention in the outer shell region of the hemostatic plug (Stalker et al, unpublished observation).

Collagen/vWf

Collagen is a potent platelet agonist. Unlike most soluble platelet agonists, the collagen receptor GPVI is not a GPCR, but instead belongs to the family of immune-type signaling receptors. GPVI is coupled to the Fc receptor gamma chain, which acts as the signaling component of the collagen receptor complex. Collagen binding to GPVI initiates a signaling cascade similar to Gq signaling in that it results in an increase in cytosolic CA^{2+} concentration and subsequent downstream platelet activation events (Figure 3). In vitro, collagen mediated platelet activation is highly dependent on secondary signaling by ADP and thromboxane A_2.

In contrast to soluble platelet agonists, collagen is an insoluble component of the vessel wall and extravascular tissue. As such, its direct contribution to platelet signaling via activation of the collagen receptor GPVI is restricted to platelets in contact with the damaged vessel wall and those that escape into the extravascular compartment. The contribution of GPVI signaling in experimental models is therefore highly dependent on the mechanism and extent of injury, as well as the amount of thrombin generated[37]. This likely explains varying reports of GPVI being a critical regulator of platelet accumulation and activation in some settings, yet completely dispensable in others[37–40]. Thus, while collagen is clearly a potent activator of platelets in vitro, its contribution to hemostasis and thrombosis in vivo is likely much more context dependent than other platelet agonists such as thrombin, ADP and TxA_2.

The contribution of signaling from the GPIb-V-IX complex (the platelet vWf receptor) to platelet activation in vivo remains unclear. In vitro studies have suggested that GPIb-V-IX may act as a mechanosensor, including the demonstration of platelet calcium transients following GPIb complex engagement under flow conditions in vitro[41,42]. However, these

results have not been directly recapitulated in an in vivo system. In fact, vWf mediated signaling appears to be dispensable for platelet activation as measured by cytoplasmic calcium concentration in the mouse cremaster laser-injury model[14]. In contrast, a mouse line in which the cytoplasmic tail of GPIb-alpha was truncated showed defective thrombus formation in a $FeCl_3$ injury model[43].

Influence of local hemodynamics

The importance of the local hemodynamic conditions on platelet accumulation has been documented using in vivo and in vitro methods. The presence of geometrical features that disrupt the local flow pattern, such as stenosis or aneurysms, can exacerbate pre-existing conditions for platelet recruitment and activation. For instance, stenotic regions or developing platelet masses restrict the volume of the vessel available to blood flow, and in so doing generate zones of fluid acceleration and deceleration[44–47]. In the deceleration zones platelet aggregation occurs via vWF/GPIb and $\alpha_{IIb}\beta_3$ integrin dependent interactions[44,45,48]. Anti-platelet agents blocking the TxA_2 and ADP signaling pathways prevent the shear-dependent aggregation occurring in the deceleration zones, indicating that platelet activation is a necessary component of the deposition process[44,47,48].

Aneurysms also present flow disturbances and are frequently associated with intramural thrombi[49]. A combination of experimental and computational studies shows that the presence of these thrombi further deteriorate the local conditions of the aneurysm via distinct mechanisms including local release of degenerative enzymes[50], flow-induced hypoxia and changes in wall shear stress distribution[51,52]. All these mechanisms contribute to vessel wall weakening and may explain why the presence of intramural thrombi are associated with aneurysm growth[53], rupture[54], and mortality[55].

Taken together these studies indicate that flow disturbances affect platelet deposition, and that platelet deposition in turn causes flow disturbances. This positive feedback can lead to disastrous consequences when platelet accumulation occurs at sites of atherosclerotic plaque formation. Platelet accumulation, however, in all cases discussed is always activation-dependent[45]. Some in vitro evidence suggests that under conditions of non-physiologic shear rates (>20,000 s^{-1}) vWF fibers become tissue plasminogen activator and ADAMTS13 resistant[56], and can support activation-independent platelet aggregation[57]. This mechanism of platelet aggregation may be particularly problematic when blood moves through left ventricular assist and other artificial devices.

The intrathrombus microenvironment shapes agonist distribution

Even as a growing platelet mass disturbs the bulk flow around it causing accelerations and decelerations, the flow conditions inside of the platelet mass are exceedingly calm. Within this calm domain many chemical reactions occur that result in complex gradients of soluble platelet agonists. Location, mode of release, stability, and ease of movement are all factors that contribute to an agonist specific gradient. The movement of a soluble agonist within the platelet mass can become restricted due to both agonist-dependent and platelet mass-dependent effects. Agonist-dependent effects include its size, charge, and binding interactions. The platelet mass-dependent effects are determined by the pore space formed

between platelets, the size of the pores, and the connectivity and plasma velocity between the pores.

While each of these factors has a physical meaning the net effect on a solute movement is estimated by measuring two multifactorial parameters, permeability and diffusivity. Permeability measures the ability of a porous material to allow the passage of fluids. Diffusivity measures how quickly a solute spreads as a function of temperature and concentration. Studies have used experimental and computational approaches to estimate the permeability and diffusivity values of a platelet mass. Clot permeability, as measured in vitro, is small, has a value close to that of the endothelium (2×10^{-18} m^2), and is determined by platelet retraction and fibrin[58]. The small and heterogeneous permeability of a clot translates to a model of hindered or absent flow within the platelet mass microenvironment[59–62].

When the contribution of flow becomes negligible diffusion becomes the controlling factor for molecular movement[58,61,62]. In the platelet mass microenvironment, a solute's diffusivity is determined by the ratio of the solute size to the size of the pores. Solutes much smaller than the size of the pores diffuse faster while solutes close to the size of the pores become excluded. Due to differences in the extent of platelet packing density, diffusivity within the platelet mass is higher in the shell and lower in the core[61–63], where it may even be absent due to size exclusion[13]. Thus, the net effect of permeability and diffusivity is reduced and heterogeneous solute transport properties in a platelet mass, with lower transport in the core[61,64,65].

Understanding how solute transport is regulated is critical to understand the delivery, removal, and depletion of pro- and anti-coagulant factors within the platelet mass[66–68]. Studies have shown that individual reactions within the coagulation cascade can switch from being reaction-to transport-limited depending on the local conditions[69]. Limited transport increases the residence time of solutes in the platelet mass, which translates into reduced generation of new thrombin, and at the same time increased biological activity of locally trapped thrombin. Evidence from experimental and theoretical studies show that as the platelet mass develops platelets physically cover tissue factor sites, which also leads to reduced thrombin generation[68,70–72].

Taken together, these observations describe a progression of coordinated events that depend on the tight interaction between the hemodynamic environment and the biological response of platelets: 1) platelets accumulate at an injury site, 2) as the platelet mass grows the transport of soluble agonists is restricted and shifts from being convection to being diffusion dominated, 3) diffusion increases the residence time of larger agonists (e.g. thrombin) more so than smaller agonists (e.g. ADP), 4) the platelet mass consolidates in response to longer exposure to agonists creating zones that are closed to solute exchange, 5) hemostasis is achieved[61,64,65]. When this balance is perturbed using mice deficient in platelet retraction, solute transport is increased resulting in fewer platelets becoming maximally activated[64].

Summary

In summary, extensive research by many investigators over the past several decades has provided a thorough understanding of the major molecular mechanisms responsible for thrombin generation and platelet activation. We are now beginning to understand how the multiple cell signaling and biochemical events involved in the hemostatic response are integrated in time and space to achieve an optimal response to injury, and how perturbations in the regulation of these events cause pathologic thrombosis. Recent findings highlight the importance of the local microenvironment, including hemodynamic and other physical factors, in shaping the biochemical gradients present at a site of injury to elicit specific cellular responses. Finally, the spatio-temporal distribution of thrombin, ADP and other platelet agonists helps explain effects of therapeutic approaches that target these specific pathways. By gaining a better understanding of how coagulation and platelet activation are regulated in time and space we may be able to better delineate differences between hemostasis and pathologic thrombosis, and identify novel targets for safer, effective anti-thrombotic treatment.

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Key Points

1. The platelet response to vascular injury involves multiple cell signaling pathways that are coordinated in both time and space.
2. Local conditions within the evolving platelet plug microenvironment result in the development of platelet agonist gradients.
3. Anti-platelet therapeutics targeting specific platelet activation pathways have disparate effects on platelet mass architecture depending on the spatio-temporal regulation of the target pathway.

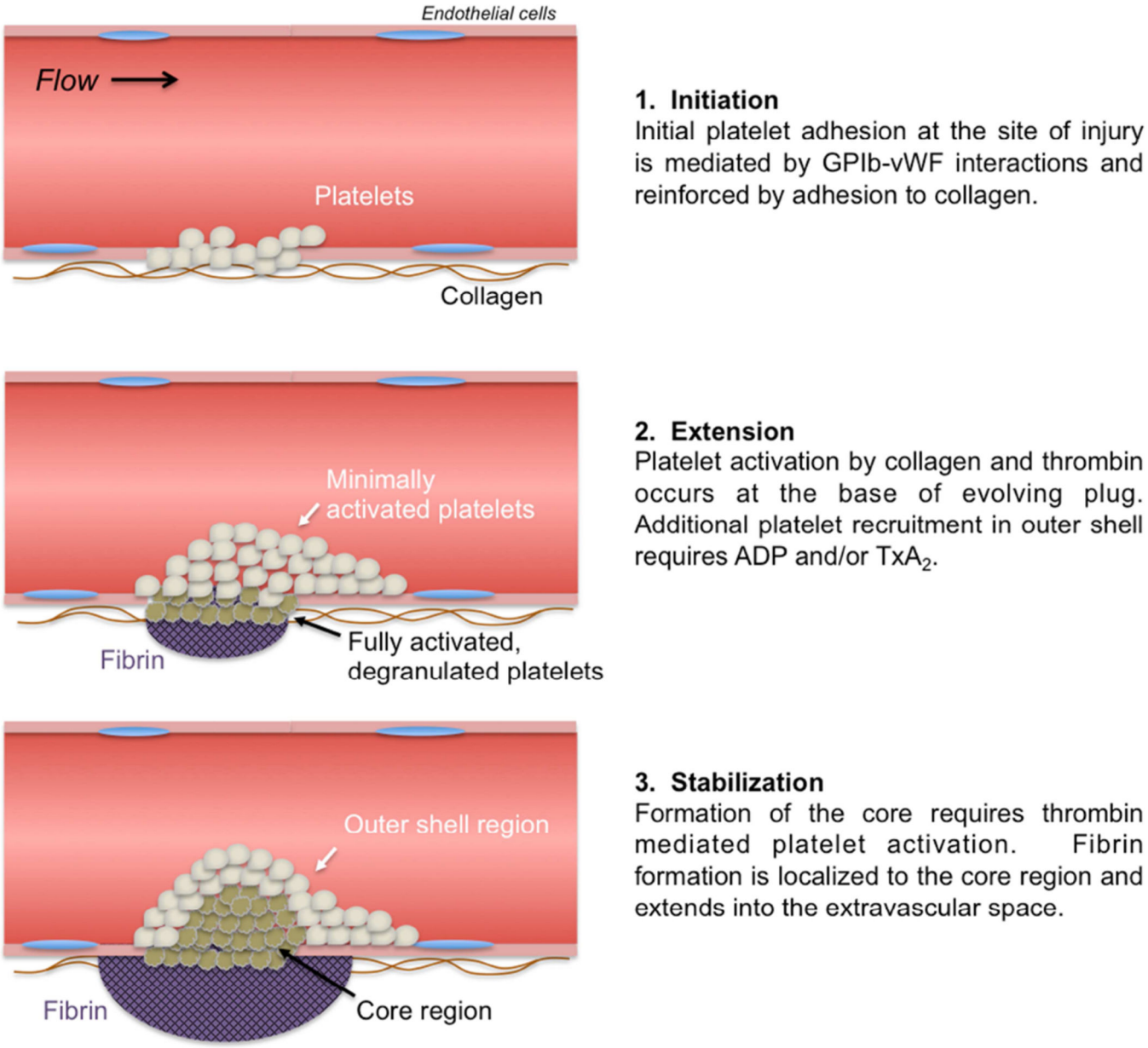

Figure 1. An updated model of the hemostatic response to vascular injury

This updated model takes into account the gradient of platelet activation observed emanating from the site of injury.

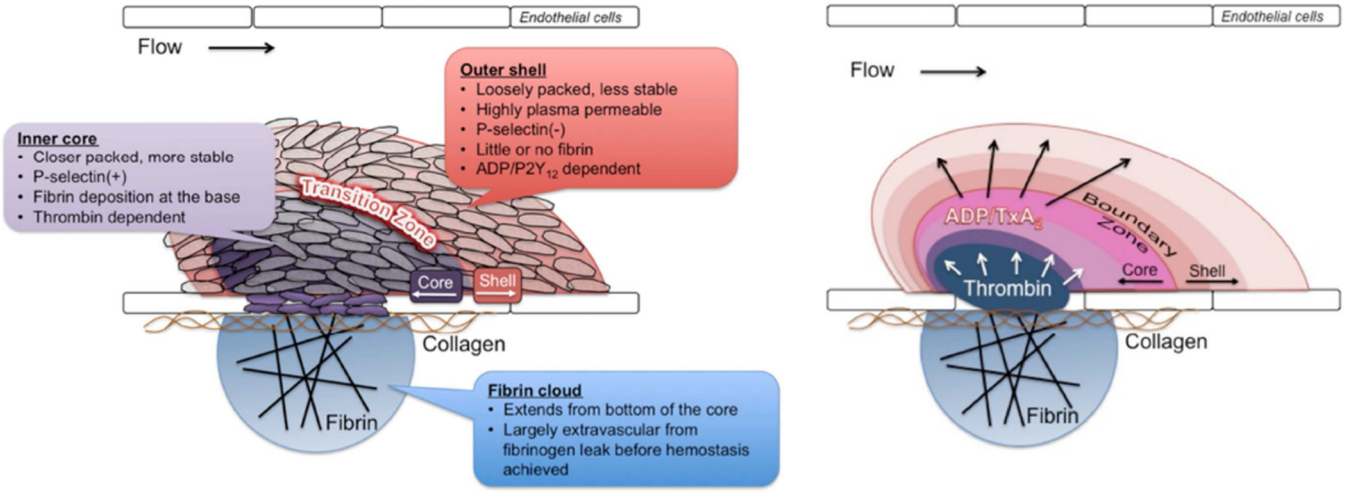

Figure 2. Regional architecture of a hemostatic plug

A) The properties of the core and shell regions of a hemostatic plug are described. **B)** The core and shell architecture develops as a result of local platelet agonist gradients that are shaped by physical forces within the platelet mass microenvironment.

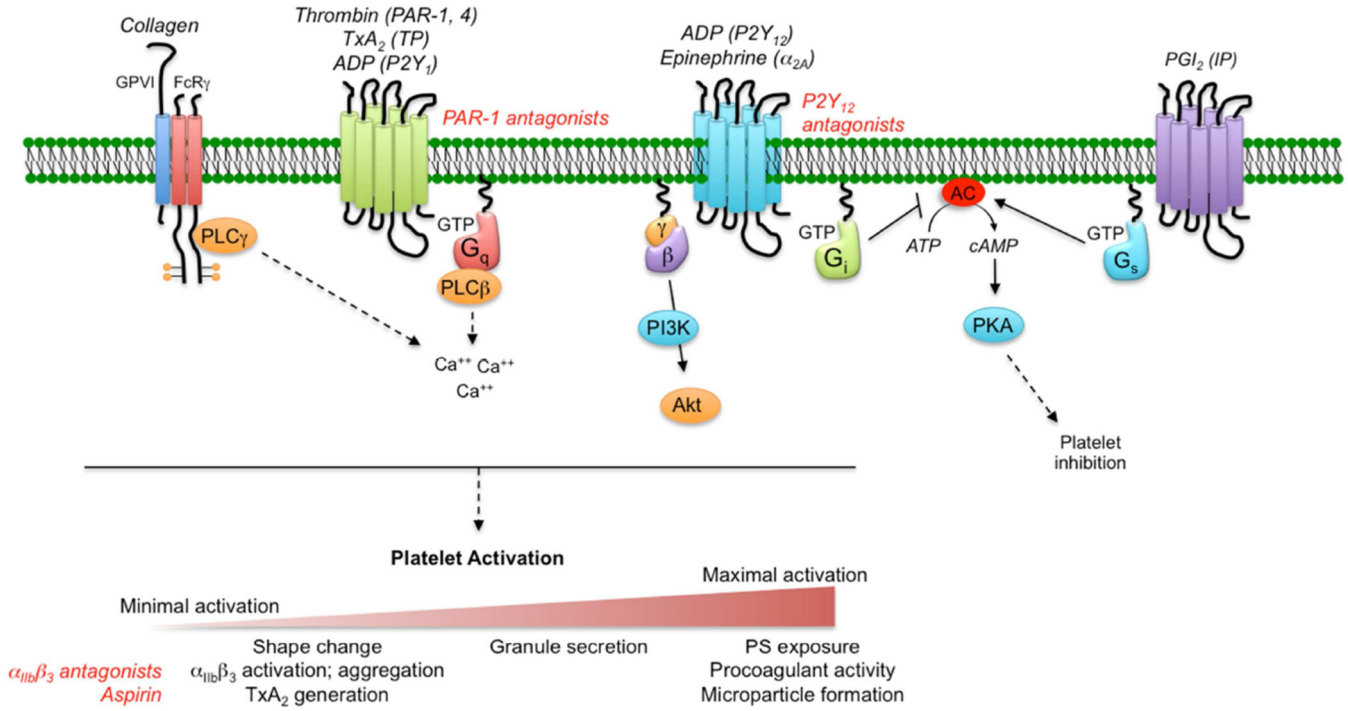

Figure 3. A simplified illustration of the platelet signaling network

Platelets respond to chemical inputs in their local microenvironment via cell surface receptors that initiate multiple intracellular signaling cascades, ultimately leading to a graded series of platelet activation events, including shape change, integrin activation, aggregation, granule secretion and procoagulant activity. The degree of activation achieved by individual platelets within a hemostatic plug or thrombus is the net result of the integration of multiple inputs. For the GPCRs shown, the text in parentheses indicates the receptor(s) name for each agonist indicated. Red text indicates anti-platelet therapeutic targets.

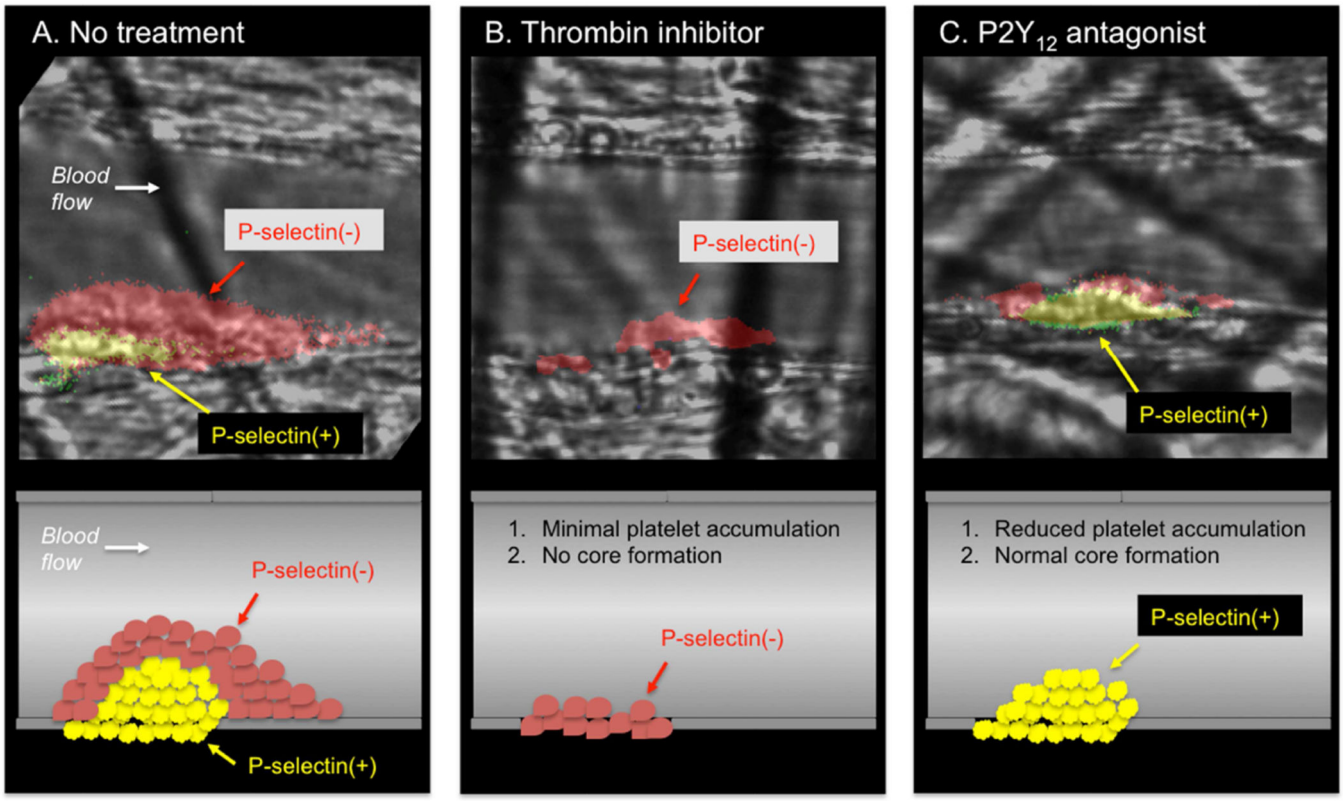

Figure 4. The effect of anti-thrombotic agents on hemostatic plug architecture in an experimental model

A) Hemostatic plug formation in the mouse cremaster microcirculation is characterized by formation of a core of P-selectin positive platelets overlaid by a shell of P-selectin negative platelets. **B)** In the presence of the thrombin inhibitor hirudin, platelet accumulation is significantly attenuated and none of the adherent platelets become P-selectin positive. **C)** In contrast, a $P2Y_{12}$ antagonist results in a decrease in platelet accumulation in the outer shell region with no effect on full platelet activation in the core region. Data from Stalker TJ, Traxler EA, Wu J, et al. Hierarchical organization in the hemostatic response and its relationship to the platelet-signaling network. Blood. 2013;121(10):1875–1885.