blood advances

POINT Platelets and hemostasis: a new perspective on an old subject

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This article has a companion Counterpoint by Kapur and Semple.

This is an exciting time for clinicians and scientists interested in platelet biology. Improved imaging methods allow platelets to be observed in action in animal models in real time at ever greater resolution. Expanding proteomic and genetic data sets lend themselves to better understanding platelet activation. New gene editing methods make it easier, faster, and less expensive to test new ideas using transgenic animal models. Combining systems biology approaches with computational methods encourages a broader perspective on platelet activation and makes it possible to develop ideas in silico that can then be tested in vivo. One result has been an opportunity to revisit prevailing wisdom about the hemostatic response, extending and occasionally refuting what has come before.

Systems biology is the study of complex interactions, some of whose properties can be understood only when multiple cells or multiple pathways are considered. Here we will consider 2 examples in which improved methods and a systems-oriented approach have provided insights into the most basic of platelet functions: participation in the hemostatic response to injury. The first example considers the ways in which the simple act of piling up of platelets at a site of injury helps to calibrate the hemostatic response by altering the environment in which platelet activation occurs. The second example considers how individual signaling events within platelets form an integrated network whose properties emerge from the individual pathways.

Achieving hemostasis: piling up platelets changes everything

Penetrating injuries trigger platelet activation by the local accumulation of platelet agonists. Some agonists, such as collagen, are stationary; others, such as thrombin, adenosine diphosphate (ADP), and thromboxane A2 (TxA_2) are mobile. Platelet activation is commonly considered with an agonist-centric perspective, but this perspective omits the impact of the local environment, which changes rapidly as platelets and fibrin accumulate. Recent evidence suggests that formation of a hemostatic thrombus first promotes and then limits platelet activation by providing a sheltered environment in which agonists can accumulate. Thus, there is a reciprocal, rather than a unidirectional, relationship between platelet activation and thrombus structure (Figure 1A). Because this relationship emerges as platelets pile up, it is worth considering how it happens.

Although platelet behavior has been studied for over a century, recent advances in intravital imaging pioneered by the Furie laboratory[1-3] and others[4-9] have made it possible to observe the hemostatic response in mice in real time at high resolution. Those studies show that platelet activation in this setting is heterogeneous. Although some platelets change shape, secrete their granule contents, and become procoagulant, others display only minimal external signs of activation. The result is a gradient of platelet activation with a core of fully activated platelets, a shell of less activated platelets, and a transition zone between them (Figure 1A).[7]

Among the properties that distinguish the core from the shell is packing density, which is greater in the core.[7] Tight packing slows the movement of soluble molecules in the gaps between platelets, which shrink as the thrombus retracts.[10,11] The core is where most of the fibrin is found and where clot retraction would be expected to have the greatest impact (Figure 1B). As packing density increases, transport becomes dominated by diffusion rather than convection, slowing movement to an even greater extent (Figure 1C).[10-12]

Regional differences in packing density also affect the distribution of platelet agonists. The result is the appearance of concentration gradients in which the distribution of each agonist is also affected by its physical properties and binding to other molecules. Individual platelets are exposed to combinations of agonists whose concentrations vary over time (Figure 1B). Submaximal concentrations of multiple platelet agonists can have additive or even synergistic effects.[13] Thrombin is the main driver of full platelet activation in the thrombus core. TxA_2 and ADP are primarily drivers for the thrombus shell.[7,14]

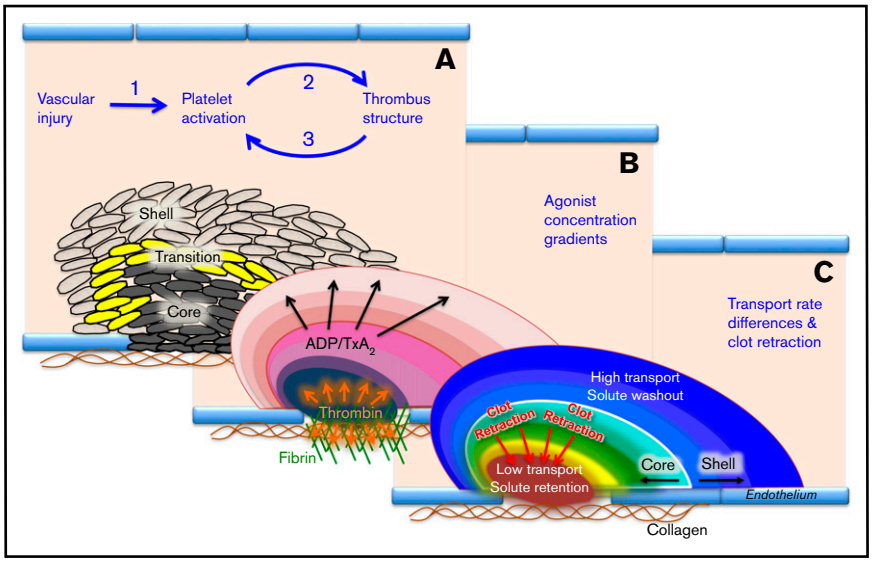

Figure 1. A systems view of the hemostatic response to penetrating injuries. High-resolution confocal fluorescence microscopy studies performed in mice show that the hemostatic structure has a characteristic architecture whose properties emerge as platelets accumulate, altering local conditions. (A) Presence of a core of highly activated, densely packed platelets overlaid with a shell of loosely packed, less activated platelets and the transition zone that exists between them. (B) Manner in which soluble platelet agonists such as thrombin, ADP, and TxA_2 form concentration gradients radiating from the site of injury. (C) Distribution of the agonists is determined in part by differences in transport rates in the narrowing gaps between platelets, gaps whose dimensions decrease as clot retraction proceeds.

The impact of packing density is demonstrated by studies showing a mutation in $\alpha_{IIb}\beta_3$ that impairs clot retraction decreases thrombin activity and reduces platelet activation.[11,15] Studies performed in silico extend the observational studies and provide hypotheses that can be tested in vivo and in vitro.[10,16,17]

Most of the studies summarized in Figure 1 were performed in the mouse microvasculature using a laser or a sharpened probe to make small holes in arterioles and venules. To what extent are the results applicable to people? Human platelets cannot readily be studied in vivo. However, when studied in a microfluidics device that incorporates collagen, tissue factor, and the transmural pressure drop that normally occurs following vascular injury, human platelets form an inner core of fully activated platelets overlaid by a shell of less activated platelets just as mouse platelets do in vivo.[18]

What about events in arteries and veins, rather than arterioles and venules? Primarily for technical reasons, high-resolution imaging studies have largely been limited to the microvasculature. However, there has been progress.[9,19-21] More work needs to be done, but the initial message appears to be the same. In both settings, the piling up of platelets changes everything by producing a local environment in which agonists accumulate.

The platelet signaling network is an integrating engine

Most of what is known about the platelet signaling network was worked out one pathway at a time. The first part of this essay shows that platelets within a growing hemostatic mass are exposed to combinations of agonists, any of which may be present below optimal concentrations. Agonist receptors are not generic. Each agonist has a unique receptor set that can couple to the platelet signaling network in different ways (Figure 2).[22] Thrombin, for example, activates 2 members of the protease-activated receptor family on human platelets, PAR1 and PAR4, allowing it to signal through the heterotrimeric G proteins, G_q, G_{12}, and, directly or indirectly in platelets, G_{i2}. PAR1 produces a quick burst of signaling; PAR4 a more sustained response. ADP activates $P2Y_1$ and $P2Y_{12}$, the latter coupled to G_{i2} and the former to G_q. Signals mediated by G_q activate phospholipase Cβ, leading to increased cytosolic Ca^{2+}, activation of Rap1b, and, ultimately, to the activation of $\alpha_{IIb}\beta_3$.[23,24] G_{i2} inhibits cyclic adenosine monophosphate (cAMP) formation, activates Akt, and promotes integrin activation by inhibiting Rap1b inactivation.[25] Once $\alpha_{IIb}\beta_3$ has been activated, integrin-dependent signaling promotes clot retraction, increasing packing density and slowing solute transport.

The platelet signaling network makes possible a measured response to agonists in part because of feedback loops and nodes within the network where signaling pathways converge. Examples include G_q, G_{i2}, and Rap1b (Figure 2). The activity state of each of these is determined by whether they are bound to guanosine triphosphate (GTP) or guanosine diphosphate (GDP), the GDP-bound state being inactive. In effect, these are on/off switches. Replacement of GDP with GTP is promoted by a guanine nucleotide exchange factor (GEF), which for G_q and G_{i2} is an agonist-occupied receptor and for Rap1b is CalDAG-GEF1. Restoration of the inactive state is accelerated by a GTPase activating proteins (GAP). For G_q and G_{i2} the primary GAPs in platelets are RGS10 and RGS18.[26-28] For Rap1b, the primary GAP is Rasa3.[25,29]

Network integration occurs in part by regulating the balance of GEF and GAP activity. The availability of RGS10 and RGS18 is regulated by spinophilin (SPL), which sequesters both in resting platelets, and by 14-3-3γ, which binds RGS proteins in activated platelets.[26,30] Dissociation of SPL/RGS complexes occurs after a brief delay, creating a negative feedback loop when platelets are activated by thrombin or TxA_2.[26] As an example of pathway convergence, dissociation of the SPL/RGS complex also occurs when endothelium-derived PGI_2 suppresses platelet activation by raising platelet cAMP levels (Figure 2).[31] For Rap1b, regulation occurs at the level of Rasa3, whose ability to act as a GAP is inhibited by signaling downstream of G_{i2}.[25,32] Rap1b[33] and CalDAG-GEF1,[34,35] like spinophilin, are targets for cAMP-dependent phosphorylation.

How can the relative contributions of these regulatory events be assessed? One way is with transgenic mice (Figure 2). Deletion of $G_{i2}\alpha$[36,37] or $G_q\alpha$[38] produces a loss of function phenotype, as does

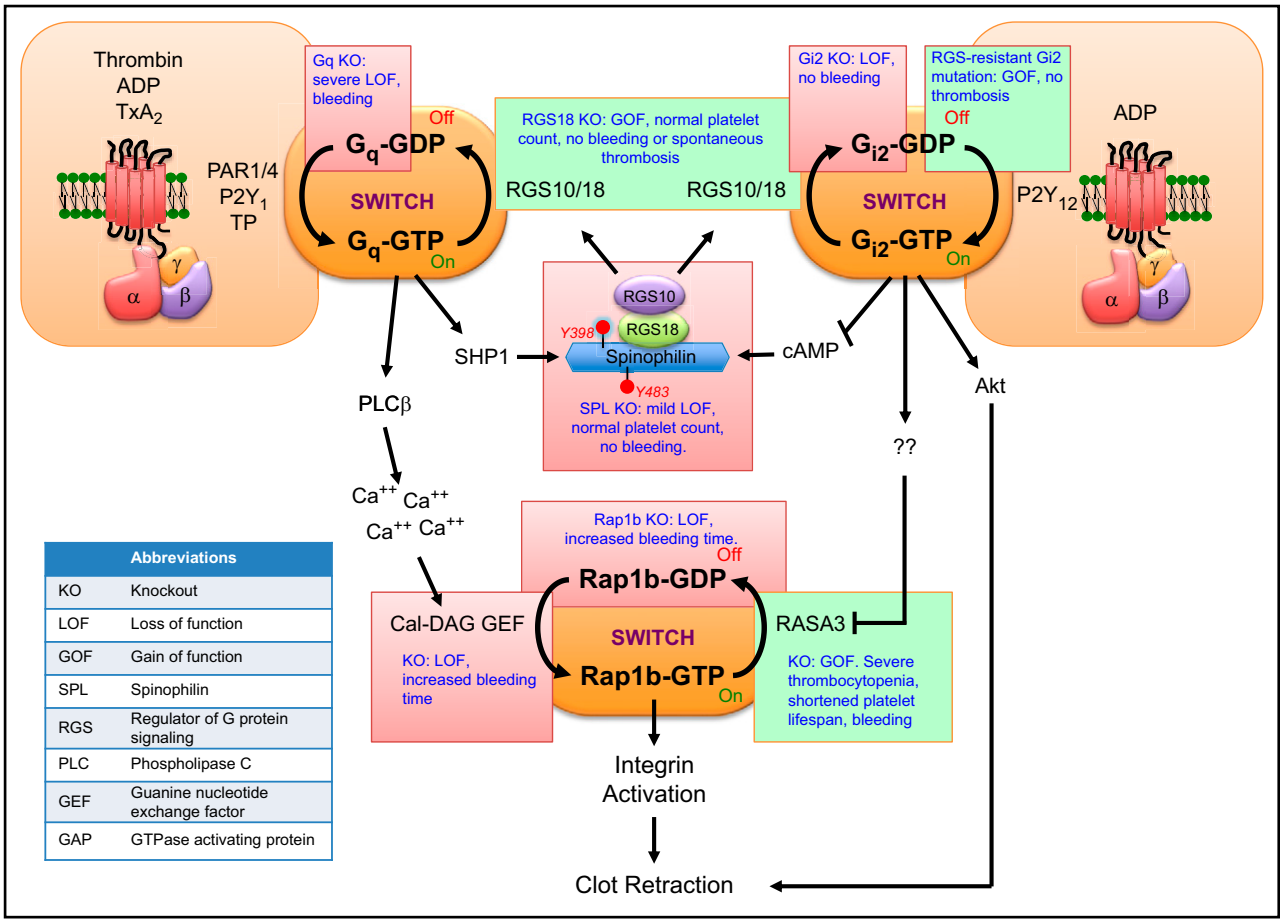

Figure 2. Integrating the platelet signaling network to obtain an optimal response. Although platelet signaling pathways were originally described one at a time, they form a closely regulated network that both promotes and limits the hemostatic response. The figure focuses on events downstream of the G protein–coupled receptors for thrombin, ADP, and TxA_2. It illustrates 2 GTP-dependent switching points or nodes in the network, crosstalk between pathways, and the presence of regulatory loops that affect information flow through the nodes. The green and red boxes summarize transgenic mouse models associated with gain or loss of function, respectively. References are in the text.

deleting spinophilin or introducing a missense mutation in spinophilin that mimics the effects of cAMP-dependent phosphorylation.[26,31] In contrast, deleting either RGS18[27,39] or RGS10 (Peisong Ma and L.F.B., unpublished observations, 2015), or introducing a mutation in $G_{i2}\alpha$ that makes it resistant to RGS proteins,[7,40] produces a gain of function. These effects are not of equivalent magnitude: deleting G_q causes spontaneous bleeding, but deleting G_{i2} does not.[36-38] Neither gain-of-function mutation appears to cause spontaneous thrombosis. The RGS-insensitive G_{i2} mutation causes expansion of the thrombus shell without affecting the size of the core.[7]

Mutations at the level of Rap1b are equally informative about network integration. Deleting Rap1b causes a loss of function phenotype with a prolonged tail bleeding time and increased time to occlusion.[41] Deleting CalDAG-GEF1 also causes a loss of function[42] as do CalDAG-GEF1 mutations in humans.[43,44] Deleting Rasa3 causes severe thrombocytopenia, bleeding, and increased embryonic and perinatal lethality.[25,29] The thrombocytopenia is believed to be due to spontaneous platelet activation and shortened platelet survival.[25] These observations speak to the importance of Rasa3 at the Rap1b network integration point.

In summary, recent studies show that platelets possess an integrated signaling network rather than a collection of independent pathways. Packing density and therefore transport rates help determine agonist distribution and concentration. Activity at network nodes determines how large the hemostatic mass will grow.

Is any of this clinically relevant?

There are several ways that the 2 examples cited here can inform decision making by hematologists, cardiologists, and pharmaceutical companies. First, they provide a context to better understand why platelets express receptors for so many different agonists. Second, they suggest that the strengths and limitations of some commonly used antiplatelet agents reflect not only their half-lives, affinities, and off-rates, but also where they work on the platelet signaling network and how well they penetrate thrombus structure. For example, observational studies performed in vivo suggest that widely prescribed $P2Y_{12}$ antagonists impair hemostasis and reduce recurrent thrombotic events by destabilizing the thrombus shell with comparatively little impact on the thrombus core, at least in the microcirculation where these studies were performed. Finally, the data suggest that tests of on-treatment platelet function in

patient taking antiplatelet agents need to be designed to better reproduce the complex conditions that the observational studies show exists within a growing thrombus. Computational studies that recapitulate platelet accumulation and transport may prove helpful in this regard, especially as the simulations become even more refined.[10,17,45-50]

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