

Contact-dependent signaling events that promote thrombus formation

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Abstract

There is increasing evidence that formation of a stable hemostatic plug requires adhesive and signaling events that continue beyond the onset of platelet aggregation. These events are facilitated and, in some cases, made possible, by the persistent close contacts between platelets that can only occur when platelets begin to aggregate. Participants include integrins and other cell adhesion molecules, secreted agonists, receptor tyrosine kinases, and protein fragments that are shed from the surface of activated platelets. Collectively, these molecules promote the continued growth and stability of the hemostatic plug.

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Introduction

The broader outlines of platelet activation have grown increasingly familiar, but numerous questions remain unanswered. For example, what happens *after* activated platelets stick to each other? Are interactions required between platelets beyond those mediated by $\alpha_{IIb}\beta_3$? What prevents destabilization of the hemostatic plug during the time required for wound healing to occur? What keeps integrins engaged with their ligands? How do events on the surface of opposing platelets compare with events on the unopposed surfaces of activated platelets?

In contrast to most cells, platelets are not normally in stable contact with each other but develop such contacts once aggregation has begun. Electron micrographs show the close proximity of the plasma membranes of adjacent platelets but do not show adherence junctions such as those formed by epithelial and endothelial cells [1–3]. Estimates for the width of the gap between adjacent platelets range from 0 to 50 nm [4]. In theory, the short distance between platelets should make it possible for molecules on the surface of one platelet to bind to molecules on an adjacent platelet. This could be a direct interaction, as when

one cell adhesion molecule binds to another in trans, or an indirect interaction, such as occurs when adhesive proteins link activated $\alpha_{IIb}\beta_3$ on adjacent platelets. In either case, these interactions can theoretically provide both an adhesive force and a secondary source of intracellular signaling. Close contacts between platelets can also limit the diffusion of molecules into and out of the gaps between platelets. Seen in this context, the phenomenon of clot retraction, which is dependent on the interaction between actin/myosin complexes and the cytoplasmic domain of $\alpha_{IIb}\beta_3$, can be viewed as a mechanism for narrowing the gaps between platelets and increasing the local concentration of soluble ligands for platelet receptors [5].

Integrins, cell adhesion molecules, and outside-in signaling

Outside-in signaling refers to the intracellular signaling events that occur downstream of activated integrins once ligand binding has occurred. Since this topic has been reviewed in depth by others [6], only a few points will be made here. Integrin signaling depends in large part on the formation of protein complexes that link to the integrin cytoplasmic domain. In the case of the dominant platelet integrin, $\alpha_{IIb}\beta_3$, some of these interactions require the phosphorylation of tyrosine residues Y747 and Y759 in the β_3 cytoplasmic domain, others do not. The binding of Shc, for example, requires Y759 phosphorylation

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[7]. Myosin binding requires phosphorylation of both Y747 and Y759 [8]. Fibrinogen binding to the extracellular domain of activated $\alpha_{IIb}\beta_3$ stimulates a rapid increase in the activity of Src family members and Syk. Studies of platelets from mice lacking these kinases suggest that these events are required for the initiation of outside-in signaling and for full platelet spreading, irreversible aggregation, and clot retraction [9–12].

Activation-dependent phosphorylation of the β_3 cytoplasmic domain is an event of particular relevance to this review. Phosphorylation is thought to be mediated by one or more Src family members and can require both activation of the integrin and its engagement with an adhesive protein [10,11]. Mutation of Y747 and Y759 in β_3 to phenylalanine produces mice whose platelets tend to disaggregate and which show diminished clot retraction and a tendency to re-bleed from tail bleeding time sites [13]. Loss of clot retraction is also a hallmark of $\alpha_{IIb}\beta_3$ -deficient platelets from patients with Glanzmann's thrombasthenia—reflecting the dependence of clot retraction on the interaction of $\alpha_{IIb}\beta_3$ with extracellular fibrin and with intracellular actin/myosin filaments.

In addition to the integrins, a number of other cell adhesion molecules which have been identified on the surface of platelets can potentially play a direct role in interactions between

platelets, either by acting as adhesion molecules or signaling molecules (or both). These include members of the immunoglobulin (Ig) superfamily (PECAM-1, JAM-A, JAM-C, ESAM, and CD226) (Fig. 1). A recent review can be found in reference [14].

Contact-dependent and contact-facilitated signaling by receptor tyrosine kinases

Direct contacts between platelets can promote signaling by more than one mechanism. In addition to signaling events that occur downstream from integrins and other cell adhesion molecules, there are receptors that interact in trans with cell surface ligands. A recent example from our own studies is the Eph kinases and their membrane-bound ligands, known as ephrins. Eph kinases are receptor tyrosine kinases with an intracellular kinase domain and a C-terminal binding domain for cytosolic proteins with an appropriate PDZ domain. The ligands for Eph kinases are cell surface proteins known as ephrins that have either a glycosylphosphatidylinositol anchor (the ephrin A family) or a transmembrane domain (the ephrin B family). The cytoplasmic domains of the ephrin B family members are comprised of 90–100 residues with several

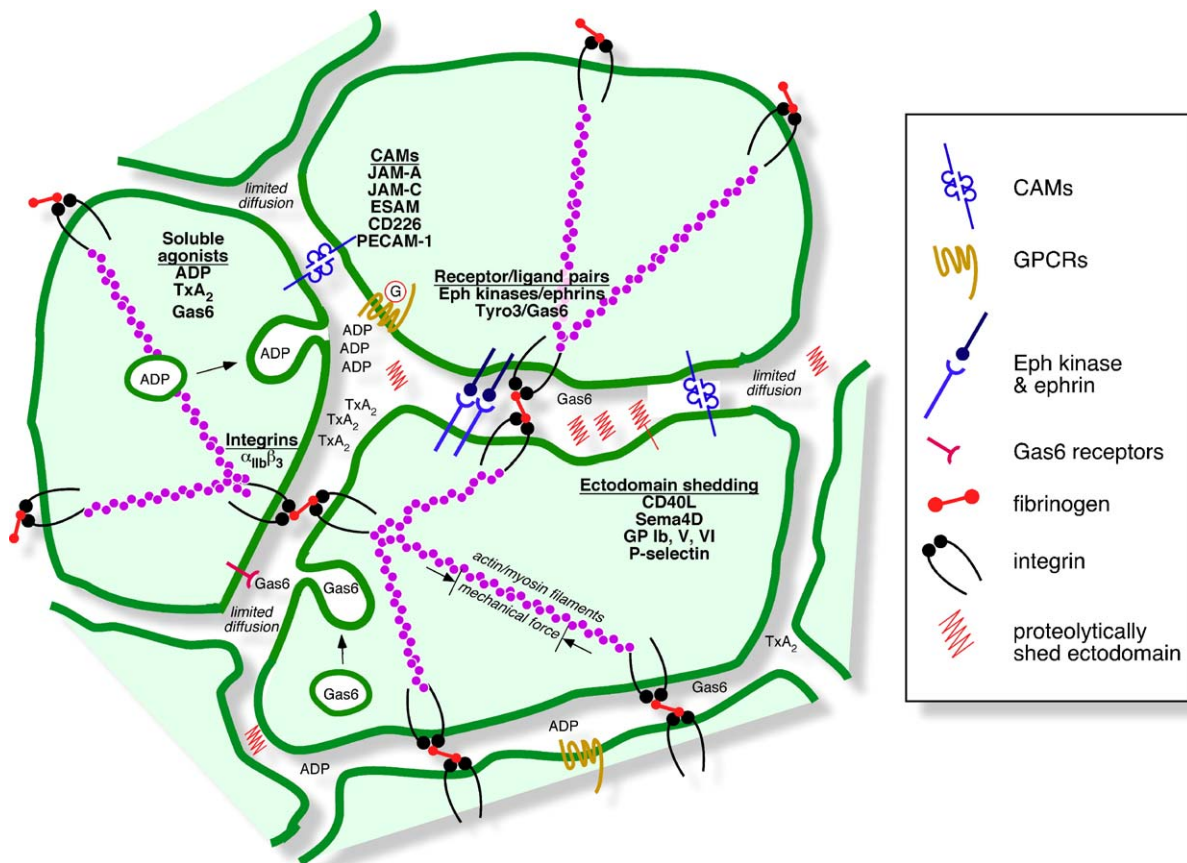


Fig. 1. Contact-dependent and contact-facilitated events during thrombus formation. The onset of aggregation brings platelets into sufficiently close contact for integrins and other cell adhesion molecules to interact and for the activation of Eph receptor kinases by their cell surface ligands known as ephrins. The space between platelets also provides a protected environment in which soluble agonists for G-protein-coupled receptors (ADP, thrombin and TxA₂) and receptor tyrosine kinases (Gas-6), and the proteolytically shed bioactive exodomains of platelet surface proteins (CD40L and sema4D) can accumulate. The mechanical forces generated by the contraction of actin/myosin filaments helps to compress the space between platelets, improving contacts and increasing the concentration of soluble agonists.

phosphorylatable tyrosine residues [15] and, like the receptors, a PDZ target domain [16–19].

Although Eph kinases and ephrins are best known for their role in neural development [20–22] and vasculogenesis [23], human platelets have now been shown to express EphA4, EphB1, and ephrinB1 [24]. Eph/Ephrin interactions are particularly relevant to contact-dependent signaling in platelets because the binding of an ephrin to an Eph kinase can cause signaling in both the receptor-expressing cell and the ligand-expressing cell [16,17,25–29]. Forced clustering of either EphA4 or ephrin B1 causes platelets to adhere to immobilized fibrinogen. Clustering of ephrinB1 also causes the activation of Rap1, a known intermediate in integrin activation in platelets [30,31], and promotes platelet aggregation [24,32]. Blockade of Eph/ephrin interactions leads to reversible platelet aggregation at low agonist concentrations and limits the growth of platelet thrombi on collagen-coated surfaces under arterial flow conditions [24,33]. It also impairs β_3 phosphorylation, thereby inhibiting the association of myosin with $\alpha_{IIb}\beta_3$ and impairing clot retraction [33]. EphA4 is constitutively associated with $\alpha_{IIb}\beta_3$ in both resting and activated platelets and co-localizes with the integrin at sites of contact between aggregated platelets [33]. Collectively, these observations suggest a model in which the onset of aggregation brings platelets into close proximity and allows ephrinB1 to bind to EphA4 and EphB1. Signaling downstream of both the receptors and the kinases then promotes further integrin activation (in part by activating Rap1B) and integrin signaling (in part by promoting β_3 phosphorylation). In turn, these events promote thrombus growth and stability.

A second example of a ligand/receptor tyrosine kinase interaction that is facilitated by platelet:platelet contacts is the binding of growth arrest specific gene 6 (Gas-6) to its receptors. Gas-6 is a 75-kDa protein related to protein S and, like protein S, contains γ -carboxylated glutamic acid residues [34]. Gas-6 is expressed in a number of tissues, including vascular smooth muscle, and levels of its expression are upregulated following vascular injury. In rodent platelets, Gas-6 is found in the α -granules [35,36]. There is disagreement about whether Gas-6 is expressed in human platelets [37]. Secreted Gas-6 can serve as a ligand for the receptor tyrosine kinases, Tyro3, Axl, and Mer [38,39], all of which are expressed on platelets [36]. Since Tyro3 family members have been shown to stimulate PI 3 kinase and phospholipase C γ [34], a reasonable hypothesis is that secreted Gas-6 can bind to its receptors on the platelet surface and cause signaling that would promote platelet plug formation and stability. Consistent with this hypothesis, platelets from Gas-6(–/–) mice were found to have an aberrant response to agonists in which aggregation terminates prematurely [36]. Furthermore, although the tail bleeding time of the Gas-6(–/–) mice were normal, the mice were resistant to thrombosis [36], as are mice lacking any one of the three Gas-6 receptors. Platelets from the receptor-deleted mice also failed to aggregate normally in response to agonists [40–42]. Curiously, this appears to occur irregardless of which of the receptors is suppressed. Biochemical studies showed that Gas-6 signaling promotes β_3 phosphorylation and, therefore, clot retraction

[41]. Secretion of Gas-6 into the spaces between platelets in a growing thrombus would be expected to allow it to achieve higher local concentrations and provide protection from being washed away.

Shedding downstream and into the gaps between platelets

In addition to secreting proteins from their storage granules, activated platelets also shed a number of surface molecules, including GP Ib α [43], GP V [44], GP VI [45,46], and P-selectin [47]. Shedding of these proteins can be prevented with inhibitors of metalloproteases and in at least two cases (GP Ib α and V), a role for a particular metalloprotease, ADAM17, has been established through studies on platelets from mice that lack it [43,44]. The advantage that the platelet derives from shedding surface proteins can sometimes only be surmised, but at least two of the molecules that are cleaved from the surface, CD40L and the semaphorin, sema4D, give rise to fragments that can stimulate platelets as well as other nearby cells.

CD40 ligand (CD40L; CD154) is a 33-kDa transmembrane protein that is present on the surface of activated platelets, but not resting platelets [48–50]. Its appearance on the platelet surface is followed by the gradual release of an 18-kDa exodomain fragment [49]. Both the surface-bound and the soluble form of CD40L (sCD40L) are trimers [51]. CD40L is a member of the TNF family and platelet-derived soluble CD40L or activated, CD40L-expressing platelets can elicit responses from endothelial cells and monocytes that appear to be pro-atherogenic [48,49,52–54]. CD40L(–/–) platelets aggregate normally, but the growth of platelet plugs on collagen-coated surfaces under shear is impaired [55,56]. CD40L(–/–) mice show delayed occlusion following vascular injury and decreased thrombus stability [55]. The extracellular portion of CD40L includes a binding domain for the CD40L receptor, CD40, as well as a KGD (RGD in mice) integrin recognition sequence. Platelets express CD40 [49,55,57]. However, although the binding of sCD40L to activated platelets can be blocked by mutating the KGD sequence, adding antibodies to $\alpha_{IIb}\beta_3$ or by suppressing expression of β_3 , loss of CD40 has no apparent effect [55]—suggesting that the effects of CD40L on platelets are mediated by the integrin rather than by CD40.

Semaphorins are a large family of structurally related proteins divided into subfamilies based in part on whether they are secreted or surface associated [58,59]. Like Eph kinases and ephrins, semaphorins are best known for their role in the central nervous system, but individual family members have been found expressed elsewhere, including hematopoietic cells. Semaphorins have in common 500-amino-acid residue extracellular “sema” domain that forms a 7-bladed propeller structure [60]. Sema4D or CD100 is a 150-kDa type I glycoprotein that forms a disulfide-linked homodimer. Each subunit has an N-terminal sema domain, an Ig domain, a lysine-rich stretch, a transmembrane domain, and a cytoplasmic tail with consensus tyrosine and serine phosphorylation sites. Sema4D has a well-described role in lymphocyte biology [61–64]. Sema4D(–/–) mice show defective B cell development, impaired T cell activation, and blunted immune responses [65]. There are two

known receptors for sema4D: plexin-B1, a high affinity receptor expressed on endothelial cells [66,67], and CD72, which is expressed on lymphocytes [68]. CD72 is a 45-kDa ITIM domain-containing type II transmembrane protein that can associate with the tyrosine phosphatase, SHP-1, and the adaptor protein, Grb2 [69,70].

We have recently determined that platelets express sema4D and are a likely source for the soluble sema4D that can affect endothelial cells at sites of injury. As in lymphocytes, platelet sema4D forms a disulfide-linked homodimer that can be found on the platelet surface [71]. Platelet activation causes the translocation of additional sema4D to the surface and the progressive shedding of its disulfide-linked exodomain. There are several possible roles for platelet-associated or platelet-derived sema4D. Western blots show that platelets express the sema4D receptor, CD72, and, therefore, may be capable of responding to, as well as releasing sema4D. If events in lymphocytes are predictive, the binding of platelet sema4D to platelet CD72 may promote platelet activation by causing the dissociation of SHP-1 from CD72, allowing it to fold into an inactive conformation. In addition, activation of plexin-B1 by soluble sema4D has been shown to cause migration and signaling by endothelial cells, suggesting that platelet-derived sema4D can also effect cells other than platelets [66,72,73].

Conclusion

There is now ample evidence that the signaling events that support platelet activation continue after integrin activation, granule secretion, and platelet aggregation have begun. Some of these events can reasonably be expected to promote the growth and stability of the hemostatic plug, support clot retraction, and help to maintain the plug in place until wound healing is complete or at least well under way. In contrast to the initiating events of platelet activation, these “late” events can take advantage of the close proximity between platelets once aggregation begins and may even extend to the contacts that develop between platelets, endothelial cells, and leukocytes as the hemostatic plug evolves. The models of contact-dependent and contact-facilitated signaling discussed in this review are just examples of what will probably turn out to be a more complex process involving additional cell surface molecules whose function remains to be identified.

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