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Platelets and coagulation in thrombus formation: aberrations in the Scott syndrome

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KEYWORDS

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Abbreviations
JAM, junctional adhesion molecule
PS, phosphatidylserine
VWF, von Willebrand factor

ABSTRACT

Platelets play key roles in thrombosis and hemostasis by forming aggregates and providing a procoagulant surface, at which thrombin is generated and fibrin fibers are formed. Here we present an overview of the different mechanisms how platelets orchestrate coagulation processes in thrombus formation in thrombosis and hemostasis. Parts of these are via Ca^{2+}-dependent activation responses, leading to phosphatidylserine exposure; swelling to form balloons with increased binding of coagulation factors; and calpain-mediated integrin $\alpha_{IIb}\beta_3$ cleavage and inactivation. Other mechanisms are secretion of (anti) coagulation factors, and $\alpha_{IIb}\beta_3$-mediated thrombus retraction, and clot retraction. In a thrombus, coagulation factors are found at both platelets and fibrin fibers. Many of the procoagulant platelet activities are altered in the Scott syndrome.

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Triggering platelet activation and coagulation in thrombus formation

Platelets have key roles in thrombosis and hemostasis by clustering into aggregates and providing a procoagulant surface, at which thrombin is generated and fibrin fibers are formed [1,2]. Similar to other platelet responses, such as adhesion, shape change, Ca^{2+} signaling, secretion and aggregation, platelet procoagulant activity is efficiently suppressed by endothelial-derived prostacyclin and nitric oxide, thus ensuring that clotting is minimalized at the sites of healthy endothelium [3,4]. Upon injury of a healthy vessel or rupture of an atherosclerotic plaque, platelets and the coagulation system are simultaneously activated by substances that become exposed to the blood stream. These include collagen and laminin, serving as scavenger sites for von Willebrand factor (VWF) [5,6]; tissue factor, which by interacting with factor VIIa stimulates the extrinsic pathway of coagulation [7,8], while exposed collagen activates factor XII and hence the intrinsic coagulation pathway [9]. The ensuing thrombus formation is also supported by traces of tissue factor from the blood [10]. As any traumatic injury also results in formation of a procoagulant surface on vascular cells with relatively high concentrations of membrane-bound tissue factor, the coagulation process will also be propagated by damaged or activated cells from the vessel wall.

The mechanism of thrombus formation is mostly studied during shear-dependent interaction of platelets to vWF/collagen via the glycoprotein Ib/V/IX complex [4,5,11]. Platelet activation in this case occurs via the signaling collagen receptor, glycoprotein VI, in a way enforced by the adhesive receptor integrin $\alpha_2\beta_1$ [12,13]. Recent evidence indicates that similarly platelet co-adhesion via other receptor sets, such as the CLEC-2 receptor and integrins like $\alpha_6\beta_1$ (laminin receptor) and $\alpha_{IIb}\beta_3$ (fibrinogen receptor) leads to formation of multilayered thrombi [14]. Recruitment of platelets from the circulation into a thrombus occurs through the release of soluble autocrine mediators - ADP, ATP and thromboxane A_2 -, which activate via G-protein coupled receptors [15,16].

Platelets orchestrating coagulation processes in thrombus formation

In vivo and *in vitro* studies of thrombus formation provide strong evidence that platelets can steer the coagulation process in multiple ways, in particular via exposure of phosphatidylserine (PS), integrin activation, secretion and the generation of fibrin.

Procoagulant activity and PS exposure. For more than three decades it is known that platelets stimulated with a Ca^{2+} ionophore respond by scrambling their membrane phospholipids and exposing the negatively charged PS [17,18]. Surface exposure of PS potently stimulates the binding and activation of vitamin-K dependent serine proteases, such as factors II, VII, IX and X, and leads to a dramatic increase in the formation of factor Xa (tenase activity) and thrombin (prothrombinase activity) [1,19]. The interest in this membrane change was boosted by the discovery that patients with the rare Scott syndrome have blood cells with greatly impaired phospholipid scrambling and PS exposure. Scott patients are characterized by a mild bleeding disorder associated with a low platelet-dependent consumption of prothrombin in coagulating plasma or serum [19].

Platelet agonists or combinations of agonists causing PS exposure are in general those inducing a potent and prolonged

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increase in cytosolic free Ca^{2+}. Next to ionophores like ionomycin, such a high Ca^{2+} flux can be achieved by stimulation of the (hem) ITAM-linked receptors glycoprotein VI or CLEC-2, particularly in combination with thrombin receptors (PAR1 and PAR4); otherwise by stimulation with the Ca^{2+}-pump inhibitor thapsigargin plus thrombin [1,4]. Events required for this high Ca^{2+} signal are inositol trisphosphate receptor-induced Ca^{2+} mobilization; Ca^{2+} entry via the Orai1 channel and stromal interaction molecule 1 (STIM1); plus Ca^{2+} release from mitochondria through the mitochondrial permeability transition pore [2].

During thrombus formation under flow conditions, platelets are subjected to combinations of physiological agonists. As a result, a population of the platelets responds by PS exposure, particularly platelets adhered to collagen and patches of platelets stimulated with thrombin [13]. A marked characteristic of PS-exposing platelets is that they undergo a dramatic change in morphology to gradually detach from the thrombi [20] and obtain a balloon-like shape [21]. This balloon formation likely serves to increase the membrane surface area for binding of coagulation factors.

Platelet integrin activation. Activation of integrin $\alpha_{IIb}\beta_3$ is a strict requirement for the formation of platelet aggregates. However, this integrin also influences the coagulation process in various ways. It is known that $\alpha_{IIb}\beta_3$ activation predisposes for contact-dependent platelet signaling – regulated for instance by junctional adhesion molecule (JAM), signaling lymphocytic activation molecule (SLAM), platelet-endothelial cell adhesion molecule (PECAM), endothelial cell-selective adhesion molecule (ESAM) and connexins –, such as occurring in the thrombus core with densely packed and degranulated platelets [13,22]. The likely mechanism is inside-out signaling by fibrinogen-bound activated $\alpha_{IIb}\beta_3$, which stimulates spreading of platelets on a surface and contraction of platelets in a thrombus or clot. Integrin-mediated platelet contraction is regulated by Src and Rho kinases, and relies on actin-myosin changes of the cytoskeleton [23]. In coagulating platelet-rich plasma, this contraction process leads to retraction of the diffuse fibrin clots. Interestingly, it appears that the majority of platelet signaling pathways that are regulatory for integrin activation also contribute to fibrin clot retraction [2]. Perhaps counter-intuitively, outside-in $\alpha_{IIb}\beta_3$ signaling can potentiate the Ca^{2+} rises in platelets and thereby stimulate PS exposure [24]. This integrin-induced PS exposure likely is confined to platelets not trapped into an aggregate and not involved in clot retraction. Recent findings suggest that platelet-dependent clot contraction allows stacks of red blood cells to incorporate into a thrombus [25].

The thrombus shell contains platelets that do not firmly adhere due to limited integrin activation [26]. However, also platelets that are adhered inside a thrombus can detach by secondary inactivation of $\alpha_{IIb}\beta_3$, for example by restriction of $P2Y_{12}$ signaling [27]. A different way of integrin inactivation occurs in high-Ca^{2+} PS-exposing platelets, namely by calpain-mediated cleavage of several cytoskeletal-linked proteins and the integrin β_3 chain itself [28]. The latter mechanism of integrin shut-off likely helps to detach PS-exposing platelets from a thrombus.

Platelet secretion. Platelets secrete numerous substances from their α- and δ-granules and lysosomes to influence their environment [29,30]. Among these, stored in the δ-granules, are procoagulant factors - fibrinogen, prothrombin, factor V, factor VIII-like, factor XIII - as well as anticoagulant factors - tissue factor pathway inhibitor, various serpins -. In addition, the α-granules contain polyphosphates, which support factor XII activation [31]. Overall, the effect of platelet secretion products is coagulation-promoting. This may explain the hemostatic insufficiency of patients with Gray platelet syndrome, lacking α-granules in their platelets [32].

Thrombin and fibrin formation. Both *in vitro* and *in vivo* studies suggest that the activity of thrombin is highest in the core of a thrombus [22]. However, the majority of thrombin is generated by prothrombinase activation on PS-exposing platelets, usually present in patches at the thrombus surface [33]. Once sufficient thrombin is formed, it produces fibrin fibers extending from the platelet surface [34]. Interestingly, these fibrin fibers rapidly bind large amounts of the generated thrombin [35], thus simultaneously reducing the level of soluble thrombin and localizing its action at the site of fibrin formation. Furthermore, inside a thrombus, strong positive feedback loops must exist between thrombin-induced PS exposure and PS-dependent thrombin/fibrin generation. This became apparent from studies where either factor Xa or thrombin was deficient or inhibited, resulting in major decreases in PS exposure as well as fibrin formation [36]. Along the same line, Scott platelets with a deficiency in PS exposure were also impaired in fibrin formation [37].

Intra-thrombus localization of coagulation factors

Recent findings have provided unexpected insight into the quite heterogeneous localization of coagulation factors in a growing thrombus, with both PS-exposing platelets and fibrin fibers as main concentration sites (Table 1).

The vitamin K-dependent coagulation factors II, VII, IX, X and the anticoagulant factors protein C and S strongly bind to PS-containing membranes via their Gla domains (Figure 1) [1,19]. Factor V, acting as protease co-factor, also binds to PS membranes but via its C2 domain. Thus, it is not a surprise that these factors co-localize with PS-exposing platelets in a thrombus [13,36].

It is known that fibrin fibers can bind multiple plasma proteins [38]. Data are accumulating that these fibers act as a scaffold for several coagulation factors, and that this binding enhances the coagulation process. Once cleaved into thrombin, prothrombin massively binds to fibrin, as described above. In addition, factor X [39] and factor XII [40] may directly interact with fibrin and change the fibrin clot structure. Another key hemostatic protein with binding sites for fibrin is vWF (via its C-domains), which then is active in stimulating platelet adhesion [41]. Detailed studies indicate that vWF, bound to collagen or fibrin, is also the main localization site of factor VIII [36,42].

The transglutaminase factor XIII cross-links fibrin fibers to increase their stability and, by implication, needs to bind to fibrin [43]. On the other hand, factor XIII has also been detected at the surface of PS-positive platelets, where it exerts anti-fibrinolytic activity by cross-linking α_2-antiplasmin to fibrin [44]. A similar binding pattern is seen for the fibrinolysis factor plasminogen, which binds to PS-exposing platelets and after cleavage into plasmin translocates to fibrin [45,46]. Following the original work of the Dale group on formation of a transglutaminase-dependent fibrin-containing coat on so-called coated platelets (with PS exposure) [47], it recently could be established that the role in fibrin formation of coated platelets relies on interactions via both integrin $\alpha_{IIb}\beta_3$ and factor XIII [48].

Recently, two independent groups demonstrated that platelet glycoprotein VI can also act as a receptor for fibrin. Fibrin binding to glycoprotein VI evokes ITAM-linked signaling, and thereby contributes to thrombus growth and stability [49,50]. Taken together, these data suggest that the interactions of coagulation factors and platelets with fibrin stimulate thrombus formation. Surprisingly little is known of the putatively suppressive action on thrombus formation of the anticoagulation factors, protein S, C and antithrombin.

Altered procoagulant state in Scott and Stormorken syndromes

As summarized elsewhere, the Ca^{2+}-induced scrambling of phospholipids and PS exposure is mediated by the transmembrane ion channel protein anoctamin-6 [51,52]. Evidence for this is based on the fact that Scott patients have one or more mutations in anoctamin-6 (gene *ANO6* or *TMEM16F,* OMIM:608663),

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