Procoagulant Platelets: Mechanisms of Generation and Action

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Abstract

During the past decades, it has been increasingly recognized that the major function of accelerating membrane-dependent reactions of blood coagulation is predominantly implemented by a subset of activated platelets. These procoagulant platelets (also called collagen- and thrombin-activated or COAT, coated, necrotic, although there could be subtle differences between these definitions) are uniquely characterized by both procoagulant activity and, at the same time, inactivated integrins and profibrinolytic properties. The mechanisms of their generation both in vitro and in situ have been increasingly becoming clear, suggesting unique and multidirectional roles in hemostasis and thrombosis. In this mini-review, we shall highlight the existing concepts and challenges in this field.

Keywords
- procoagulant platelets
- thrombosis
- hemostasis

Introduction

Platelets play several physiological roles, but their two major contributions to hemostasis and thrombosis are believed to be based on the two abilities: to form aggregates and to support membrane-dependent reactions of coagulations upon activation.[1] For many years, it was believed that these two abilities are manifested by activated platelets uniformly.[2] Even though pioneering findings of phosphatidylserine (PS) expression by a separate platelet subpopulation could be traced as early as 1990s,[3] the explosive development of the field began 10 years later with the report on procoagulant COAT platelets by Alberio et al[4] that paradoxically have low levels of active integrins.[5] Only in the past decade, the exciting fact that platelets upon activation get segregated into at least two subpopulations, each assuming one of the two earlier-described major functions, has become widely accepted.[6–8] Although many mysteries of the generation, functional properties, physiological roles, and clinical significance of platelet subpopulation remain to be deciphered, the recent years witnessed an explosive growth of studies addressing some of these issues (see recent reviews[6,7,9–11]). In this mini-review, we shall highlight the existing concepts and challenges in this field.

Procoagulant Platelets: What Are They?

Platelets, in addition to the usual variability in size and number of molecules/organelles, also have morphological and functional differences. Back in the 1960s, morphological differences in platelets subjected to hypotonic stress were described.[12] In addition, at the end of the 1990s, using flow cytometry, it was shown that Annexin V (a specific marker of PS) does not bind to all platelets activated by thrombin with collagen.[3] Also it has been shown that, upon adhesion to collagen, about half of the platelets bind to annexin V, while the addition of thrombin increases the proportion of such cells to 80%.[13] It was found that activation by thrombin with

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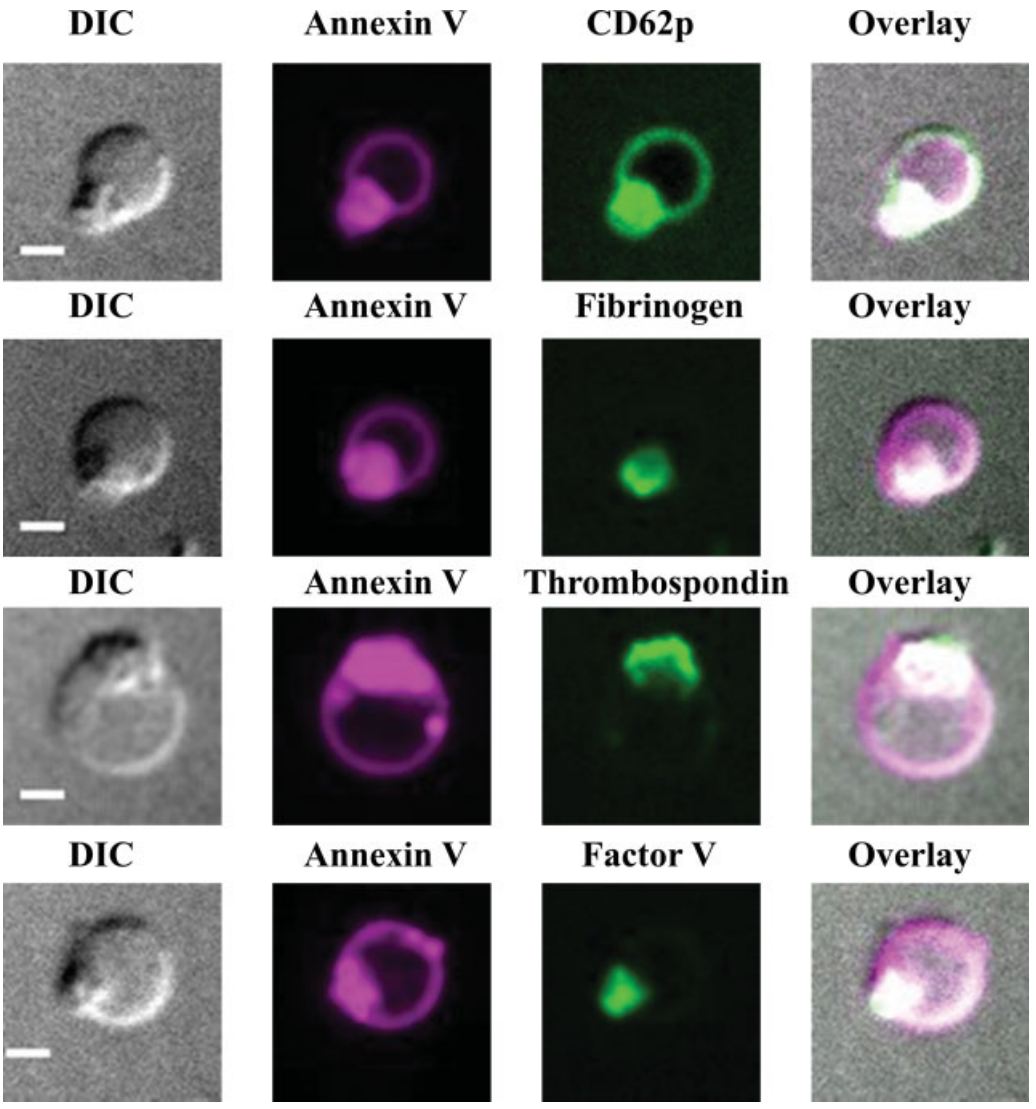

Fig. 1 Distribution of α-granule proteins on the procoagulant platelets. Platelets from healthy donors were activated at 50,000 μL^{-1} with 100 nM of thrombin and 20 μg/mL of CRP, dual labeled with annexin V, and with indicated antibodies, and imaged with confocal microscopy.[14,88] Representative confocal images of DIC; Alexa647 (magenta) fluorescence of annexin V; and FITC (green) fluorescence of anti-CD62p, anti-fibrin (ogen), anti-thrombospondin, or anti-factor V antibody are shown. Scale bar, 2 μm.

collagen results in a subpopulation of cells expressing the α-granular protein factor V on their surface.[4] In addition, the same subpopulation of cells is characterized by the presence of PS on the outer leaflet of the membrane, and also binds to other α-granular proteins (fibrinogen, von Willebrand factor, thrombospondin, fibronectin, and α_2-antiplasmin).[5] It was shown that α-granular proteins are nonuniformly distributed on the platelet membrane, and are concentrated only in its small area (►Fig. 1).[14–17] Despite the large number of works devoted to platelet subpopulations, only by the end of the 2000s, the phenomenon of the separation of platelet subpopulations was finally recognized. However, there is still no common name for subpopulations.[6,7]

It was believed that only PS-negative platelets are able to aggregate, because they have active αIIbβ3 integrin on their surface.[5,18] There is evidence in the literature that inhibition of the formation of PS-positive platelets leads to an increase in the rate of platelet plugs growth in flow chambers, and the adhesion of nonactivated platelets to the activated monolayer is significantly reduced if there are PS-positive cells in the monolayer.[19] More recent studies have shown that PS-positive platelets cannot aggregate with each other but can be recruited into aggregates by PS-negative platelets.[20] However, the main function of PS-positive platelets appears to support the plasma coagulation reactions. Rest platelets cannot to promoting coagulation reactions. Although some coagulation factors can also bind to inactivated platelets, active enzymatic complexes do not appear in this case.[21] Only after strong activation negatively charged phospholipids, primarily PS, appear on the outer leaflet of cell membrane.

Several studies have shown that coagulation factors are mainly associated with PS-positive platelets.[22–24] And it is on the surface of these platelets that the main processes of plasma coagulation are performed: activation of a complex of factors X and prothrombin of intrinsic tenase (from factors

VIIIa and IXa associated with the phospholipid membrane) and prothrombinase (from factors Va and Xa associated with the phospholipid membrane), respectively.

Another important question is where PS-positive platelets go after they have completed their function. Our body has mechanisms for removing dead cells, both apoptotic and necrotic. However, it is still not completely clear whether these mechanisms apply to platelets. It was shown that lactadherin could mediate the clearance of platelet-derived PS-positive microvesicles,[25] but whether they mediate the clearance of PS-positive platelets remains to be fully elucidated.

Procoagulant Platelets Generation and Signal Transduction

The pioneering studies reported that procoagulant platelets are formed upon potent dual stimulation with collagen and thrombin, which was one of the reasons for the name COAT.[3–5] Later reports suggested that two distinct subpopulations can be observed with thrombin alone[23] or PAR1 (protease-activated receptors) and/or PAR4 thrombin receptor agonist peptide[24,26] stimulation as well, although their fraction does not usually exceed 10 to 20% compared with 30 to 60% and more for dual stimulation. Other properties including balloon-like morphology, coagulation factor binding, the presence of the "coat" of α-granule proteins of the thrombin-produced procoagulant platelets appear similar to those produced by dual stimulation.[14,27] Thrombin's enzymatic activity is essential, in particular, for the formation of the α-granular protein "coat."[14,28] Adenosine diphosphate is not considered a strong agonist by itself, but was shown to significantly modulate, via $P2Y_{12}$ receptor, the number of procoagulant platelets produced in response to other agonists.[27,29–31] The role of other signaling receptors is not clear. To summarize, it is currently believed that procoagulant platelets are mainly produced by either thrombin (via PAR1 [or PAR3 for murine platelets] and PAR4) or collagen (via glycoprotein VI) stimulation, with ADP acting as a modulator via $P2Y_{12}$. Instead of collagen, which is very difficult during in vitro studies, convulxin[4,32] or collagen-related peptides[33] are often used. It should be noted that recent studies suggest that not only collagen and its analogs but other molecules such as polymerized fibrin may cause procoagulant platelet formation via glycoprotein VI.[34]

With regards to intracellular events, it was found quite early that high, sustained cytosolic calcium concentrations (and not single or even serial calcium spikes) are needed for procoagulant platelets to be formed,[13] but it took some time to figure out the mechanisms responsible for different scenarios of calcium signaling. The critical regulator of procoagulant platelet formation turned out to be mitochondrial permeability transition pore (mPTP) opening,[35] and the pathway to procoagulant platelet formation upon stimulation was found to be caspase independent.[36] Beginning from this stage, the use of mice knockouts (in cyclophilin D, mitochondrial calcium uniporter (MCU), and anoctamin 6 as well as in Scott's syndrome patients,[26,29,37] single-cell microscopy of shape change, calcium signaling and mitochondrial potential monitoring,[15,38–40] imaging flow cytometry,[41] and continuous flow cytometry)[26,42,43] and the use of low-affinity calcium dyes[44] allowed significant insights into the nature of procoagulant platelet generation upon stimulation.

The current concept suggests that procoagulant platelet generation begins with classic cytosolic calcium increase, which is caused by its efflux from internal stores in its turn induced by inositol triphosphate released by phospholipase C action at the plasmatic membrane (►Fig. 2). This increase may have a form of spikes, and additional calcium may come from store-operated calcium entry or store-independent pathways.[43,45] The cytosolic calcium is uptaken into the

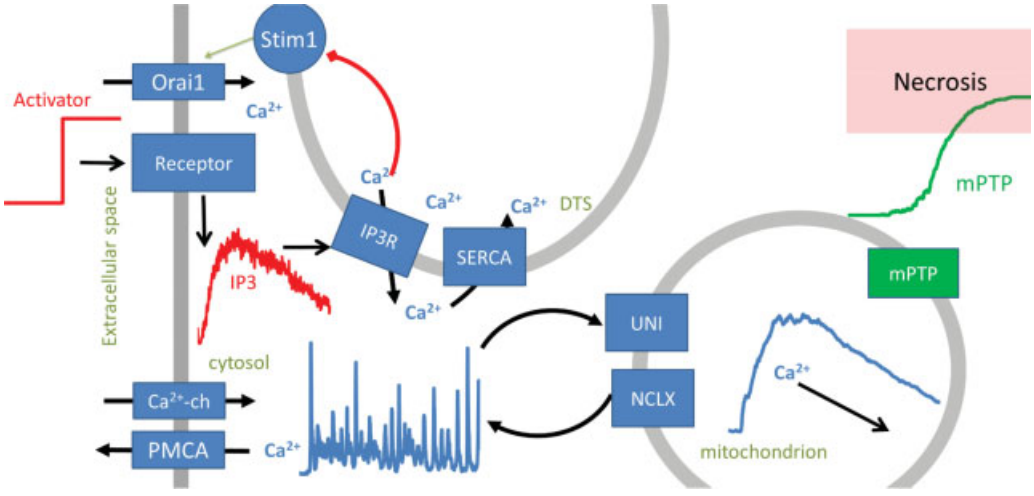

Fig. 2 Procoagulant platelet generation. Activation of platelets via PAR1 (protease-activated receptors) and/or PAR4 and/or glycoprotein VI leads to IP3 (inositol trisphosphate) increase, which initiates calcium spiking in the cytosol controlled by the DTS (dense tubular system). Sufficiently high calcium spikes lead to calcium uptake by the mitochondria, supposedly via calcium uniporter, so that calcium signal is "integrated" over time. If sufficient level is accumulated, then cyclophilin D–dependent mPTP (mitochondrial permeability transition pore) opening leads to the mitochondrial collapse and cell necrosis. IP3R, inositol trisphosphate receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; STIM1, stromal interaction molecule 1; NCLX, Na/Li/Ca exchanger; UNI, calcium uniporter.

mitochondria mainly via MCU, leading to the overloading of mitochondria matrix with calcium and mPTP opening, resulting in further cytosolic calcium increase and rapid ATP depletion as a result of inner mitochondrial membrane potential loss. The stable opening of mPTP in all mitochondria results in a typical necrotic cell death associated with rapid loss of plasma membrane integrity and collapse of any ion homeostasis. The sustained calcium increase at the supraphysiological level appears to be a consequence of this chain of events.[39,43,44] PS externalization by anoctamin 6, calpain-dependent cytoskeleton degradation with ballooning and microvesicle formation, and integrin inactivation appear to be mostly downstream events after the commitment to necrosis. Other signaling pathways may essentially modulate the upstream events leading to the platelet death and affect chances of a platelet to become procoagulant.

It is essential that the formation of procoagulant platelets is all-or-nothing process. Platelets are not predestined to become procoagulant: depending on the presence and concentration of thrombin (or thrombin receptor peptide), collagen (or collagen-related peptide, or convulxin), and ADP in the activation mixture, the number of platelets to become procoagulant can be from below 1% to above 90%.[20] However, there could be factors that predispose platelets to become procoagulant rather than non-procoagulant: the reported ones include age,[4] size,[46,47] baseline calcium concentration,[39] and the number of mitochondria per platelet.[38]

Generation of Procoagulant Platelets In Situ

The published data on the localization of both PS-positive platelets and fibrin in thrombi in vivo are rather controversial, and probably depend on the conditions of the experiments. The early studies on real-time confocal intravital imaging of laser-induced microvasculature thrombosis reported fibrin in the center of the thrombi,[48] in line with the latest reports suggesting the core/shell concept of thrombus architecture in both microvasculature and macrovasculature.[49,50] The first data on procoagulant platelets localization in thrombi came from Munnix et al,[51] who described procoagulant platelets to be distributed as separate patches throughout the thrombi formed either in vitro or in vivo. However, at about the same time, other study reported that both procoagulant platelets and fibrin are colocalized in the core region of thrombi formed in vivo following a laser-induced injury of mesenteric venules.[52] Another in vivo study showed that there are no procoagulant platelets following laser-induced injury of cremasteric arterioles in mice, but they are present and partially colocalized with fibrin in FeCl3-induced occlusive injury of the same vessels (though, their specific spatial distribution is hard to judge).[53] In marked contrast to previous studies on fibrin, another article on laser injury of mesenteric venules reported that fibrin formed a "coat" on the surface of platelet thrombus, rather than being in the center.[54] Data coming from in vitro parallel-plate flow chamber experiments revealed that procoagulant platelets were also distributed on the surface of thrombus.[14]

Recent study on the origin of pulmonary thrombosis after gut ischemia revealed the importance of neutrophil interactions with procoagulant platelets on the surface of thrombi.[55] Interestingly, early reports on the structure of the hemostatic plugs in dogs and humans described both ballooned platelets and fibrin on the surface of these plugs.[56,57] Another study reported predominant surface localization of PS-positive in thrombi formed on collagen under flow conditions in vitro and in small platelet aggregates formed in vitro without controlled flow.[15] In the same study, TEM images of in vivo thrombus formed in response to ferric chloride–induced injury depicted ballooned platelets near the interface of thrombus and injured vessel wall. Detailed analysis of platelet plugs formed after penetrating injury of jugular vein revealed significant difference in both platelets and fibrin composition of the intraluminal and extravascular sides of the plug.[58] Interestingly, numerous small spherical platelet fragments were described at the boundary of the injury site—possibly the fragments of procoagulant platelets.

Localization of procoagulant platelets on the surface of thrombi reported in many studies seemed paradoxical, as far as local biochemical microenvironment near thrombus surface is not expected to drive the formation of PS-positive, which requires potent activation. Recent study demonstrated predominant surface localization of PS-positive formed in two in vivo models of thrombosis and reported outward translocation of PS-positive during thrombus formation on collagen in vitro.[59] Redistribution of procoagulant platelets to thrombus surface in vitro was driven by thrombus contraction, suggesting that the same mechanism might be responsible for surface localization of procoagulant platelets in vivo. Interestingly, redistribution of procoagulant platelets to thrombus periphery in vitro was followed by enhanced surface generation of fibrin, suggesting that mechanically induced redistribution of PS-positive may impact both spatial and temporal character of plasma coagulation reactions.

Thus, analysis of the published data regarding localization of procoagulant platelets in thrombi suggests that most of the in vivo models of thrombus formation in large vessels (like arteries), in vitro models of thrombus formation on collagen and hemostatic plugs formed in vivo, share a common feature of surface localization of procoagulant platelets. Enhanced deposition of fibrin on the thrombus surface, reported for some of these models, is likely mediated by surface distribution of procoagulant platelets; however, the details on the interplay between spatiotemporal features of procoagulant platelets formation and generation of both thrombin and fibrin are still poorly understood.

Generation of Procoagulant Platelets in Patients

Among the inherited platelet disorders, Scott's syndrome is probably the single well-known primary pathological consequence of impaired platelet procoagulant activity[60] caused by a deficiency of anoctamin 6, which normally exposes PS on

the outer surface of activated platelets.[61] However, decreased generation of procoagulant platelets was reported for a cohort of patients with bleeding,[62] suggesting that they contribute in a much wider range of cases.

In contrast, Stormorken's syndrome, another inherited platelet disorder, is characterized by preactivated (with elevated surface PS exposure) and, at the same time, much less responsive to stimulation platelets[63] due to increased basal calcium level caused by autosomal dominant mutations in the *STIM1* (stromal interaction molecule 1) gene.[64] Another platelet disorder potentially related to procoagulant platelets is Wiskott–Aldrich syndrome for which it has been shown that platelets readily expose PS via mitochondria-dependent necrotic mechanism caused by their smaller size, which could contribute to the development of thrombocytopenia.[38] An acquired platelet disease immune thrombocytopenia is characterized by elevated PS exposure on unstimulated platelets[65,66] and increased resting intracellular calcium[67] which likely contribute to the decreased platelet counts. Levels of circulating procoagulant platelets were significantly higher in colon cancer patients[68] and patients with stage III/IV of gastric cancer[69] compared with healthy control, which may play a role in cancer-associated venous thrombosis.

Procoagulant platelets can be considered biomarkers for the clinical assessment of thrombotic or bleeding tendency. Several studies have shown that a higher level of the procoagulant platelets was correlated with transient ischemic attack and stroke[70,71] and with stroke recurrence.[72,73] Moreover, elevated procoagulant platelet levels identify asymptomatic carotid stenosis patients at high risk for stroke or transient ischemic attack.[74] Likewise, procoagulant platelet level has been suggested for use in risk stratification for stroke at 30 days after transient ischemic attack.[75] In another study, procoagulant platelets rise after aneurysmal subarachnoid hemorrhage was associated with adverse clinical outcomes.[76] On the contrary, a low level of the procoagulant platelets has been shown to be associated with more severe hemorrhage and increased mortality after spontaneous intracerebral hemorrhage.[77–79]

Studies in hemophilia A have shown that procoagulant platelet levels were significantly lower in hemophilia patients compared with healthy controls. Moreover, in hemophiliac patients with frequent bleeding episodes, the level of procoagulant platelets was relatively low.[80]

The level of procoagulant platelets can be affected not only by the disease itself but also by therapy. One example is the use of tyrosine kinase inhibitors for cancer treatment. It was shown that in dasatinib-treated patients with chronic myeloid leukemia, procoagulant platelet formation was significantly suppressed,[81,82] which may be the cause of gastrointestinal bleeding on dasatinib treatment.[83,84] Ibrutinib treatment further reduced procoagulant platelet formation upon strong stimulation, which was already reduced in patients with chronic lymphocytic leukemia which could also contribute to the development of bleeding.[85] On the other hand, recent study suggests that procoagulant platelets are upregulated by desmopressin and may play a role in its mechanism of action of desmopressin.[86] Another study[87] showed the ability of procoagulant platelets to bind factor VIIa and promote factor X activation by it, which may be a mechanism of action of recombinant-activated factor VII.

Conclusions

The existing evidence suggests that the main mechanism of procoagulant platelet generation is mitochondrially driven necrosis. It begins with "normal" platelet activation and intracellular calcium increase. This leads in turn to the accumulation of calcium by mitochondria. In some of the platelets (depending on size, age, and the number of mitochondria per platelet), this "normal" signaling results in mitochondrial collapse and necrotic process, with downstream formation of the procoagulant "coat" (or "cap") and integrin inactivation. Agonists under the influence of which formed PS-positive platelets have a significant effect on the presence of the procoagulant "coat" (or "cap"). But there is no convincing data that the type of activation affects the other functional properties of platelets or their fate. Interplay of thrombi, collagen (fibrin/fibrinogen), and ADP receptors determine the quantity of procoagulant platelets produced. Within thrombus, these properties lead to a redistribution of procoagulant platelets to the outside of the dense core as a result of contraction, which affects fibrin distribution in the thrombus. Currently, there is an active accumulation of knowledge about the relationship between the level of procoagulant platelets with various diseases and some types of therapy. However, it is still too early to say that we clearly understand the pathophysiological role of these platelets.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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