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Chapter

Procoagulant Platelets

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Abstract

There are two well-known subpopulations of activated platelets: pro-aggregatory and procoagulant. Procoagulant platelets represent a subpopulation of activated platelets, which are morphologically and functionally distinct from pro-aggregatory ones. Although various names have been used to describe these platelets in the literature (CoaT, CoaTed, highly activated, ballooned, capped, etc.), there is a consensus on their phenotypic features including exposure of high levels of phosphatidylserine (PSer) on the surface; decreased aggregatory and adhesive properties; support of active tenase and prothrombinase complexes; maximal generation by co-stimulation of glycoprotein VI (GPVI) and protease-activated receptors (PAR). In this chapter, morphologic and functional features of procoagulant platelets, as well as the mechanisms of their formation, will be discussed.

Keywords: procoagulant, platelet, mitochondria, necrosis, apoptosis, coagulation

1. Introduction

Blood has different components, like plasma, red blood cells (RBC), white blood cells (WBC), and platelets. Platelets, although being only "tiny fragments of megakaryocytes (mother cell)," are essential for life. We need them, together with about two dozen of coagulation factors, to keep all that nutrient-rich liquid plasma, infection-fighting WBCs, and oxygen-carrying RBCs in our bodies in the case of trauma and bleeding. Platelets and coagulation get activated immediately upon being exposed to things they normally do not have contact with (e.g., during blood vessel wall rupture, collagen gets exposed and activates platelets). Upon activation, platelets form a mesh-like structure using another plasma protein called fibrinogen as bridges between them. This process forms a so-called "unstable clot." At later stages of blood clotting, generated thrombin converts soluble fibrinogen to insoluble fibrin, stabilizing the initial platelet plug. These processes are collectively called hemostasis. Every aspect of hemostasis has its history. Although exploring history can sometimes be tedious, studying the bridge between past and present is essential in basic understanding of the subject, the subject of procoagulant platelets in this case.

Unlike WBCs, which exist as functionally and morphologically distinct subpopulations, it has been thought for years that platelets are rather simple in their function, being just "cell particles." It was later revealed that platelets, although lacking nuclear material, are indeed very complex not only in their nature but also in function. Nowadays, the existence of two different subpopulations of activated platelets, pro-aggregatory and procoagulant, is a widely accepted fact. Pro-aggregatory platelets, historically known as activated, have been a major focus since the initiation of platelet research. The history of procoagulant platelets, on the other

hand, can be subdivided into two periods. The first one is the discovery of platelet procoagulant functionality, whereas in the second period, procoagulant platelets were discovered and characterized as a distinct subpopulation of activated platelets.

In 1912, long before we learned that prothrombinase supporting platelets are distinct from pro-aggregatory ones, Howell discovered that unsaturated cephalin is a phospholipid factor that triggers clotting [1]. This discovery was followed by decades of controversial results of what phospholipid or what mixture of phospholipids is responsible for this effect until, in 1960, Karl Slotta demonstrated the presence of phosphatidylserine (PSer) is an absolute requirement [2]. This discovery established the central role of PSer in prothrombinase activity, which in a way, paused research activities in the procoagulant platelet field.

The history was resumed when two decades later, Bevers and colleagues spectrophotometrically showed that thrombin and collagen co-stimulated platelets possessing about 5-fold higher prothrombinase activity than those stimulated with thrombin or collagen alone. This effect was even more pronounced in the presence of exogenous factor Va [3]. Later that year, the same group confirmed their spectrophotometric observations in one-stage prothrombinase assay [4]. In 1985, Rosing et al. more thoroughly described the role of platelet PSer exposure in prothrombin (FII) and factor X (FX) activation [5]. They revealed that collagen and thrombin co-stimulation significantly increases prothrombinase and tenase activity, while this increase was abolished in the presence of phospholipase A_2 (PLA_2) from *N. naja*. Also observing no cellular lysis in these conditions, they concluded that PSer exposure, which is followed by thrombin and collagen co-stimulation, is responsible for this phenomenon. In 1993, with the discoveries of annexin V and advancement in flow cytometry, Dachary-Prigent et al. established a protocol to detect PSer exposing platelets, the methodology that has overtime become fundamental in procoagulant platelet field and has been widely used since then [6]. In 1997 Heemskerk and colleagues discovered that a percentage of platelets adhering to collagen, but not fibrinogen, balloon and expose PSer [7]. In parallel to these findings, there was a series of publications determining the extent and ultrastructure of PSer exposure, as well as the essential role of calcium in this process [8–10].

Although numerous studies described platelet procoagulant function in response to dual stimulation [3, 5, 11, 12], a breakthrough discovery that identified procoagulant platelets as a distinct subpopulation of activated platelets is the work by Alberio et al., where they demonstrated that only a certain percentage of activated platelets retain factor Va (FVa) on their surface [13]. This subpopulation was generated by co-stimulation with convulxin/collagen (GPVI agonists) and thrombin; hence they were named CoaT platelets. A few years down the road, Kulkarni and Jackson introduced a new term—'sustained calcium-induced platelet morphology (SCIP)' by discovering the fact that procoagulant platelets require prolonged elevation in cytosolic calcium to form [14]. Diversifying terminology of procoagulant platelets did not stop there, in 2005 revealing that procoagulant platelets retain multiple α-granule proteins on their surface in transglutaminase-dependent manner led to the introduction of a new term—'coated' (by α-granule proteins) platelets [15, 16]. Further studies by Panteleev et al. revealed that this subpopulation of activated platelets binds high levels of factors IXa and Xa [17]. In 2008, Jobe et al., characterizing molecular mechanisms of procoagulant platelet formation, introduced another term—highly activated platelets [18]. There are few more terms used in the literature like ballooned [7], ballooned and procoagulant-spread [19], and super-activated platelets [20].

Just like in a famous Indian parable, where blind men try to describe an elephant they have never encountered before by touching different parts of it, researchers have been describing different (morphological and functional) features of

procoagulant platelets and introducing different terms based on their discoveries. Whereas indeed, everyone has been describing "different parts of the same elephant."

2. Mechanisms of procoagulant platelet formation

The discovery of procoagulant platelets as a distinct subpopulation of activated platelets at the beginning of the twenty-first century triggered research activities into cellular and molecular mechanisms of their formation. As shown by Alberio et al. and confirmed in later studies, procoagulant platelets are maximally generated upon co-stimulation of glycoprotein VI and PAR1/4. In 2005, Jobe and colleagues discovered that the absence of FcRγ, a key component responsible for glycoprotein VI signaling, ablates procoagulant platelet formation almost to baseline levels, evidencing GPVI stimulation is the major component of their generation [21]. The same year, Remenyi et al. demonstrated the role of mitochondrial permeability transition (MPT) in procoagulant platelet formation [16]. MPT is a Ca^{2+}-dependent molecular process that leads to mitochondrial swelling and cell death [22, 23]. During the onset of MPT, large pores are formed on the mitochondrial inner membrane making it non-specifically permeable to all solutes and molecules of molecular weight up to 1500 Da [24, 25]. It is a very well-known fact that mitochondrial Ca^{2+} overload can induce MPT, although the structure of MPT pore remains unknown.

Ca^{2+}, being a key signaling molecule in most cells, is important for many processes including platelet shape change and integrin α2bβ3 activation [26, 27]. In resting platelets, free Ca^{2+} is tightly regulated and maintained at about 100 nM in both the cytosol and mitochondria through the action of Ca^{2+}-ATPases in the plasma and cell membranes. Thus, platelet cytosolic-free Ca^{2+} is substantially lower than the blood Ca^{2+} levels, which are around 2 mM. With stimulation, however, cytosolic Ca^{2+} increases instantaneously. As outlined in **Figure 1**, this increase is mediated by activated phospholipase C (PLC). There are two major isoforms of PLC in human platelets, PLCβ and PLCγ. PLCβ is only activated downstream of Gq protein-coupled receptors (GPCRs). Whereas, PLCγ is activated downstream of numerous receptors like GPVI, glycoprotein Ib-IX complex (GPIb-IX), Fcγ receptor IIa (FcγRIIa), and C-type lectin-like receptor 2 (CLEC-2). As illustrated in **Figure 1**, both isoforms of PLC induce the release of Ca^{2+} from the dense tubular system (DTS) as well as activating transient receptor potential channel 3 (TRPC3). DTS release of Ca^{2+}, in turn, triggers its extracellular entry through store-operated calcium entry (SOCE). For some time, it remained mysterious on how the release of DTS calcium stores into the cytosol induces more Ca^{2+} to flow into the cell, further increasing its cytosolic concentrations. However, with the discovery of core components of SOCE, everything falls into place. STIM1 and ORAI1 are parts of the same complex. When STIM1 senses drop in Ca^{2+} levels within the DTS, it signals to ORAI1 located on a cell membrane to allow Ca^{2+} passage from extracellular space into the cell. As evidenced by the study in the early 2000s, increased cytosolic Ca^{2+} levels are one of the requirements of procoagulant formation [14]. Mitochondria, being the major and perhaps the only Ca^{2+} buffering system within platelets, equilibrate increased cytosolic Ca^{2+} following PAR and GPVI co-stimulation. The ensuing fate of mitochondrial Ca^{2+} overload is the opening of the mitochondrial permeability transition pore (MPTP), followed by PSer exposure and integrin deactivation (**Figure 1**), ultimately leading to a cell death. However, in this case cell death shall be considered physiologic considering the essential role of procoagulant platelets in hemostasis.

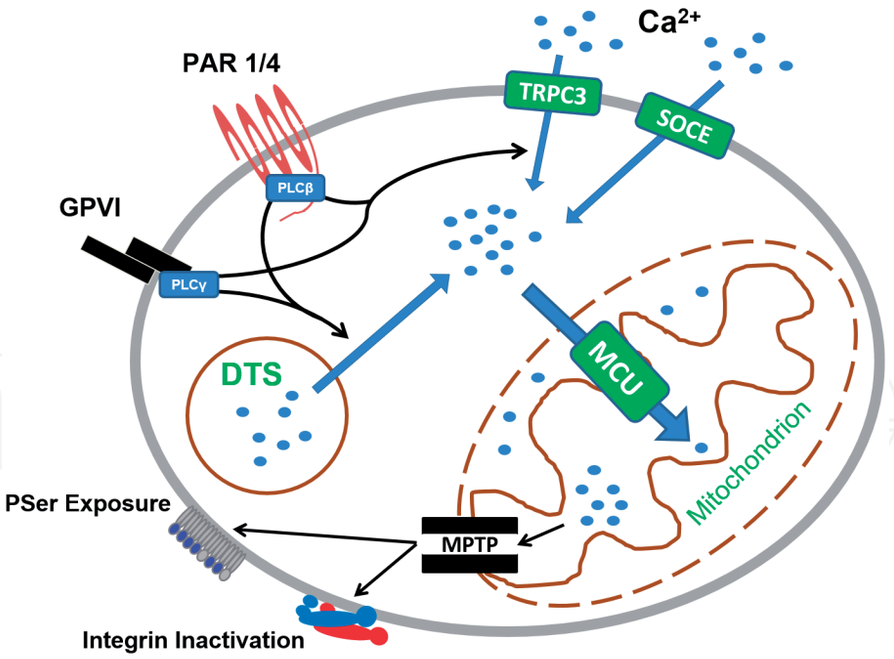

Figure 1.
Molecular mechanisms of physiologic agonist-induced procoagulant platelet formation. In resting platelets, low cytosolic Ca^{2+} is maintained by cell and plasma membrane Ca^{2+}-ATP-ases. Co-stimulation of protease-activated receptor 1/4 and glycoprotein VI leads to a sustained increase of cytosolic Ca^{2+} through activation of PLCβ and PLCγ, respectively. Increased cytosolic Ca^{2+} drives mitochondrial Ca^{2+} entry through the mitochondrial calcium uniporter complex. The resulting increase in mitochondrial Ca^{2+} opens mitochondrial permeability transition pore, which in turn leads to necrotic cell death and exposure of phosphatidylserine accompanied by integrin inactivation. DTS, dense tubular system; GPVI, glycoprotein VI; MCU, mitochondrial calcium uniporter; MPTP, mitochondrial permeability transition pore; PAR, protease-activated receptor; PSer, phosphatidylserine; SOCE, store-operated calcium entry; TRPC3, transient receptor potential channel 3. Adapted from [28] with modifications.

It brings us to another aspect of procoagulant platelet research, debated since their discovery, which is whether procoagulant platelets are necrotic or apoptotic. Cell death, both necrosis and apoptosis, is an essential event in the normal life of many cells in the human body, including platelets. Apoptosis, or programmed cell death, occurs in many organs throughout human lifetime. Necrosis, although considered to be mostly a catastrophic uncontrolled cell death, can also occur physiologically, as in the shedding of decidual endometrium during human menses. Necrosis and apoptosis differ in many aspects. The ultimate event during necrotic cell death is the osmotic swelling of the cell followed by the rupture of a cell membrane, whereas in apoptosis, cell shrinkage with preserved cell membrane is evident in later stages of this process.

Although the proposal that PSer is not homogeneously distributed on the surface of activated platelets was made back in 1985 [5], it took three decades to visualize that experimentally due to the complex nature of procoagulant platelets. In 2016, Podoplelova et al. presented a detailed structure of procoagulant platelets and cellular changes that give them the characteristic morphology. They elegantly demonstrated how the balloon is blown out from the platelet, leaving it as a "cap" [29]. The existence of bulges in a phospholipid bilayer, known as the open canalicular system (OCS), is essential, as it provides platelet a fair amount of surface reserve for ballooning. Morphologic appearance, in this case, resembles the classical osmotic swelling of necrotic cell death, as schematically presented on the left side of **Figure 2**. Another feature of necrosis, which is a collapse of energy production, is present in procoagulant platelets, as they lack energy-requiring contractile function [18]. Moreover, depletion of oxidative

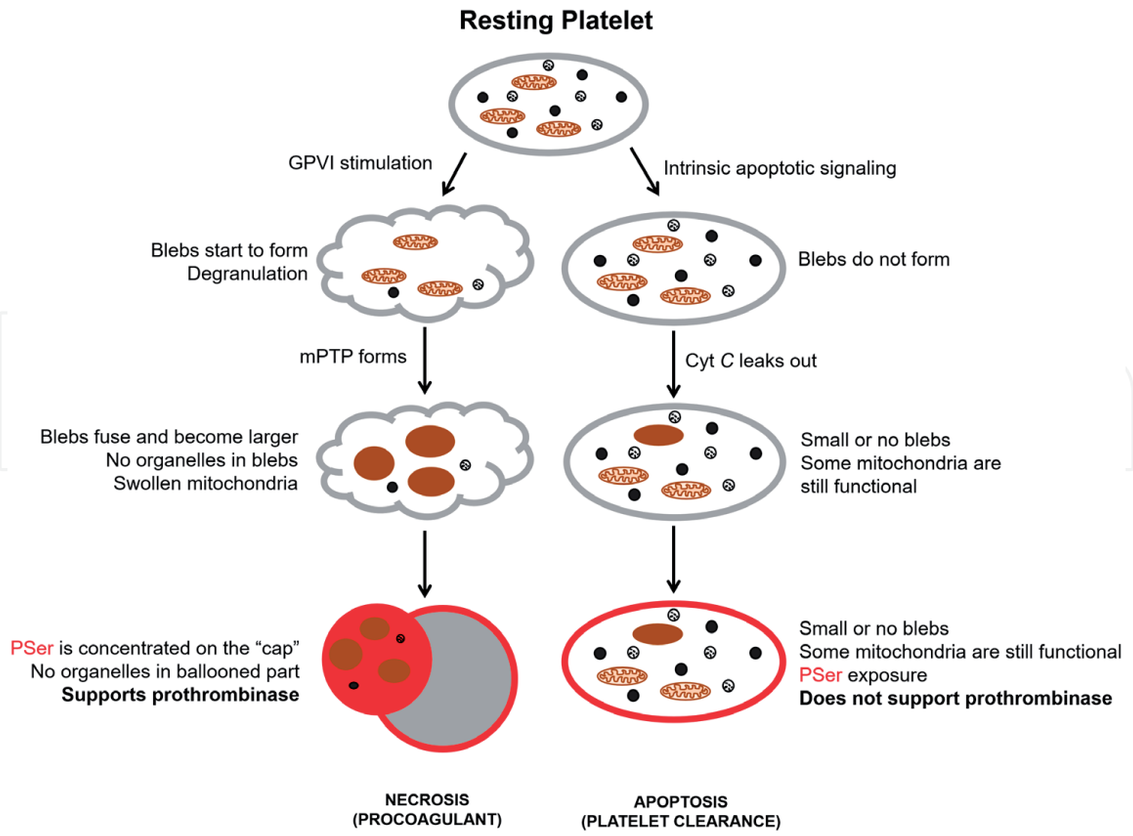

Figure 2.
Cellular mechanisms of platelet cell death. Cyt C, cytochrome C; GPVI, glycoprotein VI; mPTP, mitochondrial permeability transition pore; PSer, phosphatidylserine. PSer exposure is shown in red.

phosphorylation due to MPT, deactivates flippases that normally maintain the asymmetry of phospholipids. After the onset of MPT, contents of mitochondrial matrix get released to the cytosol leading to the second wave of cytosolic Ca^{2+} increase. This in turn leads to scramblase activation. Synergistically with deactivated flippases, scramblase performs its raison d'etre, which is flipping negatively charged PSer out in order to equilibrate its concentration. And finally, recent work demonstrated that platelets undergoing cell death through necrotic pattern and not apoptotic are functionally procoagulant as measured by prothrombinase support [30].

Platelet apoptosis (**Figure 2**, right side), on the other hand, has been widely implicated in platelet lifespan via the action of the intrinsic mitochondrial apoptosis pathway [31, 32]. Normally, antiapoptotic members of the BCL-2 family (e.g., BCL-xL) restrain the activity of proapoptotic Bax and Bak proteins, which are present within the cytosolic fraction of a platelet. When BCL-xL wears off, oligomerized Bax and Bak translocate to the mitochondrial outer membrane and permeabilize it, which leads to cytochrome *C* leakage into the cytosol, triggering apoptosome formation and eventually cell death. This is supported by the fact that the genetic ablation of murine BCL-xL reduces platelet life span from about 5 days to 5 hours [33, 34].

Thus, procoagulant platelets are indeed necrotic, while apoptosis is essential in platelet clearance. It should, however, be mentioned that the presence of PSer on the surface of a platelet is a signal for the reticuloendothelial system in the spleen and liver for clearance. Therefore, any procoagulant platelet that happened to escape the site of the active hemostatic process will be cleared out of the system by liver or spleen, just like aged apoptotic platelets do.

3. Functions of procoagulant platelets

The physiological relevance of procoagulant platelets had been questioned for a long time. In recent years, however, after the demonstration of a procoagulant platelet being predictive of bleeding or ischemic complications in patients with coronary artery disease, brain hemorrhage, traumatic brain injury, stroke, etc. [35–42] it is gaining more and more recognition. The importance of this subpopulation is further highlighted on a novel *ex vivo* model (which integrates all the core components of hemostasis [43]), where pharmacologic inhibition of platelet transition to a procoagulant state without affecting pro-aggregatory phenotype results in a decreased thrombus stability [44].

It was initially thought that the only function of procoagulant platelets is to support coagulation. However, with recent advances in the field, we learn that depending on their localizations, procoagulant platelets can play different functions within the thrombus. For general consideration, these two functionalities will be discussed separately here.

3.1 Coagulation support

Coagulation, a cascade of serine protease enzymatic reactions, is achieved by cleaving fibrinogen to fibrin, which transforms blood from a liquid to a gel-like state. Although platelet contribution to coagulation has been known for decades, the exact role of platelet phospholipids has been a matter of major debate. Dependence of hemostasis on biological membranes is very extensive, ranging from subendothelial membranes triggering coagulation and platelet activation to procoagulant platelet surface assembling tenase and prothrombinase complexes. It is very well known that at least two coagulation reactions are highly dependent on phospholipid surface, namely the activation of factor X and prothrombin by intrinsic tenase and prothrombinase, respectively. Both intrinsic tenase and prothrombinase are composed of the serine protease (FIXa for tenase and FXa for prothrombinase) and its protein cofactor (FVIIIa for tenase and FVa for prothrombinase). It is important to know that although both proteases alone are capable of activating their substrates, the presence of cofactors profoundly amplifies the catalytic process for up to 10,000 fold.

The current understanding of this process, based on numerous studies including mathematical modeling, is that phospholipid surface increases the rate of reactions by increasing the local concentration of coagulation factors, and thus increasing the probability of their interaction [29, 45–51]. This increase in a local concentration of factors, essential for tenase and prothrombinase complexes, is accomplished by the interaction of negatively charged gamma-carboxyglutamic acid (GLA) residues of the coagulation factors with negatively charged phosphatidylserine on the surface of procoagulant platelets. It is supported by the fact that GLA domain-containing (also known as vitamin K-dependent) factors predominantly bind to a procoagulant subpopulation of activated platelets [17, 29, 30]. Both enzymatic factors for intrinsic tenase (FIX) and prothrombinase (FX) are GLA domain-containing proteins and bind to PSer in a calcium-dependent manner. Whereas cofactors (FVIII and FV) structurally are not GLA proteins and bind to a procoagulant surface by different mechanisms.

In the case of FVIII, it has been shown by Gilbert et al. that it is not specific to a procoagulant surface [52]. It was further confirmed by Podoplelova et al. in their efforts exploring procoagulant platelet characteristics, they demonstrated that both (pro-aggregatory and procoagulant) subpopulations of activated platelets bind FVIIIa [29]. The fact that PS increases the catalytic activity of the intrinsic tenase

complex by about 1500-fold [53] can be explained by FIXa's specificity to PSer. As outlined in **Figure 3A**, initially FIXa and FX bind PSer. This binding reaction is calcium-dependent for all GLA domain-containing proteins. FIXa possesses enzymatic activity to convert X to Xa, whereas in the presence of its cofactor (FVIIIa), the catalytic activity raises 100,000-fold.

As for the FV—there are two different probabilities of binding to the procoagulant surface, as demonstrated in **Figure 3B**. In the first one, prothrombinase forms by the surface Xa reacting with factor Va in solution. Whereas, in the second case, both FVa and FXa form binary complexes with PSer first, and then lipid-protein rearrangement leads to prothrombinase formation [50]. The addition of exogenous FVa to procoagulant platelets increases the velocity of prothrombinase reaction [4], whereas in the absence of exogenous FVa, procoagulant platelets are still prothrombinase active [54]. These findings indicate that both pathways are physiologically important.

3.2 Limiting thrombus growth

During hemostasis, there is a time point when a thrombus needs to stop growing in order not to occlude the lumen of the vessel, which may compromise the blood supply to an end organ. Stoppage of the thrombus growth is probably the most intriguing part of thrombogenesis, although being the most understudied at the same time. When coagulation is initiated by subendothelial tissue factor, it leads to the formation of small amounts of thrombin. Thrombin then, via a positive feedback mechanism, by activating FXI and FVIII triggers the contact activation pathway and amplifies its production. This self-accelerating process is essential within the hemostatic plaque as it is the only way to overcome the anticoagulant nature of plasma, which is due to the presence of antithrombin III (ATIII). Besides the presence of ATIII in the active form, other mechanisms limit coagulation beyond its border zone. Another one of high importance is the presence of thrombomodulin, an integral membrane protein that is expressed ubiquitously on the surface of endothelial cells. Thrombomodulin converts procoagulant thrombin to an anticoagulant enzyme. Not only these mechanisms limit coagulation to the injured site but also degrade activated factors that happened to escape the hemostatic plaque.

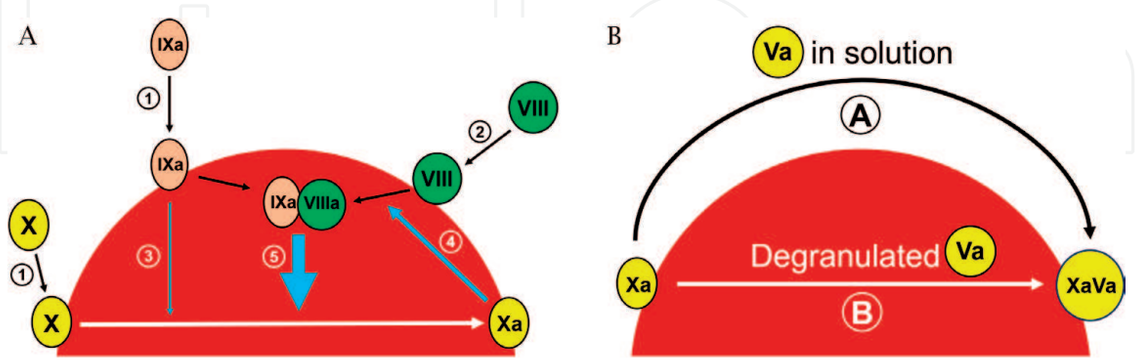

Figure 3.
Factor X activation (A) and prothrombinase assembly (B) on the "cap" of procoagulant platelet. (A). Initially FIXa and FX are bound to PSer on the surface of procoagulant platelet in calcium-dependent manner (①), whereas co-factor VIII binding is not specific to PSer and does not require calcium (②). FIXa possesses enzymatic activity to convert X to Xa (③). Although reaction ③ is very slow in the absence of factor VIIIa, it is efficient to generate small amounts of FXa. Initial FXa then activates VIII to VIIIa (④), this leads to intrinsic tenase complex assembly, which in turn amplifies Xa formation (⑤). (B). There are two possibilities for the assembly of prothrombinase on the surface of procoagulant platelet. Pathway Ⓐ forms prothrombinase by the surface Xa reacting with factor Va in solution. Pathway Ⓑ depicts the possibility for prothrombinase complex formation by already bound Xa and Va. In this case, both FVa and FXa form binary complexes with PSer first and then lipid-protein rearrangement leads to prothrombinase formation.

These processes outline the general principles of limiting coagulation to its border zone. However, not only coagulation but also processes of platelet adhesion, aggregation, and activation have to stop in order at a certain timepoint. How and when thrombus stops growing concerning cellular component had been a mystery for a long time.

In 2007, Maroney et al. demonstrated that procoagulant platelets express active tissue factor pathway inhibitor (TFPI) on their surface [55]. But why platelets expressing procoagulant PSer would also need a strong anticoagulant (TFPI) on

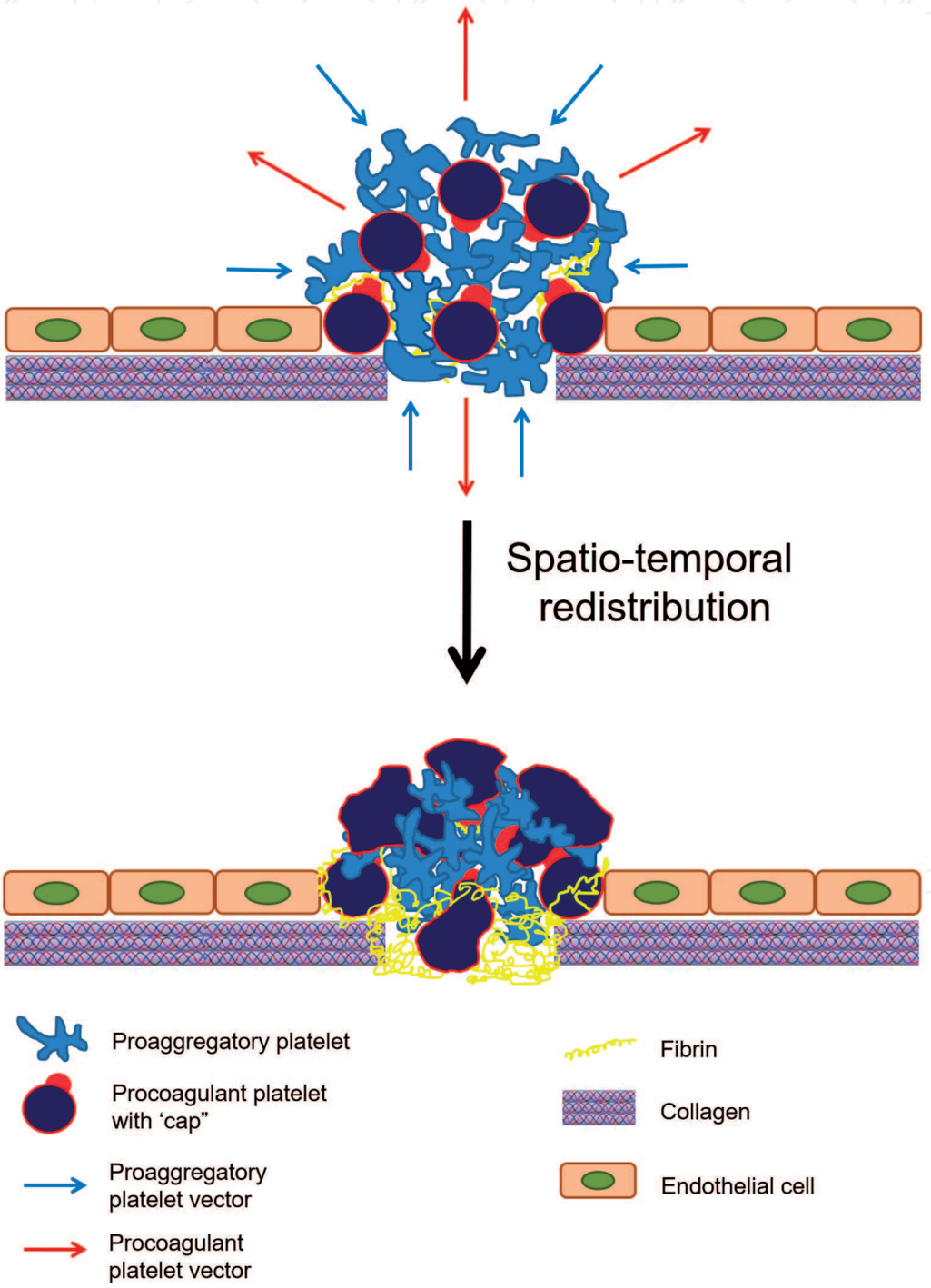

Figure 4.
Schematic representation of platelet translocation within the evolving hemostatic plug. After vascular injury, platelets adhere and aggregate at the wound site. A subpopulation of platelets within the hemostatic plug transitions to a procoagulant state. Procoagulant and pro-aggregatory platelets have opposing vectors of translocation. Clot retraction, driven by platelets with a pro-aggregatory phenotype, squeezes procoagulant platelets to the periphery. Adapted from [56] with minor modification.

their surface remained a question for about a decade until in 2019 a breakthrough work by Nechipurenko et al. characterized a phenomenon, together with its mechanisms, of procoagulant platelets translocating to the thrombus periphery [57]. They demonstrated that during clot retraction procoagulant platelets are squeezed out to the periphery of the thrombus, as shown in **Figure 4**. Another important study in this context is the 2008 work by Jobe et al., which demonstrated that procoagulant platelets do not possess contractile function [18]. This explains why pro-aggregatory and procoagulant platelets have different translocation vectors within the hemostatic plaque (**Figure 4**). Being bound to the thrombus by its "cap," procoagulant platelets do not get detached but rather are squeezed out during pro-aggregatory platelets contraction. At the luminal surface of the hemostatic plaque, procoagulant platelets limit its further growth not only by expressing low adhesive and aggregatory surfaces [58–60], but also with TFPI terminating any extrinsic tenase and prothrombinase activity.

The fact of procoagulant platelet being non-adhesive, however, gives rise to a legitimate question. If procoagulant platelets are not capable of adhesion and aggregation, how do they get attracted to a thrombus? It turns out to be, as described in recent studies, that procoagulant platelets do not form *de novo* from a resting state, but rather temporally transitioning from pro-aggregatory phenotype by the onset of the MPT and regulated necrosis within a hemostatic plaque [61, 62].

Many are aware of an ancient Roman god of duality — Janus, who had two faces as he was able to look to the future and the past. Procoagulant platelets can also demonstrate a functional duality by supporting coagulation on one side and limiting thrombus growth on the other, making them be "Janus" of hemostasis.

Conflict of interest

The author has no conflict of interest to disclose.

Abbreviations

APC	activated protein C
ATIII	antithrombin III
Cyt C	cytochrome c
DTS	dense tubular system
FcRγ	FC receptor gamma
FV	factor V
FVa	activated factor V
FVIII	factor VIII
FVIIIa	activated factor VIII
FIX	factor IX
FIXa	activated factor IX
FX	factor X
FXa	activated factor X
GPIb-IX	glycoprotein Ib and IX complex
GPVI	glycoprotein VI
MCU	mitochondrial calcium uniporter
MPT	mitochondrial permeability transition
MPTP	mitochondrial permeability transition pore
OCS	open canalicular system
PAR	protease-activated receptor

PC	protein C
PLA_2	phospholipase A_2
PS	protein S
PSer	phosphatidylserine
RBC	red blood cell
SOCE	store-operated calcium entry
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TRPC3	transient receptor potential channel 3
WBC	white blood cell

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