Platelet response heterogeneity in thrombus formation

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Summary

Vascular injury leads to formation of a structured thrombus as a consequence of platelet activation and aggregation, thrombin and fibrin formation, and trapping of leukocytes and red cells. This review summarises current evidence for heterogeneity of platelet responses and functions in the thrombus-forming process. Environmental factors contribute to response heterogeneity, as the platelets in a thrombus adhere to different substrates, and sense specific (ant)agonists and rheological conditions. Contraction of platelets and interaction with fibrin and other blood cells cause further response variation. On the other hand, response heterogeneity can also be due to intrinsic differences between platelets in age and in receptor and signalling proteins. As a result, at least three subpopulations of platelets are formed in a thrombus: aggregating platelets with (reversible) integrin activation, procoagulant (coated) platelets exposing phosphatidylserine and binding coagulation factors, and contracting platelets with cell-cell contacts. This recognition of thrombus heterogeneity has implications for the use and development of antiplatelet medication.

Keywords

Aggregation, coagulation, heterogeneity, platelets, thrombus formation

Introduction

Platelets have a multitude of physiological functions. They control haemostasis and mediate thrombosis by acting in concert with coagulation factors and other cells. Platelets, in addition, contribute to the regulation of vascular tone, interact with leukocytes in inflammatory reactions, promote vascular repair, and stimulate cellular proliferation by the supply of growth factors. Given these many distinct functions it is unlikely that every platelet will contribute to all these processes. A relevant question therefore is whether platelets in the circulation represent different populations, each predisposed to a certain response, or whether it is the local activating environment that determines their ultimate fate.

In recent years, a large body of evidence has been collected for the existence of platelet response heterogeneity. In the present paper we aim to summarise the current state of knowledge, with emphasis on the response variation in the various phases of thrombus formation. We discuss how differences in the platelet environment and intrinsic differences between platelets, together with obvious genetic factors, can contribute to this heterogeneity. A summary of these determinants is given in Table 1.

Temporal and spatial heterogeneity in flow-induced thrombus formation

Studies by intravital imaging in vivo and flow-chamber experiments ex vivo indicate that individual platelets within an (arterial) thrombus can markedly differ in morphology and in surface characteristics. The outer layer of a growing thrombus often consists of loosely bound platelets with discoid shape, suggesting a low degree of activation. Platelets in inner layers have developed pseudopods, lost their granule contents, and can form close contacts, all of which are markers of increased activation (1–4). Staining for specific surface markers is often used to determine the platelet activation stage in a thrombus: fibrin(ogen) and antibodies such as PAC1 to detect an activated integrin conformation and increased adhesion capacity; anti-P-selectin antibody to mark secretion responses; and annexin A5 or lactadherin as indi-
Table 1: Determinants of heterogeneity in platelet responses in thrombus formation.

<table>
<thead>
<tr>
<th>Environmental factors (temporal and spatial differences)</th>
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<tbody>
<tr>
<td>Local rheology around platelet in a thrombus:</td>
</tr>
<tr>
<td>– arterial vs. venous shear rate</td>
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<tr>
<td>– disturbance in shear rate</td>
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<tr>
<td>Different adhesive surface:</td>
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<tr>
<td>– inhibiting endothelial surface</td>
</tr>
<tr>
<td>– activating extracellular matrix components: collagen, VWF, fibrinectin, tissue factor</td>
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<tr>
<td>– activating thrombus components: platelet-bound VWF, fibrinogen</td>
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<tr>
<td>Local availability of soluble (ant)agonists:</td>
</tr>
<tr>
<td>– inhibiting endothelial factors: nitric oxide, prostacyclin</td>
</tr>
<tr>
<td>– activating autacoids: ADP, TxA2, ATP, serotonin</td>
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<tr>
<td>Platelet-platelet contacts in thrombus:</td>
</tr>
<tr>
<td>– formation of filopods, spreading, contracting</td>
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<tr>
<td>– integrin outside-in signalling</td>
</tr>
<tr>
<td>– contact signalling: JAM, ESAM, SLAM, PECAM-1, Eph kinase</td>
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<tr>
<td>– synaptic signalling: CD40L, Gas6 and receptors</td>
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<tr>
<td>Hetero-type of platelet contacts in thrombus:</td>
</tr>
<tr>
<td>– interactions with leukocyte subsets, erythrocytes</td>
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<tr>
<td>Local exposure to modifying plasma factors:</td>
</tr>
<tr>
<td>– proteases: ADAM, ADAMTS</td>
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<tr>
<td>– cross-linking enzymes: PDI, transglutaminases</td>
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<tr>
<td>Local activity of coagulation:</td>
</tr>
<tr>
<td>– coagulation factor activation</td>
</tr>
<tr>
<td>– thrombin and fibrin formation</td>
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<tr>
<td>Platelet activation history:</td>
</tr>
<tr>
<td>– prior (in)activation, (de)sensitisation, adhesion or embolisation</td>
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<table>
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<tr>
<th>Intrinsic factors (intra-subject platelet differences)</th>
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<tbody>
<tr>
<td>Platelet shape and size:</td>
</tr>
<tr>
<td>– discoid, spindle-shaped</td>
</tr>
<tr>
<td>Sub-cellular composition and structure:</td>
</tr>
<tr>
<td>– actin and tubulin cytoskeleton organisation</td>
</tr>
<tr>
<td>– number/type of storage granules and lysosomes</td>
</tr>
<tr>
<td>Protein expression levels:</td>
</tr>
<tr>
<td>– adhesive and signalling receptors (αIIbβ3, αIβ2, GP Ib-V-IX, P2Y11/12, PAR1/4, GPVI)</td>
</tr>
<tr>
<td>– intracellular signalling, cytoskeletal and granule proteins</td>
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<tr>
<td>Platelet age:</td>
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<tr>
<td>– apoptotic programme</td>
</tr>
<tr>
<td>– loss of function, e.g. receptor clustering and shedding</td>
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<tr>
<td>– altered metabolic activity</td>
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<tr>
<td>– sensitivity to clearance</td>
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By its surface- and flow-dependent nature, the process of thrombus formation inevitably exposes platelets to continuous changes in their local environment. A number of models have been developed to describe the temporal and spatial characteristics of thrombus formation (3–5). Common to these is a separation in distinct phases: initiation (platelet interaction with extracellular matrix components like collagen), extension (platelet-platelet interactions triggered by autocrine agonists like ADP and thromboxane A2 (TxA2), and perpetuation (stabilisation of the platelet aggregate by contraction, late signalling events and formation of a fibrin network). Below we discuss key environmental factors that contribute to inter-platelet response heterogeneity during the phases of thrombus formation. Figure 2 gives an overview of the different types of platelet responses.

Phase 1: platelet heterogeneity in the initiation of thrombus formation

Local rheology plays an important role in the initial steps of thrombus formation. The role of shear stress is resolved in pioneering studies, where anticoagulated human or mouse blood was flowed over collagen type I or III, to which plasma-derived von Willebrand factor (VWF) avidly binds (6, 7). At intermediate or high arterial shear rates (>800 s^-1), platelets initially adhere to VWF via the glycoprotein (GP)Ib-V-IX complex by a fast association. The adhesion is stabilised by additional interaction via integrin αIIbβ3. The presence of collagen fibres is considered to be required for platelet activation, as the high-shear interaction to VWF alone causes only weak responses, limited to a series of short transient Ca^{2+} spikes and little platelet aggregation (8). The relative importance of the VWF-GP Ib axis for adhesion, however, increases with the local shear rate, so that a pure VWF surface mediates aggregate formation at very high shear rates (7). Recent observations show that platelets can keep their discoid morphology during high-shear capture on VWF, but that this interaction is facilitated by the rapid formation of membrane tethers that can range in length from 1–30 µm (9, 10). Given the dramatic influence on platelet morphology, one would expect that early tether formation affects the later response of a platelet, but this has not been investigated.

Under high-shear flow conditions, both GP Ib-V-IX and αIβ2 are also implicated in mediating platelet-platelet interactions via VWF and fibrinogen (11, 12). Stable platelet adhesion to VWF/collagen is achieved by the collagen receptors, GPVI and integrin αIβ2 (6, 13, 14). Studies using inhibitors and receptor-deficient mice point to an interplay of GPIb-V-IX with either receptor in order to achieve stable platelet adhesion and activation under high-shear conditions (14–16). However, on the level of individual platelets, the roles of these collagen receptors can differ from cell to cell. It appears that one fraction of platelets stably adheres via GPVI and immediately responds by an increase in intracellular Ca^{2+} (17). This response relies on GPVII/Src kinase activation and is not influenced by αIβ2 inhibition. Another fraction of platelets reacts differently by showing a delayed increase in Ca^{2+} (up to 30 s) after initial adhesion. In the latter cells, αIβ2 mediates the early adhesion, while signalling via the GPVII/Src kinase pathway occurs later. The response heterogeneity is explained by assuming that individual platelets bind to different motifs within collagen fibers (GPVI- or αIβ2-binding motifs). On the other hand, it is also possible that the platelets of either fraction differ in intrinsic activation properties. The recognition of this response heterogeneity has led to a unifying model of platelet adhesion to collagen (17), explaining why in some studies αIβ2 is redundant (18), whereas in other studies αIβ2 contributes to platelet adhesion in a way as described by the two-site two-step model (19).

Single-cell measurements under flow conditions demonstrate that collagen-adherent platelets considerably vary in intracellular Ca^{2+}
The Ca\(^{2+}\) rises and formation of procoagulant platelets were inhibited by cAMP elevating agents (20). An obvious cause for the Ca\(^{2+}\) signal heterogeneity in flow-dependent adhesion is the gradual change in adhesive substrate, in that collagen fibres are rapidly covered with platelets, after which newly incoming platelets will bind to the first layer of cells. The platelets initially adhering to and spreading out on collagen have been categorised as vanguard platelets, while those binding to fibrinogen via activated α\(\text{IIb}\)β\(\text{3}\) were called follower platelets (21). Autocrine adenosine diphosphate (ADP) is implicated in the priming of the adhesion of the follower cells.

In comparison to flow chambers, the situation in vivo is more complex, since platelets from the circulation will not only interact with vascular VWF/collagen, but also with other extracellular matrix components, including fibronectin, laminin, thrombospondin and fibrin(ogen) (7). This surface complexity further adds to the response heterogeneity of adherent platelets.

**Phase 2: extension of thrombus formation with aggregating and procoagulant platelets**

The extending phase of thrombus build-up relies for a considerable part on release of autocooids by primary adhered and (GPVI-)activated platelets. Next to ADP and TxA\(_2\), several other soluble release products are described with a proaggregatory effect, such as ATP, Gas6, serotonin and cytokines (4, 22, 23). Also, traces of platelet-activating thrombin can be formed at this stage (2). The response of flowing platelets to these autocooids is dependent on multiple factors, such as the rheological situation (disturbances in flow and shear rate), the density and activation state of primary adhered platelets, the levels of autocooid formation, and the nature and area of vascular damage. Although little is known on the precise sites of ADP and TxA\(_2\) release in a thrombus, it is likely that most autocooids are present in gradients, also because they have a short half-life time in the circulation. In vivo, the platelet-inhibiting effects of endothelial-derived nitric oxide and prostacyclin will further compete with the activating effects of autocrine agents.

Platelet-collagen interaction under flow appears to result in two markedly different types of responses, both of which rely on GPVI activation. Some platelets develop filopods and assemble into aggregates, but other platelets remain solitary and change in morphology by forming blebs and shedding microparticles (24). It is unclear how microparticles contribute to the thrombus forming process. The platelets in aggregates have α\(\text{IIb}\)β\(\text{3}\) in an active conformation and bind fibrinogen. Interestingly, under certain conditions, e.g. if P2Y\(_12\) signalling is restricted, this integrin is secondarily inactivated, which results in disaggregation (25). Under flow, disaggregation is mostly observed for loosely bound platelets present at the surface of a thrombus.

**Figure 1: Inter-platelet response heterogeneity.** A) Variation in α\(\text{IIb}\)β\(\text{3}\) expression in unstimulated platelets, as detected by flow cytometry (anti-β\(\text{3}\) integrin antibody). Histograms of variable expression of P-selectin (anti-P-selectin antibody) and active α\(\text{IIb}\)β\(\text{3}\) (binding PAC1 mAb) in convulxin-stimulated platelets. B) Heterogeneity in spiking Ca\(^{2+}\) responses of two adjacent platelets adhered to fibrinogen and stimulated with ADP. C) Separate subpopulations of platelets with exposed PS (population 1, binding annexin A5) and active α\(\text{IIb}\)β\(\text{3}\) (population 2, binding PAC1 mAb) after stimulation with convulxin.
The second type of (blebbing) platelets expose procoagulant PS, are capable of binding to coagulation factors (prothrombin, factors Va and Xa), and generate thrombin at their outer surface (24, 26). Although PS-exposing platelets are high in Ca\(^{2+}\), their \(\alpha_{\text{IIb}}\beta_{3}\) is secondarily inactivated by a mechanism that is still not understood. A marked finding is that in a developing thrombus, the aggregating and procoagulant platelets cluster in separate micro-domains, likely because of differences in integrin adhesive properties (26). Also the onset of coagulation results in PS exposure, but the separation of PS-exposing platelets from aggregated platelets is maintained under coagulant conditions.

The signalling pathways responsible for procoagulant platelet formation have been extensively studied. Collagen either alone or in combination with thrombin leads to PS exposure via a high and prolonged rise in Ca\(^{2+}\) through GPVI-induced stimulation of phospholipase C (PLC)\(_{\gamma2}\) (15, 20). In agreement with this, Jackson and colleagues define the same subpopulation of PS-exposing platelets as having a sustained calcium-induced platelet (SCIP) morphology, characterised by membrane ruffling and fragmentation (microparticle formation) (27). The SCIP morphology is reported to require prior activation of calpain (a cysteine protease) and fragmentation (microparticle formation) (27). The SCIP morphology is required for the development of procoagulant platelet formation (27). The SCIP morphology is reported to be required for the development of procoagulant platelet formation (27).

**Phase 3: heterogeneity during the perpetuation of thrombus formation**

There is increasing evidence that platelets continue to signal even after their incorporation into a thrombus. Likely, these ‘late events’ help to stabilise the platelet aggregate within the high-shear environment of an artery (4, 30). Many platelets that are trapped into a growing thrombus gradually form filopods and lamellipods, increase their number of contact sites with other platelets, and change accordingly in activation state. The surface of these activated platelets expresses several transmembrane receptor proteins that enhance the interactions with neighbouring platelets. These receptor proteins establish homophilic or heterophilic contacts between the adjacent cells, which are often accompanied by trans-cellular signalling events (3). Some of the receptor proteins are positive regulators of thrombus formation, like junctional adhesion molecule (JAM) and signalling lymphocytic activation molecule (SLAM), whereas others are negative regulators, e.g. platelet endothelial cell adhesion molecule (PECAM-1) and endothelial cell-selective adhesion molecule (ESAM). Further inter-cellular contact signalling occurs via interactions of ephrins and Eph kinases; CD40 and (soluble) CD40 ligand (CD154); semaphorin 4D (CD100) with its ligand plexin B1; and Gas6 with its receptors (3, 31). Most probably, these signalling events predominate in the core of a thrombus, where the platelets are most tightly packed.

Not much is known of the functions of other blood cells (leukocyte subsets and erythrocytes) in thrombus formation. As reviewed elsewhere, leukocytes can interact with activated platelets via a number of adhesive receptors (32). A recent paper reports that procoagulant platelets (SCIP) produce proinflammatory platelet-activating factor, which promotes the interaction with neutrophils in a shear-dependent manner (33). Red cells are trapped into fibrin networks, especially under low-shear conditions.

The outer shells of platelets in a thrombus are in most extensive contact with the flowing blood and, hence, are easily accessible to modifying plasma enzymes that change the platelet surface. Several plasma proteases are described, which cause shedding of platelet receptors, e.g. disintegrin and metalloproteinases (ADAMS) mediate the cleavage of glycoprotein (GP)Ib\(
\text{a} \), GPV, GPVI, CD40L and semaphorin 4D (34). Plasma-derived ADAMTS-13 uses WVF as a substrate, although it preferentially cleaves multimeric VWF that is bound to platelets, thereby reducing the thrombogenic effect of WVF multimers (35). Also the reported capability of protein disulfide isomerase and transglutaminase (factor XIIIa) in cross-linking of platelet receptors (27, 36, 37) may differ within a thrombus.

Another cause of inter-platelet response heterogeneity is localised coagulation activity, i.e. restricted sites of formation of thrombin and fibrin. High-speed imaging of thrombus formation in damaged mouse arterioles shows that the first traces of fibrin appear already soon after vascular damage, which implies a rapid onset of thrombin generation close to the damaged vessel wall (2). At a later stage, once a full fibrin network is formed, this consolidates the thrombus not only passively but also in an active way, as fibrin provides a substrate for platelets to contract (3, 4). The generation of thrombin is greatly promoted by the formation of PS-exposing platelets (26, 38), which bind activated coagulation factors, promoting the cleavage and activation of factor X and prothrombin (39, 40). Thrombin, in turn, is a potent platelet agonist, can enhance PS exposure, and so promote its own formation.

Several research groups describe that the relative contribution of thrombin (and fibrin) to thrombus formation in vivo varies with the type of arterial thrombosis model. In laser-induced vessel wall damage, tissue factor exposure and thrombin generation appears to be relatively important (41, 42). On the other hand, in ligation- and FeCl\(_3\)-induced arterial damage, platelet activation by collagen is a more important thrombogenic trigger (13, 43). The possibility of localised, non-homogenous coagulation within a developing (arterial or venous) thrombus has not been considered in any detail. However, one can assume that local differences in rheology will provoke such heterogeneity, since these lead to altered delivery of coagulation factors, washout of thrombin, and thrombin inactivation by antithrombin or thrombomodulin. An additional environmental factor that can contribute to response variation is the platelet (in)activation history. For instance, embolised platelets that previously have been trapped into a thrombus may be more prone to later activation than circulating ‘virgin’ platelets.

**Heterogeneous formation of procoagulant platelets in suspension**

Evidence for intrinsic, non-environmental factors contributing to platelet response heterogeneity comes from studies performed with platelets in suspension, where all cells are subjected to the same activation condition. For instance, flow cytometry shows that washed platelets in suspension respond to the GPVI ligand
convulxin by a variable, Gauss-shaped extent of P-selectin exposure or $\alpha_{\text{IIb}\beta_3}$ activation (Fig. 1A). Also platelets adhered to the same substratum show response heterogeneity (44), e.g. by varying in peak profile of Ca$^{2+}$ rises, for instance after triggering with ADP (Fig. 1B).

On top of these quantitative differences, platelet responses can differ in a qualitative way. This is particularly true for responses that rely on a threshold level of activation, such as secretion and surface expression of PS. Flow cytometric experiments, where PS exposure is detected with fluorescently-labelled annexin A5 or lactadherin (45), unequivocally demonstrate that this response relies on threshold, potent increases in Ca$^{2+}$ (46). These are reached in almost all platelets stimulated with Ca$^{2+}$ ionophore (A23187 or ionomycin), but only in a subpopulation of the cells stimulated with (combinations of) Ca$^{2+}$-mobilising agonists. For example, platelet stimulation with GPVI ligand (convulxin, collagen-related peptide, collagen) alone or in the presence of thrombin, or with thrombin/thapsigargin or thrombin/ADP results in response heterogeneity with subfractions of PS-exposing platelets (47–50). In general, the population size of PS-exposing cells appears to increase with the dose of agonist(s), which agrees with the concept of threshold activation levels for this response. Furthermore, authors comparing the platelets from subjects with different GPVI expression levels, have found that variation in GPVI expression correlates with the extent of GPVI-induced PS exposure (51).

The coagulation-stimulating activity of the population of PS-exposing platelets is demonstrated in dual-labelling flow cytometric studies. Thrombin-stimulated platelets with a high intracellular Ca$^{2+}$ appear to bind factor IXa, and facilitate factor X activation (52, 53). Dual-labelling studies using annexin A5 and coagulation factors have shown major overlap of the subpopulations of platelets that expose PS and bind the coagulation factors Va, IXa and Xa (26, 54).

Remarkably, the PS-exposing platelets, regardless of the agonist(s) used, do no longer express $\alpha_{\text{IIb}\beta_3}$ integrin in activated conformation, as demonstrated by the absence of PAC1 binding (Fig. 1C). Kinetic studies have indicated that GPVI stimulation initially leads to $\alpha_{\text{IIb}\beta_3}$ activation of all platelets, but that only the PS-exposing cells show secondary inactivation at unchanged $\alpha_{\text{IIb}\beta_3}$ expression level (25, 26). Similarly, in platelets stimulated with Ca$^{2+}$ ionophore, PS exposure is accompanied by a loss of fibrinogen binding to $\alpha_{\text{IIb}\beta_3}$, implicating inactivation of the integrin (55). Summarising, it is clear that the subpopulation of PS-exposing platelets characteristically binds to and activates coagulation factors and, thereby, primes for coagulation. This does not rule out the possibility that membrane components other than surface-exposed PS contribute to coagulation stimulation. Given the many new proteins detected in the platelet proteome, some of these may become expressed on the surface of PS-exposing cells.

**Fibrin(ogen)-coated platelets**

In 2002, flow cytometric studies led to the identification of a population of platelets with a role in coagulation (56). Dale et al. originally termed these as collagen- and thrombin-activated (COAT) platelets, according to the agonist combination required for this platelet type, but later redefined these as ‘coated’ platelets (57). They report that a subpopulation of gel-filtered platelets, when activated with thrombin and another strong agonist (collagen, convulxin or FcRIIA agonist) are capable of binding various granule proteins, including fibrinogen, VWF, factor Va, thrombospondin and $\alpha_2$-antiplasmin, via a mechanism involving transglutaminase activity (56, 58). Although this has been directly demonstrated for fibrinogen only, it is proposed that the transglutaminase acted by the cross-linking of platelet-produced serotonin to these granule proteins. In a molecular model of the coating, the concept is that the serotonin-conjugated proteins interact with serotonin binding sites on fibrinogen and thrombospondin to thereby form a two-dimensional coating matrix around individual platelets (58). The same group has shown that coated platelets expose PS (59). It is concluded that PS by interaction of factor Va contributes to attachment of the coating to the platelet membrane, in addition to fibrinogen binding to $\alpha_{\text{IIb}\beta_3}$, and thrombospondin binding to CD36 (58). Interestingly also, the subpopulation of coated platelets shows decreased binding of PAC1, while the surface expression of $\alpha_{\text{IIb}\beta_3}$ is unchanged (56). Nevertheless, it was found that integrin antagonists are unable to displace fibrinogen bound to coated platelets, suggesting that the binding occurs via other receptors than $\alpha_{\text{IIb}\beta_3}$ (57). The involvement of transglutaminases in coated platelet formation was challenged in later studies. For instance, platelets from mice lacking factor XIII, which is the main transglutaminase in platelets, have normal surface binding of fibrinogen (60). Other authors report that pharmacological inhibition of transglutaminase leads to incomplete reduction of the platelet population with ‘coated’ characteristics (26, 27).

Flow cytometric studies by Jobe et al. have led to further characterisation of this subpopulation of activated platelets. PS-exposing platelets from wild-type mice, formed by co-stimulation with thrombin and GPVI agonist convulxin, show high binding of fibrinogen (ogen) and VWF, while their $\alpha_{\text{IIb}\beta_3}$ integrins are in the inactive conformation (60). In mouse platelets deficient in Fc receptor γ-chain and GPVI, such high fibrinogen (ogen) binding is not detectable, thus pointing to a requirement for signalling via the GPVI/Src kinase pathway. Interestingly, when wild-type platelets are stimulated with convulxin alone without thrombin, the result is PS exposure in the absence of high fibrinogen (ogen) binding (60, 61). Together, this suggests that thrombin and, hence fibrin formation, is required for the formation of coated platelets. Whereas exposure of PS and the binding of coagulation factors clearly occurs in the absence of thrombin, integrin-independent fibrinogen (ogen) binding seems to rely on the generation or presence of thrombin. The likely role of thrombin is the assembly of a coat of fibrin around platelets. The fibrin coat may incorporate other plasma proteins like VWF and thrombospondin as well. However, more work is needed to confirm this. In addition, it is still unclear how fibrinogen (ogen) can bind to PS-exposing platelets in the absence of active $\alpha_{\text{IIb}\beta_3}$.

Recently, novel activation pathways have been identified leading to procoagulant platelet formation. Using platelets from mice deficient in cyclophilin D, which is a component of the mitochondrial permeability transition pore, it was concluded that formation of this pore is required for platelet activation responses including PS exposure, procoagulant activity, high fi-
Platelet ageing and other intrinsic factors contributing to heterogeneous activation

Only a limited number of studies have addressed which intrinsic platelet factors contribute to inter-platelet response variation. Platelets vary in size and morphology, with most platelets having a discoid shape and small populations with a rounded and spindle shape that are altered in organisation of the actin and tubulin cytoskeleton, respectively (65). Whether these morphological differences are accompanied by changed activation is unclear. Electron micrographs point to different numbers of \( \alpha \)-granules, dense granules and lysosomes between platelets (66), which might be released in an agonist-specific way (67). How this relates to response heterogeneity is also unclear.

On the other hand, it is likely that inter-platelet variation in expression of receptor and adhesive surface proteins, as apparent from flow cytometric analyses, is linked to response variation. For instance, platelets with a high expression of GPVI receptors or \( Ca^{2+} \) signalling proteins may be more prone to PS exposure than platelets with lower expression levels. Similarly, expression differences in signal (de)sensitisation proteins may be related to altered functional responses (68). Since maturing megakaryocytes show heterogeneity in \( Ca^{2+} \) signalling properties, the origin of a platelet is another relevant factor in response variation, (69).

It is presumed that the platelet age is a cause of response heterogeneity, but there is limited evidence for this idea. Recent findings show that the life span of platelets is limited by the start of an apoptosis-like programme of anuclear cell death, which is controlled by a gradual altering of the Bcl-xL/Bak ratio (70). Ageing platelets also appear to change in surface characteristics by clustering and shedding of GPIb-V-IX (71). These and other time-dependent changes may be accompanied by alterations in activation tendency. There is indeed evidence that prolonged storage of platelets leads to a gradual decrease in responsiveness, in part by reduced metabolic activity (72, 73). However, even after prolonged storage the response variation to PS-exposing and non-PS-exposing platelets is maintained (74). Thus the findings so far indicate that platelet age is not the only factor in functional heterogeneity.

Concluding remarks

Taken together, the literature provides sufficient evidence that both environmental and intrinsic factors contribute to response heterogeneity of platelets, even when incorporated in a thrombus. However, given the variation in environmental conditions of individual platelets in a thrombus, the precise location is the
most likely determinant of the ‘fate’ of a platelet: aggregating,
perhaps transiently attaching at the thrombus surface; pro-
coagulant (coated), in patches around the thrombus where con-
tact with coagulation factors is possible; and contracting,
with tight cell-cell contacts in the thrombus core (Fig. 2). The con-
sequence of this heterogeneity is formation of a stable contracted
thrombus with a regulated extent of clotting and a size restricted
to the area of vascular damage. This recognition of heteroge-
neity has implications for the development of antiplatelet medi-
cation, which may be directed to only one of the subpopulations
of activated platelets in a thrombus, with the aim to limit throm-
buss growth without affecting haemostasis. For instance, selec-
tively targeting of PS-exposing platelets may reduce excessive
clot formation, without affecting the capability of platelets to
aggregate.

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References
1. Van Gestel M, Heemskerk JWM, Slaaf DW, et al. Real-time detection of activation patterns in individual
platelets during thromboembolism in vivo: differences between thrombus growth and embolus formation. J
of thrombus assembly in a living mouse by confocal and widefield intravital microscopy. J Thromb Haemost
2003; 1: 60–68.
115: 3385–3392.
4. Jackson SP. The growing complexity of platelet ag-
3415–3425.
6. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific
synergy of multiple substrate-receptor interactions in
platelet thrombus formation under flow. Cell 1998; 94:
657–666.
7. Ruggeri ZM. Platelets in atherothrombosis. Nat
2008; 28: s43-s50.


53. London FS, Marcinkiewicz M, Walsh PN. PAR1-stimulated factor IXa binding to a small platelet subpopulation requires a pronounced and sustained increase of cytoplasmic calcium. Biochemistry 2006; 45: 7290–7298.


