Platelet Signaling in Primary Haemostasis and Arterial Thrombus Formation*: Part 1

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Abstract

Platelets react immediately in response to traumatic vascular injury by adhesion, activation, aggregation and subsequent haemostatic plug formation. While this reaction pattern is essential for haemostasis, platelet responses can also cause occlusive thrombi in diseased arteries, leading to myocardial infarction or stroke. Initially, flowing platelets are captured from the circulation to vascular lesions. This step is mediated by glycoprotein (GP) Ib-IX-V interacting with immobilized von Willebrand factor (VWF) on exposed subendothelial components. Tethered platelets can now bind to collagen through GPVI and integrin α2β1. Outside-in signals from the adhesion receptors act synergistically with inside-out signals from soluble stimuli and induce platelet activation. These mediators operate through G protein–coupled receptors and reinforce adhesion and activation. Typical manifestations of activated platelets include calcium mobilization, procoagulant activity, cytoskeletal reorganization, granule secretion and aggregation. This requires activation of integrin αIIbβ3 with shifting into a high-affinity state and is indispensable to bind soluble fibrinogen, VWF and fibronectin. The multiple interactions and the impact of thrombin result in firm adhesion and recruitment of circulating platelets into growing aggregates. A fibrin meshwork supports stabilization of haemostatic thrombi and prevents detachment by the flowing blood. This two-part review provides an overview of platelet activation and signal transduction mechanisms with a focus on αIIbβ3-mediated outside-in signaling in integrin variants. In the first part, a three-stage model of platelet recruitment and activation in vivo is presented. Along with that, platelet responses upon exposure to thrombogenic surfaces followed by platelet-to-platelet interactions and formation of haemostatic thrombi are discussed. Moreover, several determinants involved in pathological thrombosis will be reviewed.

Keywords

- platelet adhesion
- activation
- aggregation
- thrombus formation
- integrin αIIbβ3

* *Dedicated to the memory of Prof. Ernst F. Lüscher (1916–2002), who was my postdoc mentor.*

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Zusammenfassung

Bei traumatischer Gefäßwandverletzung reagieren Plättchen unverzüglich mit Adhäsion, Aktivierung, Aggregation und nachfolgender Bildung eines hämostatischen Pfropfs. Dieses Reaktionsmuster ist essentiell für die Hämostase, kann aber in krankhaft veränderten Gefäßen okkludierende Thromben hervorrufen, die einen Herzinfarkt oder Schlaganfall auslösen. Initial werden zirkulierende Plättchen im Bereich von Gefäßwandläsionen eingefangen. Dieser Schritt wird durch Wechselwirkung von Glykoprotein (GP) Ib-IX-V mit immobilisiertem von-Willebrand-Faktor (VWF) auf freigelegten Subendothelkomponenten vermittelt. Die 'angegurteten' Plättchen binden nun über GPVI und Integrin α2β1 an Kollagen. Die von Adhäsionsrezeptoren ausgelösten 'Outside-in'-Signale wirken in Synergie mit 'Inside-out'-Signalen diffusibler Agonisten und aktivieren die Plättchen. Diese Mediatoren wirken über G-Protein-gekoppelte Rezeptoren und verstärken Adhäsion und Aktivierung. Typische Funktionsäußerungen aktivierter Plättchen sind Kalziummobilisierung, prokoagulatorische Aktivität, Umorganisation des Zytoskeletts, Granulasekretion und Aggregation. Letzteres setzt Aktivierung von Integrin αIIbβ3 mit Übergang in einen hochaffinen Zustand voraus und ist unabdingbar für die Bindung löslichen Fibrinogens, VWF und Fibronektins. Die multiplen Interaktionen führen unter Einfluss von Thrombin zur Verfestigung der Adhäsion und durch Einbeziehung zirkulierender Plättchen zur Größenzunahme des Aggregats. Ein Fibrinnetz fördert die Stabilisierung hämostatischer Thromben und wirkt ihrer Ablösung durch den Blutstrom entgegen. Diese zweiteilige Übersicht erörtert Abläufe bei Plättchenaktivierung und Signaltransduktion und rückt αIIbβ3-vermittelte 'Outside-in'-Signalvorgänge bei Integrinvarianten in den Mittelpunkt. In Teil I wird ein 3-stufiges Model zur Plättchenrekrutierung und -aktivierung vorgestellt. Thrombozytäre Funktionsäußerungen bei Exposition thrombogener Oberflächen und Plättchen-Plättchenwechselbeziehungen mit nachfolgender Thrombusbildung werden im Detail besprochen. Auch wird auf verschiedene Einflussgrößen eingegangen, die unter pathologischen Bedingungen eine Thrombose hervorrufen.

Schlüsselwörter

- Plättchenadhäsion
- Aktivierung
- Aggregation
- Thrombusbildung
- Integrin αIIbβ3

Introduction

Platelets contribute essentially to survey the integrity of the vascular system. Apart from haemostasis, platelets play a pivotal role in arterial thrombogenesis.[1,2] More recent studies have highlighted a different but equally relevant contribution of platelets to other processes in biology and pathology. The platelet contributions beyond haemostasis and thrombosis include immune-mediated responses to microbial and viral pathogens,[3–5] inflammation,[6] embryonic development,[7] angiogenesis,[8] tumour cell growth,[9] and cancer metastasis.[10–14] In particular, multiple functions of platelets in innate and adaptive immunity have been identified.[15]

Upon release by megakaryocytes, their hematopoietic precursor cells residing in the bone marrow, circulating platelets respond immediately to vascular lesions by becoming adherent within seconds and by forming aggregates at sites of injured endothelial cells (ECs) and exposed subendothelial matrix structures. Following activation, platelets provide a highly effective catalytic membrane surface for the generation of α-thrombin that in turn accelerates the recruitment of circulating platelets and the formation of fibrin necessary to stabilize thrombi and to prevent their detachment by the flowing blood.[16] Once stimulated, platelets respond uniformly and do not distinguish between traumatic injury and atherosclerotic or inflammatory damage of the vessel wall.[2,17] While their physiological function is to support arrest of bleeding, to contribute to host defence and wound healing, and to restore vessel wall integrity, platelets can form occlusive thrombi as a consequence of vascular diseases, such as atherosclerosis. Thus, under pathological conditions, platelet responses may result in acute ischaemic syndromes of the heart, brain, and other organ systems.

Platelet Activation

Platelet Activation In Vitro

Platelets can be stimulated in response to a variety of chemically distinct fibrillar or soluble compounds, including collagen, α-thrombin, adenosine diphosphate (ADP), epinephrine, thromboxane A_2 (TXA_2), and serotonin (5-HT). This property provided the basis for platelet aggregometry that was introduced by Born in 1962.[18] Since then, light transmittance aggregometry (LTA) has become a popular technique of platelet function testing in vitro. LTA monitors agonist-induced changes in the optical density of platelet-rich plasma (PRP) or isolated platelets in suspension while

stirring the sample. However, apart from insufficient standardization, LTA represents a rather incomplete scenario of the situation in vivo. Specifically, the model is lacking vessel wall components, circulating blood cells other than platelets, blood flow and resulting shear stress.

Despite these evident limitations of LTA, it has been believed for many years that platelet activation in haemostasis and thrombus formation in vivo may occur in a similar way as in the in vitro system. Several investigators have rejected this contention for good reasons. For example, upon addition of an excitatory agonist to PRP, the platelet aggregation response begins only after a lag phase of approximately 1 minute. However, the formation of a haemostatic plug has to be an extremely fast process[19] to cover an endothelial defect by a monolayer of attached platelets at sites of vascular lesions, to limit blood loss and, eventually, to arrest bleeding.

In a straightforward experimental approach using formaldehyde-fixed platelets covered with patches of tightly packed fibrinogen, Lüscher and Weber provided evidence that resting platelets immediately adhere onto surface-bound fibrinogen.[19] This process mediated by integrin αIIbβ3 (GPIIb-IIIa) in the resting (low-affinity) state subsequently leads to platelet activation with a conformational change of αIIbβ3 into its active state, which in turn can trigger high-affinity interactions between the integrin and soluble fibrinogen or other fluid-phase adhesive proteins. Thus, propagation of the activation process appears possible without a requirement for other external activators.[19] However, such agonists released or secreted by activated platelets, and most importantly the generation of α-thrombin, are nonetheless of vital importance for the consolidation of a haemostatic platelet plug.

Using an analogous experimental setting, Savage and Ruggeri also demonstrated that quiescent platelets interact with immobilized fibrinogen and insoluble fibrin strands.[20] Again, the initial attachment was independent of platelet activation followed by spreading and irreversible adhesion of platelets in the absence of exogenous agonists. Moreover, it was shown that αIIbβ3 on unstimulated platelets displays selective recognition specificity for attachment onto immobilized adhesive proteins, which is distinct from that seen following platelet activation.[20] This selectivity of αIIbβ3 for different adhesive proteins may represent a relevant mechanism for controlling the initiation and propagation of a haemostatic thrombus. It should be noted, however, that these findings were obtained under static conditions.

In perfusion systems, simulating flow conditions, platelets adhere to immobilized fibrinogen or fibrin in a shear rate–limited fashion, thereby displaying a decreasing efficiency at increasing shear rates (up to 1,800–2,000 per second).[21–23] In normal circulation, shear rates between 500 and 5,000 per second (with a median value of 1,700 per second) are present,[24] which can raise about 10-fold in severely stenosed arteries.[25,26] Therefore, as discussed later, other interactions than fibrinogen- or fibrin-mediated processes are essential in vivo to initiate platelet adhesion and activation upon vascular injury.

A Three-Stage Model of Platelet Recruitment and Activation In Vivo

Over the past two decades, abundant data have become available on how circulating platelets are being activated under flow-dynamic conditions in response to vascular lesions. For the complex mechanisms and versatile interactions involved, a three-stage model of platelet activation has been proposed. The principle of this model is summarized schematically in ►Fig. 1. Of note, the three stages, involving platelet adhesion, activation and aggregation, develop in successive but partially overlapping or closely integrated and redundant processes.

Under physiological conditions, circulating platelets are left as 'innocent bystanders'. They are protected from highly reactive subendothelial extracellular matrix (ECM) components by an intact EC lining. Moreover, several EC-derived inhibitors keep platelets quiescent. Such 'protectors' include prostaglandin I_2 (PGI_2, prostacyclin), nitric oxide (NO) and CD39, an *ecto*-ADPase on the surface of ECs that hydrolyses trace amounts of ADP, which can otherwise cause platelet activation (►Fig. 1A). Upon vascular injury, PGI_2, NO and thrombomodulin (TM), which bind thrombin and thereby change the substrate specificity, essentially contribute to the limiting of platelet thrombus formation within the boundaries of a lesion in the vessel wall.

Key receptors of cell adhesion (►Fig. 1A) expressed by circulating quiescent platelets remain unresponsive to fluid-phase adhesive plasma proteins or do not interact with collagen or other ECM components unless exposed. This regulation prevents intravascular platelet activation and subsequent aggregation, but the 'on stand-by' mode of receptors to function irrespective of cellular activation ensures immediate interactions, whenever 'active' ligands are present. For example, plasma von VWF and fibrinogen undergo conformational changes when being adsorbed ('immobilized') onto biological or artificial surfaces. These active conformations are recognized by cognate receptors on the platelet membrane and permit immediate platelet attachment at sites of endothelial lesions.

Initiation (tethering and adhesion). Upon vascular injury with exposure of the subendothelium, circulating platelets are rapidly captured ('tethered') from the bulk blood flow through binding of GPIb-IX-V to subendothelial VWF or formed VWF–collagen complexes[2] (►Fig. 1B). In this initial stage, VWF originates from plasma and/or Weibel-Palade bodies, the EC organelle storing multimerized VWF,[27,28] while, in later phases, platelet secretion from α-granules provides an endogenous source of VWF. Both EC- and platelet-secreted VWF are characterized by ultra-large multimers, which display an intrinsically high affinity with GPIbα.[17]

Despite this feature of VWF, it is relevant for subsequent interactions that the bonds between GPIbα and VWF (A1 domain) have a limited half-life and cannot support stable adhesion[17] but are rather 'catch' and 'flex' bonds.[29,30] In other words, the fast off-rate of the interaction between immobilized VWF and GPIbα cannot mediate irreversible platelet adhesion by itself.[31] This results in detachment of single platelets or continuous translocation of others in the direction of flow, while other individual platelets undergo a

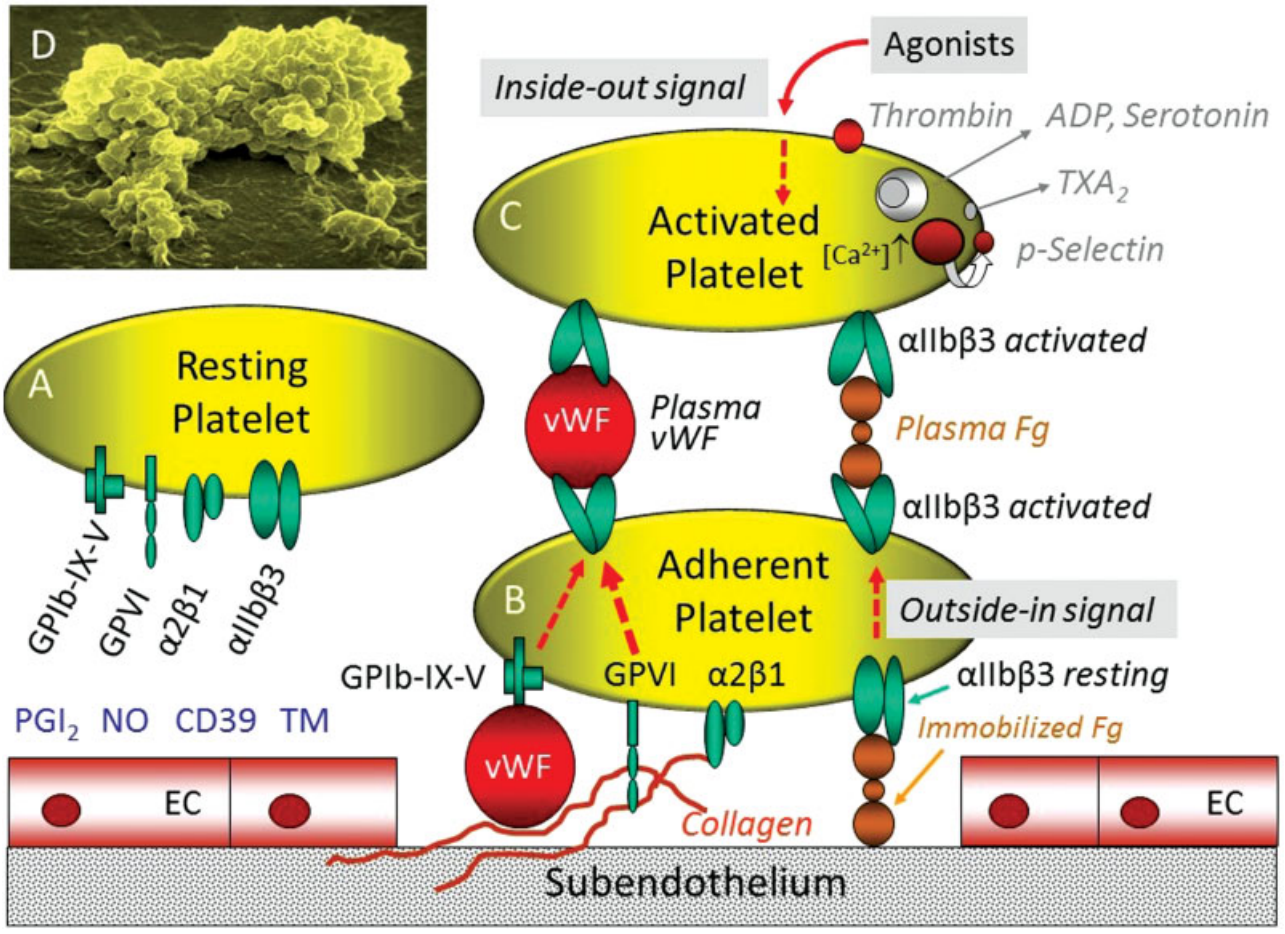

Fig. 1 Three-stage model of platelet activation and thrombus formation. In this schematic representation, major mechanisms and phenotypic manifestations of adhesion, activation and aggregation are depicted. (**A**) Circulating *quiescent* platelet with key receptors of cellular adhesion expressed on the membrane surface. Also displayed are endothelial cell (EC)-derived platelet inhibitors, including prostacyclin (PGI_2), nitric oxide (NO) and CD39, an *ecto*-ADPase, and thrombomodulin (TM), a receptor for thrombin that, following binding to TM, activates protein C. (**B**) ***Stage 1: Initiation (tethering and adhesion)***. Upon injury with loss of the endothelial cell barrier, circulating platelets are rapidly captured ('tethered') through binding of GPIb-IX-V to VWF or VWF–collagen complexes. For this very first step, exposure of the subendothelium is the major trigger. Collagen is an essential substrate for adhesion by concerted interaction of the platelet collagen receptors. Outside-in signals (indicated by dotted arrows) originating from adhesion receptors, mainly GPVI and α2β1, act in synergy with signals from soluble agonists and induce platelet activation through a network of signaling pathways. For reasons of clarity, inside-out signaling by thrombin and other agonists is shown in **C**. Of note, these stimuli are present *within seconds*, notably thrombin, and amplify platelet adhesion and activation. Not displayed are integrin receptor clustering and cytoskeletal rearrangements. (**C**) ***Stage 2: Propagation (activation and aggregation)***. Acquisition of high-affinity binding properties of αIIbβ3 is a major event in this phase. The active conformation (indicated by spreading of the integrin heterodimers) allows binding of soluble adhesive proteins. As indicated for VWF and fibrinogen (Fg), bridges between adjacent platelets are formed, resulting in platelet-to-platelet cohesion. Aggregation is *the* amplification step that, *within minutes*, leads to accumulation of activated platelets. Several phenotypic manifestations of activated platelets are depicted, such as rise in cytosolic [Ca^{2+}] concentration, procoagulant activity, synthesis and release of thromboxane A_2 (TXA_2), secretion from dense granules (ADP, serotonin), and surface expression of P-selectin (upon translocation from α-granules). Not displayed is the cytoskeletal reorganization along with the actin polymerization. (**D**) ***Stage 3: Stabilization.*** Anchoring and consolidation of a growing platelet thrombus are supported by a meshwork of insoluble fibrin fibres (not identifiable in the displayed false-colour electron micrograph). Recent findings by the author's group document micro-architectural heterogeneity in thrombi, exhibiting fully activated (degranulated, P-selectin positive) platelets in the thrombus core but less activated, albeit adherent, platelets on the thrombus surface *(V.R. Stoldt et al, unpublished data, 2017)*. This observation is in accord with that of other investigators.[70]

rotational forward movement ('rolling', not depicted in Fig. 1) due to the torque imposed by the flowing blood.[16] When VWF is bound to collagen, transition from rolling to stable adhesion occurs within seconds, indicative of rapid platelet activation,[17] as discussed later. This process may be supported by signals originating from biomechanical stimulation of VWF–GPIbα bonds exposed to tensile stress.[32]

Importantly, while translocating at low velocity along the subendothelium, tethered platelets undergo additional adhesive interactions. At this phase, ligation of two adhesion receptors, GPVI and α2β1 (GPIa-IIa), by subendothelial fibrillar collagen, a highly thrombogenic substrate, is of major relevance (►Fig. 1B). The original 'two-step/two-site' model of platelet–collagen interactions, suggesting that α2β1 is responsible for adhesion while GPVI induces activation of adherent platelets, has been revised in recent years by placing GPVI in a central position in the receptor interplay with collagen.[33,34] However, similar to GPIb-IX-V, GPVI is

unable to mediate platelet adhesion by itself. Moreover, neither GPVI nor α2β1 is capable of initiating and propagating thrombus formation under arterial flow-dynamic conditions unless platelets are initially tethered to the reactive surface through the interaction between GPIbα and VWF bound to collagen.[21,31] The VWF–GPIbα pathway becomes an absolute requirement for platelet adhesion above a threshold shear rate on the order of 1,000 per second.[16,17,35]

In accord with seminal studies, it is now generally accepted that GPVI and α2β1 closely act in a cooperative way and display mutual reinforcement in the adhesion and activation steps.[16,34,36] Thus, both receptors, finely tuned to each other, undergo activation by a series of signaling events. Thereby, GPVI mediates cellular activation, while platelet integrins are shifted from a low- to high-affinity state either by inside-out or outside-in signaling (see Part 2 of this review in this issue). Recently, fibrin (and also fibrinogen) has been proposed as novel ligand(s) of GPVI[37–39]; however, this is debated controversially at present.[40,41]

Additional adhesive interactions during platelet attachment can involve binding of αIIbβ3 to immobilized fibrinogen or fibrin, at shear rates less than 1,000 per second (►Fig. 1B) and ligation of other β1 integrins by their respective ligands (α5β1, fibronectin; α6β1, laminin).[16] In particular, fibrinogen that is not a normal component of the vessel wall may promote platelet adhesion upon binding to fibulin-1 on the exposed ECM matrix.[42] Insoluble fibrin strands are rapidly formed as a consequence of locally generated α-thrombin. Remarkably, as discussed, αIIbβ3 in its resting (low-affinity) state can selectively mediate platelet adhesion to both immobilized fibrinogen and fibrin with a greater specificity on quiescent platelets than after stimulation.[20,21] As a result of the multiple interactions, outside-in activation in synergy with inside-out signaling is induced (indicated by dotted arrows in ►Fig. 1), followed by firm platelet adhesion, spreading and formation of a platelet monolayer.

Propagation/Extension (activation and aggregation). A crucial outcome in the dynamic platelet response to vascular injury is a structural and functional change of αIIbβ3,[43] now displaying high-affinity ligand-binding characteristics (depicted at the luminal platelet surface in ►Fig. 1B). This property renders the integrin into a competent multispecific receptor for fluid-phase adhesive proteins. Consequently, plasma fibrinogen (via dodecapeptide sequence in the γ-chain) and VWF (via RGD motif in the C1 domain) bind to αIIbβ3 and form bridges between αIIbβ3 heterodimers on adjacent platelets (►Fig. 1C). Thereby, within minutes, platelet-to-platelet cohesion or aggregation occurs.

Activated αIIbβ3 largely contributes to stable adhesion and mediates immobilization of soluble plasma proteins (notably VWF, fibrinogen and fibronectin) on the surface of firmly attached platelets. As the αIIbβ3-bound macromolecules also undergo conformational changes upon ligation, flowing platelets recognize the immobilized proteins as substrates onto which more and more platelets are being recruited. Accordingly, platelet aggregation can be considered as perpetuation of initial platelet adhesion, as described in stage 1.

In fact, irreversible platelet adhesion and extension of the aggregate with subsequent formation of a haemostatic thrombus require additional stimuli with rapid platelet responses. Such stimuli are locally generated α-thrombin and other soluble excitatory mediators that are being released or secreted by activated platelets, including TXA_2, ADP, epinephrine, and also serotonin,[44] a rather weak effector. Upon binding to cognate receptors on the platelet membrane, these agonists act synergistically on platelet activation by amplifying and sustaining initial platelet responses and also by recruiting circulating platelets from the flowing blood into a growing haemostatic thrombus. Indeed, for propagation, platelet adhesion, activation and aggregation ('primary haemostasis') and initiation of coagulation ('secondary haemostasis') are closely integrated and dynamic events.

Importantly, the regulated surface expression of phosphatidylserine on the plasma membrane of activated platelets provides 'docking sites' for the assembly of several enzymes and cofactors of the coagulation system into functional complexes such as the tenase (FIXa-FVIIIa-Ca^{2+}) or the prothrombinase complex (FXa-FVa-Ca^{2+}). Close assembly of the reaction partners and, likewise, their sterically optimal position leads to highly efficient catalysis in α-thrombin generation ('thrombin burst').[45–47] Moreover, locally exposed tissue factor (TF) results in the rapid generation of α-thrombin at sites of endothelial defects.

Apart from mediating fibrin formation, α-thrombin is the only coagulation factor that can directly stimulate platelets but, more importantly, it represents the most potent activator of platelets.[48,49] Platelet activation by α-thrombin induces rapid shape change, secretion and aggregation. No other platelet mediator appears to be as efficiently coupled to phospholipase C (PLC) β as α-thrombin, leading to a fast and highly effective cytosolic Ca^{2+} elevation.[48] Platelet responses to α-thrombin are largely mediated by members of the PAR (protease-activated receptors) family. PAR-1 is the main thrombin receptor in human platelets together with PAR-4,[50,51] whereas mouse platelets express PAR-3 and PAR-4. In addition to interacting with PARs, evidence exists that ligation of the N-terminal high-affinity binding site in GPIbα of the GPIb-IX-V complex by α-thrombin can also activate platelets.[35] However, the resulting signaling pathway of this interaction remains to be elucidated.

Stabilization. Consolidation of formed platelet aggregates (►Fig. 1D) and haemostatic or pathologic thrombi is as important as their growth rate. In either setting, stability of platelet aggregates has a major impact on the outcome. Thus, anchoring and consolidation of the platelet plug ('clot retraction') significantly contribute to arrest bleeding in response to traumatic vascular injury. In clinical conditions, stability of formed aggregates and rheological factors are relevant in determining whether a thrombus will occlude an artery and stay in situ, or lead to embolization.

Although both platelet activation and blood coagulation are important for the formation and stability of arterial thrombi, the special and temporal regulation of the events can vary considerably. Thus, platelet recruitment preferentially occurs at areas of high shear, whereas maximal fibrin

formation occurs in areas, in which both blood flow and shear are comparatively less high.[52]

In summary, whether initial platelet adhesion results in a monolayer of platelets or in thrombus formation depends largely on several distinct determinants that include, among others, (1) nature of the exposed substrate(s), (2) prevailing rheological conditions and (3) the presence of functionally competent platelets.

Determinants Causing or Exaggerating Pathological Thrombosis

Rupture of an atherosclerotic plaque is the major trigger for partial or complete thrombotic vessel occlusion.[35,52–54] Apart from inflammatory processes other than atherosclerosis,[55] several mechanisms or determinants have been identified, contributing to thrombosis in pathological conditions.

For example, ultra-large VWF multimers have an enhanced thrombogenetic potential due to their greater number of interaction sites.[35] Normally, these VWF species are under the control of a specific metalloprotease (ADAMTS-13 [a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13]).[56–58] Impaired size regulation of VWF due to acquired or congenital ADAMTS-13 deficiency results in the persistence of ultra-large VWF multimers, causing thrombotic microangiopathies (thrombotic thrombocytopenic purpura or haemolytic-uraemic syndrome).[59–62]

P-selectin, present on the plasma membrane of activated EC or platelets upon translocation from endothelial Weibel-Palade bodies or platelet α-granules (►Fig. 1C), interacts with the P-selectin glycoprotein ligand-1 (PSGL-1), which is expressed by neutrophils and monocytes. Consequently, this interaction can tether leukocytes to activated platelets and activated EC.[63] P-selectin also induces TF expression on circulating monocytes and microparticles. Thereby, blood-born TF essentially contributes to subsequent fibrin formation.[64] P-selectin also mediates rolling interactions between leukocytes and the vessel wall, a relevant mechanism of leukocyte recruitment to sites of inflammation or infection.[65,66]

Elevated levels of soluble CD40 ligand (sCD40L), a member of the tumour necrosis family, can be a prognostic indicator in high-risk patients with acute coronary syndromes.[67] sCD40L binds to platelets by an αIIbβ3-mediated mechanism and stabilizes arterial thrombi, while absence of sCD40L delays arterial occlusion.[68] Further work suggests that sCD40L induces phosphorylation of a tyrosine motif at residue 759 in the cytoplasmic domain of β3, leading to platelet activation by outside-in signaling through Tyr759 phosphorylation (pY759).[69]

Conflict of Interest

The author states that he has no conflict of interest.

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