Differentiating haemostasis from thrombosis for therapeutic benefit

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Summary

The central role of platelets in the formation of the primary haemostatic plug as well as in the development of arterial thrombosis is well defined. In general, the molecular events underpinning these processes are broadly similar. Whilst it has long been known that disturbances in blood flow, changes in platelet reactivity and enhanced coagulation reactions facilitate pathological thrombus formation, the precise details underlying these events remain incompletely understood. Intravital microscopy studies have highlighted the dynamic and heterogeneous nature of thrombus development and demonstrated that there are considerable spatiotemporal differences in the activation states of platelets within a forming thrombus. In this review we will consider the factors regulating the activation state of platelets in a developing thrombus and discuss how specific prothrombotic factors may influence this process, leading to excessive thrombus propagation. We will also discuss some potentially novel therapeutic approaches that may reduce excess thrombus development whilst minimising bleeding risk.

Keywords

Thrombosis, atherothrombosis, antiplatelet agents

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Introduction

Atherothrombosis, which refers to the disruption of an atherosclerotic lesion with superimposed arterial thrombus formation, is now the leading cause of death globally, accounting for > 25% of all deaths (1, 2). The growing awareness of the central role of platelets in promoting atherothrombosis has led to the widespread use of antiplatelet agents in the management of a broad range of cardiovascular diseases (3). Newer and more potent antiplatelet agents are emerging that are more effective at preventing arterial thrombosis (3, 4). Moreover, combination antiplatelet therapies, typically aspirin and a P2Y12 receptor antagonist, are increasingly being employed in the clinic (5-7). However, the downside of these more intensive antithrombotic approaches is an increased risk of bleeding, which can partially undermine the therapeutic benefit of these approaches (8). Thus, there is a need to identify new therapeutic approaches that are effective at reducing thrombus propagation and vascular occlusion without undermining the physiological approaches underlying haemostasis. This is a challenge, given that the molecular events responsible for arterial thrombosis are similar to those mediating haemostasis.

In this review we will briefly summarise the important molecular events required for haemostasis and thrombosis, highlighting the major pathways that have been targeted therapeutically. We will also describe recent experimental findings indicating that some of the processes driving arterial thrombus propagation may be less critical for haemostasis. This will include recent insights into the role of specific blood coagulation reactions in regulating thrombus propagation and stability, the impact of hyperlipidaemia and diabetes on platelet reactivity and the effects of localised disturbances in blood flow in promoting platelet accumulation onto the surface of growing thrombi.

Molecular events underlying the haemostatic and prothrombotic function of platelets

The initiating event for arterial thrombus formation, particularly in the coronary circulation, is typically the rupturing or fissuring of an atherosclerotic plaque (9). Disruption of the endothelium leads to the exposure of a number of highly reactive subendothelial matrix proteins. In the context of platelet adhesion, the principle matrix proteins are von Willebrand factor (VWF), collagen (type I, III and VI), laminin and fibronectin – all of which engage specific platelet receptors to facilitate stable platelet adhesion (10-12). The relative contribution of the receptors is a function of the prevailing rheological conditions (10, 13). Under high shear, which is a feature of arterioles and stenotic arteries, the VWF-platelet glycoprotein (GP)Ibα interaction is the predominant receptor-ligand interaction initiating platelet adhesion (14, 15). VWF-GPIbα bonds have intrinsically rapid binding kinetics, rapid 'on-off' rates, that on their own support reversible platelet adhesion with the vessel wall (15-17). Stable platelet adhesion requires a second adhesion

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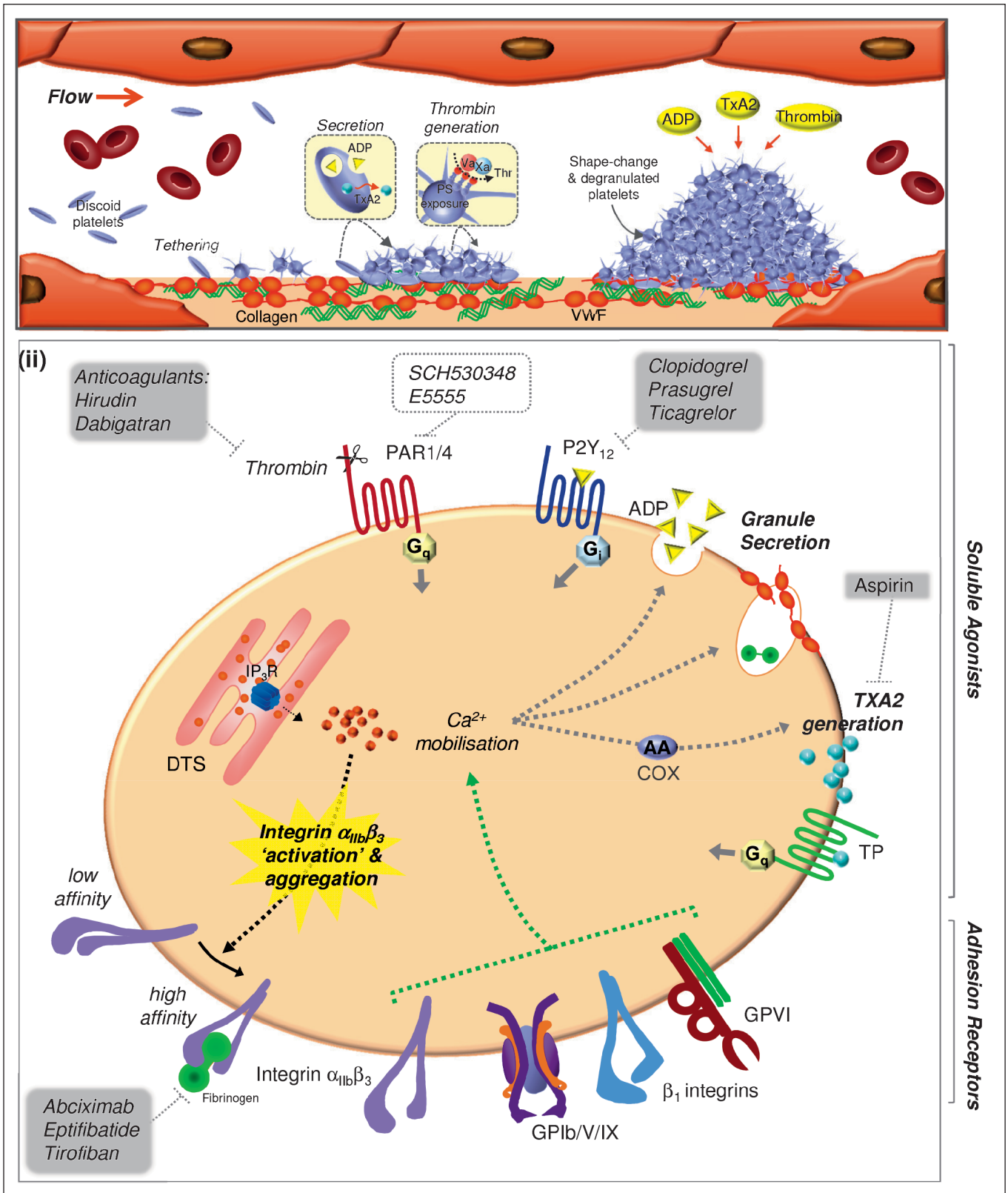
Figure 1: A 'traditional' model of thrombus development where platelets adhere to damaged endothelium, rapidly adhere and aggregate with one another in a process driven primarily by the release or generation of soluble agonists such as ADP, TxA$_2$ and thrombin. Platelet stimulation by soluble agonists results in an increase in intracellular calcium and activation of integrin $\alpha_{IIb}\beta_3$ (inside-out signalling), allowing platelets to form high affinity interactions with adhesive proteins, such as fibrinogen and vWF, thus promoting stable platelet aggregation and thrombus formation. Highlighted are the current antithrombotic therapies and their respective therapeutic targets.

step mediated by the collagen receptors integrin $\alpha_2\beta_1$ and GPVI, the fibronectin receptor integrin $\alpha_5\beta_1$ and potentially the laminin receptor $\alpha_6\beta_1$ (12, 13).

Once adherent, platelet activation is amplified by the release and production of a number of soluble agonists, principally thromboxane A_2 (TxA_2) and ADP (18, 19). TxA_2 is produced in platelets from the conversion of arachidonic acid to endoperoxidases by cyclo-oxygenase (target of aspirin and non-steroidal anti-inflammatory drugs [NSAIDS]) and their subsequent metabolism to TxA_2 by thromboxane synthetise. TxA_2 is lipid soluble and diffuses through the plasma membrane to induce autocrine and paracrine activation of platelets through the G protein-coupled receptors TPa and TPb (19). Another key soluble agonist is the water-soluble purine, ADP which is released from the dense granules of activated platelets and stimulates platelet activation through the P2Y1 and P2Y12 receptors (target of clopidogrel, prasugrel and ticagrelor) (18, 20). Although these soluble agonists have distinct receptors, they ultimately converge into common intracellular signalling events that lead to the mobilisation of intracellular calcium to instigate platelet shape change, degranulation and upregulation of the adhesive function of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) (►Figure 1). $\alpha_{IIb}\beta_3$ is the major platelet receptor for fibrinogen and undergoes a conversion from a 'low affinity' state to activated state upon platelet activation – so called 'inside-out' signalling induced by intracellular second messengers (21). The interaction of $\alpha_{IIb}\beta_3$ and fibrinogen and VWF is central to the generation of a stable platelet thrombus and antagonists of $\alpha_{IIb}\beta_3$ (GPIIb-IIIa inhibitors) have been demonstrated to be highly effective at preventing thrombus development in patients undergoing percutaneous coronary interventions (22, 23).

Blood coagulation and α-thrombin generation

Stabilisation of the platelet haemostatic plug – which is essential to prevent excess blood loss from sites of vascular injury – is critically dependent on localised thrombin generation. α-thrombin is amongst the most potent stimulators of platelets, inducing activation through the proteolytic cleavage of the Gq-linked receptors PAR1 and PAR4 on human platelets (24). Furthermore, thrombin cleavage of fibrinogen and subsequent fibrin polymerisation leads to the generation of a fibrin mesh that anchors the platelet mass to the site of vascular injury. Thrombin generation at the site of endothelial injury is initiated by the exposure of tissue factor, which then forms a catalytic complex with factor VIIa, initiating the 'extrinsic' pathway of blood coagulation (25). As discussed below, recent experimental evidence has suggested a potentially important role for the intrinsic pathway of blood coagulation (26), particularly factor XII and factor XI, in promoting thrombin generation throughout the body of the developing thrombus, through a process that is partially dependent on the procoagulant function of platelets (27). Thus, coagulation reactions, in concert with specific platelet activating events regulate the rate, extent and stability of thrombus growth.

Dynamic and heterogeneous nature of thrombus development *in vivo*

Whilst the central importance of soluble platelet agonists in promoting thrombus development is well defined, recent in vivo studies have suggested that the processes regulating thrombus development may be more complicated than previously anticipated (28, 29). For example, it has long been assumed that once platelets are recruited into a developing thrombus, they rapidly become activated by soluble agonists, undergo marked morphological alterations (shape change), as well as a series of complex biochemical events that lead to degranulation and the formation of highly stable adhesive interactions between adjacent activated platelets, ultimately leading to stable platelet aggregation (►Figure 1).
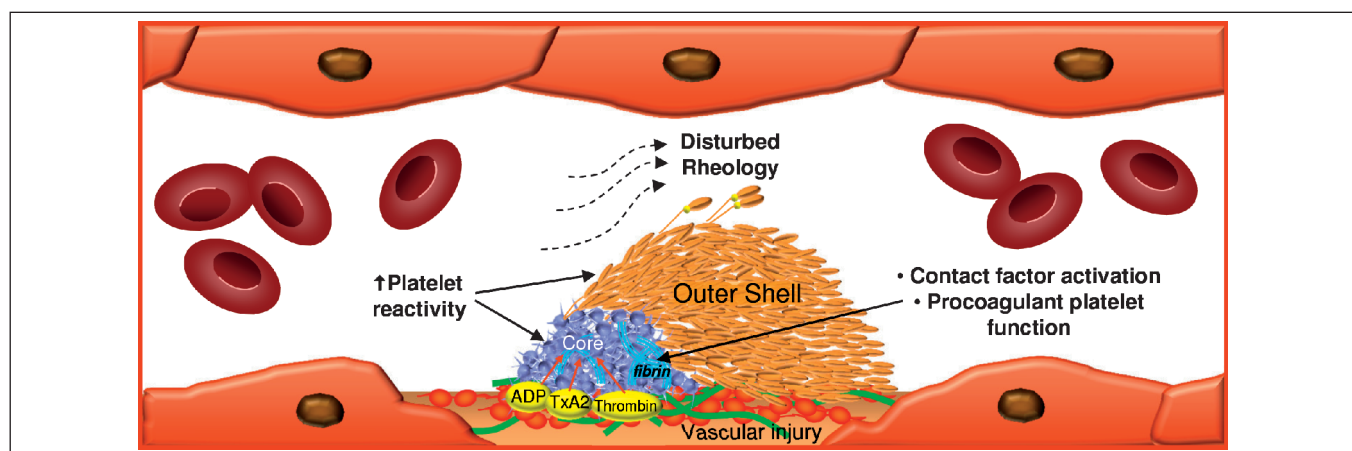
Figure 2: A revised model of thrombus development demonstrating the heterogeneous nature of the activation state of platelets in a propagating thrombus. The stable 'core' of the thrombus is composed of fully activated platelets and is driven by soluble agonists and reinforced by fibrin polymerisation. The outer shell of the propagating thrombus is composed by discoid platelets. The emerging players in thrombus propagation are highlighted. Local rheological conditions influence the recruitment of discoid platelets to the growing thrombus while the intrinsic reactivity of platelets modulates the growth of the core and propagating thrombus. Stabilisation and propagation of the thrombus is partly dependent upon thrombin generation, which requires contact factor activation and the provision of a PS positive surface – which is contingent upon the procoagulant function of the platelets.

However, key features of this model have recently been challenged by a series of *in vivo* experiments utilising intravital microscopy (28, 29). These studies have revealed that a high proportion of platelets that are initially recruited into developing aggregates retain their discoid morphology (30), do not elicit a sustained calcium response (31), do not release α-granule contents (such as P-selectin) (32), and the developing aggregates are sensitive to localised alterations in blood flow (28). Real-time analysis of thrombus development has revealed that thrombi appear to have an inner core of 'highly' activated platelets and an outer shell composed of 'weakly' activated discoid platelets (29). The former are critically dependent on soluble agonist stimulation of platelets and the inner core is stabilised by thrombin generation and fibrin polymerisation (29). In contrast, the outer shell largely consists of aggregates of discoid platelets which are sensitive to changes in local rheological conditions and remain unstable in the absence of soluble agonist stimulation and thrombin generation (▶ Figure 2). It is likely that the molecular processes that underpin the development of the thrombus core are critical for haemostasis, while the factors influencing the propagation and stabilisation of the outer shell of the thrombus may have greater relevance to the propagation of pathological thrombi. In the remaining sections of this review we will discuss some of the important processes promoting sustained platelet-platelet adhesion interactions during thrombus development, with specific emphasis on the role of disturbed blood flow, increased platelet reactivity and coagulation reactions linked to thrombus propagation and stabilisation. For detailed reviews on the role of collagen and vessel wall-derived tissue factor in promoting thrombus development, the reader is referred to several recent extensive reviews on this subject (33, 34).

Factors promoting excess thrombus propagation
Disturbed rheology

The demonstration that discoid platelets rapidly accumulate onto the surface of a developing thrombus at sites of localised flow disturbances is of interest, given the known prothrombotic effects of disturbed rheology. The impact of flow disturbances on platelet adhesion function is complex and incompletely understood. For example, it is well established that flow disturbances at sites of atherosclerosis enhance platelet deposition at the apex of the stenosis, as well as in recirculation regions and flow reattachment points (35, 36). Physical effects, such as the trapping of platelets at recirculation sites as well as the enhanced transport of platelets to reattachment points may partly explain these phenomena (37, 38). Direct shear effects on platelets is also likely to contribute to excessive platelet accumulation and activation (28, 39).

Insight into the effects of shear on platelet adhesion dynamics has recently been gained from the development of high magnification imaging techniques that can monitor platelet morphological changes during primary adhesion and thrombus development.

These studies have suggested that a key mechanism by which discoid platelets adhere and aggregate under shear is through the formation of membrane tethers (28, 40). These structures consist of smooth cylinders of lipid bilayer that are pulled from the surface of platelets by haemodynamic drag forces (40). Whilst membrane tether formation is primarily a passive phenomenon (i.e. not requiring platelet activation), tethers have the capacity to physically restructure through an activation-dependent mechanism that leads to localised cytoskeletal remodelling (28). Restructured tethers can sense and respond to rapid changes in blood flow, such that with shear acceleration (elongational force) membrane tethers extend, whereas with shear deceleration, tethers physically restructure and contract (28). This latter phenomenon appears to be important for strengthening the adhesion contacts between discoid platelets.

These recent findings on membrane tether dynamics have led to the hypothesis that biomechanical platelet activation, induced by microscale shear gradients, may play an important role in promoting platelet aggregation and thrombus growth (28). Such a process may facilitate the accumulation of locally generated soluble agonists such as thrombin, ADP and TXA_2 within the confines of the developing aggregate and reduce the 'wash-out' effect of flowing blood. This has led to the concept that platelet aggregation and thrombus growth may be primarily driven by rheology-dependent platelet aggregation mechanisms, with soluble agonists playing a secondary role, stabilising forming aggregates.

Molecular events promoting shear-induced platelet activation

The molecular basis by which shear induces platelet activation has been extensively investigated using cone-and-platelet viscometers and various flow-based devices (parallel-platelet chambers, microcapillary tubes). The details of these studies have been reviewed elsewhere (10) and will only be briefly summarised here. Central to shear activation of discoid platelets is the co-operative adhesive and signalling function of platelet GPIb and integrin $\alpha_{IIb}\beta_3$ (41). Shear-induced binding of the VWF A1 domain of GPIb stimulates a transient calcium signal that is important for localised integrin $\alpha_{IIb}\beta_3$ activation and for the subsequent binding of the integrin to the C1 domain of VWF (42, 43). Integrin $\alpha_{IIb}\beta_3$ ligation of VWF induces a more sustained calcium signal (28), however in general the signals stimulated by the VWF-GPIb-integrin $\alpha_{IIb}\beta_3$ axis are relatively weak, with full platelet activation requiring the release of dense granule ADP (44). Notably, the adhesive and signaling function of both GPIb and integrin $\alpha_{IIb}\beta_3$ appear to be sensitive to haemodynamic shear forces, suggesting potential mechanosensory role for these receptors (45, 46). Inhibitors of cytosolic calcium flux (47), Src kinases (48) and PI 3-kinases (49) are all highly effective means of reducing shear activation of platelets. PI 3-kinase inhibitors are particularly effective in this context as they inhibit signals downstream of GPIb, integrin $\alpha_{IIb}\beta_3$ and the ADP purinergic receptor, P2Y12 (49) (▶ Figure 3).
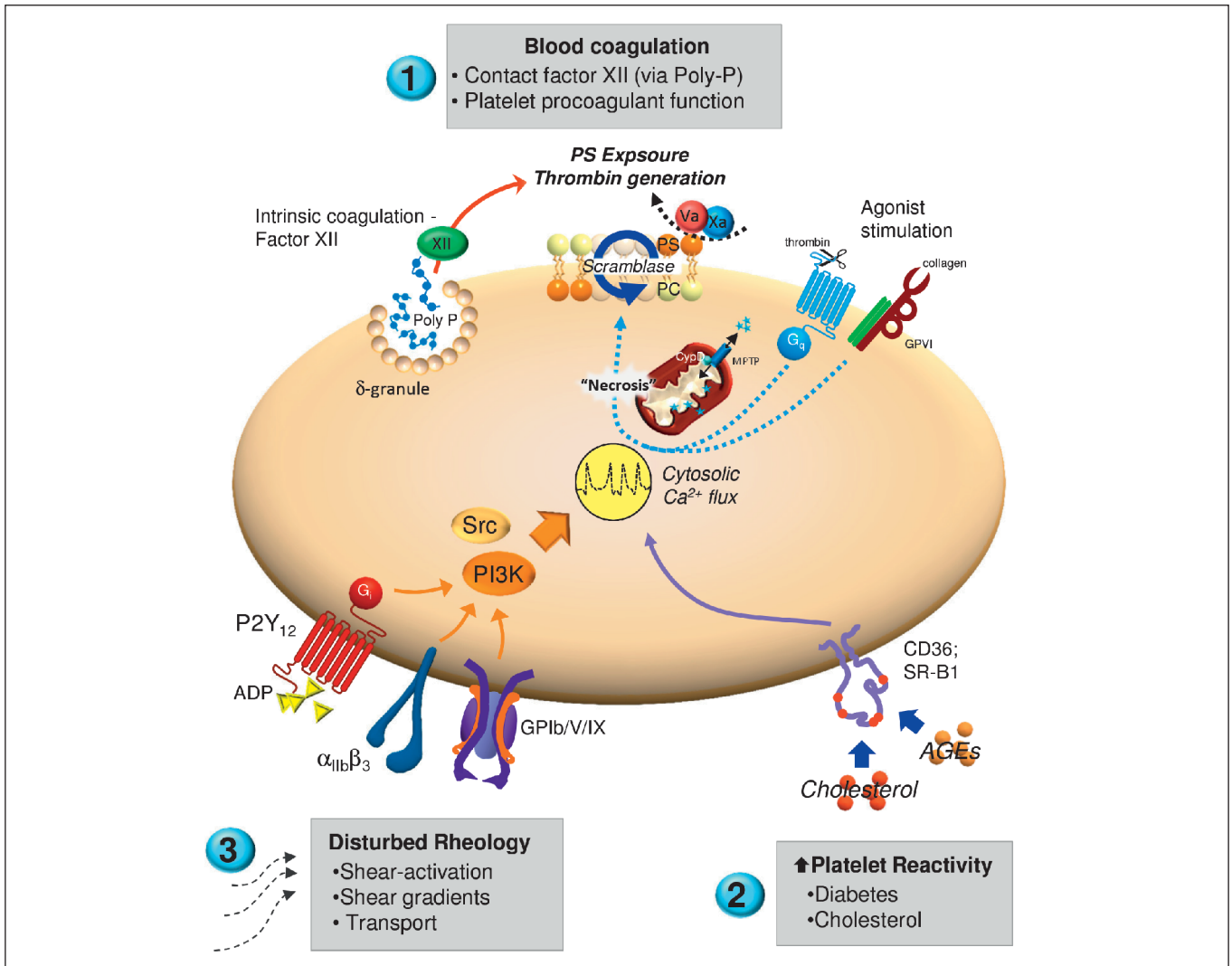
Figure 3: Factor XII is activated by PolyP released from the dense granules of platelets resulting in thrombin generation required for the stabilisation of a propagating thrombus. Preclinical trials have demonstrated inhibition of the intrinsic coagulation pathway to be a potentially safe and effective antithrombotic approach. The provision of a PS positive surface necessary for thrombin generation is regulated by the platelet cell death pathways, in particular the necrotic cell death pathway which is triggered by potent platelet stimuli such as collagen engaging platelet GPVI. The reactivity of platelets is enhanced in diabetes and hyperlipidaemia in a process that is partly mediated by the platelet scavenger receptor CD36. PI3K p110β operates downstream of the platelet receptors P2Y12, GPIbα and $\alpha_{IIb}\beta_3$ and plays a key role in regulating the adhesive function of $\alpha_{IIb}\beta_3$ under shear. Isoform selective inhibitors of PI3K p110β are in phase 1 clinical trials and represent one approach to abrogating shear induced platelet activation.

Heightened platelet reactivity

Real-time intravital analysis of thrombus development in living mice has highlighted the dynamic nature of platelet recruitment to the surface of thrombi, in which a high proportion of platelets tethering to the thrombus surface form unstable adhesion contacts and typically translocate over, or detach from, the thrombus surface. It is likely, although not formally tested *in vivo*, that the intrinsic reactivity of platelets plays a major role in regulating the reversibility and/or stability of these adhesion contacts. Increased platelet reactivity is a well-known feature of diabetes mellitus (50), hyperlipidaemia (51), cigarette smokers (52), obesity and hypertension (53), all important risk factors for atherothrombosis and cardiovascular disease. Platelets from individuals with diabetes and dyslipidaemia are more sensitive to stimulation by threshold concentrations of soluble agonists and form larger thrombi on thrombogenic surfaces (54). Whether these platelets are more sensitive to biomechanical stimulation remains unclear.

Fundamental new insights into the effects of hyperlipidaemia on platelet reactivity have been elucidated from recent studies utilising mouse models of hyperlipidaemia. The scavenger receptors CD36 and scavenger receptor class B member 1 (SR-BI) are both expressed by platelets. Podrez et al. demonstrated that in the context of dyslipidaemia (high low-density lipoprotein [LDL] and low high-density lipoprotein [HDL]), pathophysiological levels of oxidised choline plycerophospholipids (oxPC$_{CD36}$) accumulate,

stimulate platelets via the CD36 receptor and give rise to a pro-thrombotic phenotype (55). Similarly, the scavenger receptor SR-BI also induces platelet hyperactivity in the context of hyperlipidaemia by scavenging plasma cholesterol, thus altering the cholesterol loading in the platelet membrane (56). CD36 is also known to bind molecules that are associated with diabetes, including advanced glycation end products (AGEs) (57), which enhance platelet activation and thrombus growth, which has raised the possibility that scavenger receptors may play a potentially important role in promoting thrombus propagation in both diabetes and the metabolic syndrome (▶Figure 3).

Intrinsic pathway of blood coagulation and platelet procoagulant function enhance stabilisation of the propagating thrombus

The role of the contact factor or intrinsic pathway of blood coagulation in haemostasis and thrombus development has long been debated (58). This has been fuelled by the lack of, or variable bleeding phenotypes, seen in patients with factor XII and factor XI deficiency, respectively. *In vivo* studies on mice have suggested a major role for both factor XI and factor XII in promoting arterial thrombosis (59, 60). The mechanisms regulating contact factor activation are currently being delineated, with recent evidence suggesting a potentially important role for polyphosphates in the platelet dense granules promoting factor XII activation (61).

Platelets also play a key role in propagating coagulation reactions by providing a phosphatidylserine (PS) surface for the assembly of the tenase and prothrombinase complexes – requisite steps for the efficient generation of thrombin. Recent progress in our understanding of the procoagulant function of platelets has been gained through the identification of the calcium dependent platelet membrane protein TMEM16F (62) which has an essential role in phospholipid scramblase activity – deficiency of which results in the rare bleeding disorder Scott syndrome.

The intracellular pathways that mediate procoagulant platelet function are also starting to be elucidated. Platelet PS exposure is regulated by programmed cell death pathways, including apoptosis and necrosis (necroptosis) (63, 64). The necrotic cell death pathway is partly mediated through the opening of the cyclophilin D-dependent mitochondrial permeability transition pore, leading to loss of mitochondrial membrane potential (65, 66). This ultimately causes bioenergetic failure of the cell (ATP depletion), leading to rapid loss of plasma membrane integrity and the release of cellular contents in to the extracellular environment. In contrast, the apoptotic pathway is regulated by the Bcl2 family members Bak and Bax (63, 67), which forms oligomers in the outer mitochondrial membrane, leading to mitochondrial outer membrane permeabilsation (MOMP) and release of cytochrome C (CytC). Once released from the mitochondria, CytC initiates apoptosome assembly and caspase activation (68). The morphological and biochemical profile of agonist-stimulated platelets are akin to those seen in programmed cell necrosis of other cells, suggesting that this pathway may contribute to platelet procoagulant function and stabilisation of the propagating thrombus (▶Figure 3).

Potential solutions to a sticky problem – novel therapeutic approaches

The principal problem with conventional antithrombotic approaches is the inherent risk of bleeding, as the processes targeted by these drugs are important for haemostasis and thrombosis (8). The relative risks and benefits of the currently used anti-platelet agents have been reviewed elsewhere (69). With progress in the understanding of the factors promoting thrombus propagation and stabilisation, it may be possible in the future to develop therapeutics that primarily target thrombosis with less impact on the haemostatic process.

Inhibitors of factor XII and factor XI

Inhibition of factor XI production by antisense nucleotides (70), specific irreversible inhibitors of factor XIa (71) and inhibitors of factor XIIa (72) have been developed and have demonstrable antithrombotic efficacy in preclinical models of venous and arterial thrombosis with no associated increase in bleeding. Similarly, PolyP inhibitors have recently been identified and have shown efficacy in mouse models of arterial and venous thrombosis (73), without increasing bleeding risk. Thus, specific inhibition of coagulation reactions linked to thrombus propagation and stability may have a wider therapeutic window than global inhibitors of coagulation that are currently employed in the clinic.

Reducing platelet hyperactivity

Targeting specific prothrombotic mechanisms that enhance platelet activation is also a potentially attractive antithrombotic option. In principle, platelet activation by $oxPC_{CD36}$ in the setting of dyslipidaemia could be reduced by decreasing plasma levels of $oxPC_{CD36}$ or by blocking its interaction with CD36. CD36 deficiency in humans and mice seems to be well tolerated and does not cause any overt platelet defects, suggesting that the targeting of CD36 is unlikely to cause bleeding. Indeed, CD36 null mice display protection from arterial thrombosis *in vivo* with no increase in bleeding (74). However, CD36 is widely expressed therefore therapeutic attempts at blockade may have systemic effects. There are reports that CD36-deficient individuals show features of the metabolic syndrome, including dyslipidaemia and mildly elevated blood pressure. Thus, selectively blocking the binding of $oxPC_{CD36}$ and/or AGEs with CD36 might be the best option for minimising metabolic disturbances.

One of the challenges of targeting CD36 would be the identification of individuals who are likely to benefit most from this form of therapy. Personalised antithrombotic medicine will most likely require the development of specific assays which can accurately and reproducibly detect platelet hyperactivity related to CD36, and be capable of monitoring the response to therapy. Thus far, platelet

monitoring has not proven useful for guiding optimal antithrombotic approaches, therefore it remains to be seen whether such an approach would be beneficial.

Decreasing the prothrombotic effects of disturbed blood flow

Each of the key receptors promoting shear activation of platelets, including GPIb, integrin $\alpha_{IIb}\beta_3$ and the ADP receptor, P2Y$_{12}$, also play a key role in promoting the haemostatic function of platelets. The risk of minor and major bleeding is increased with the GPIIb-IIIa inhibitors such as abciximab (75); however, novel inhibitors of integrin $\alpha_{IIb}\beta_3$ that only target the 'activated' conformation of $\alpha_{IIb}\beta_3$ have demonstrated antithrombotic efficacy without increased bleeding in preclinical studies (76). Recent studies have suggested that inhibition of GPIbα binding to VWF may also hold promise as a therapeutic approach without increasing bleeding risk (77). Some of these compounds, such as the aptamer ARC1779 demonstrate a greater degree of platelet inhibition under high shear, which may account for the apparent wider therapeutic window (78). An alternative strategy to decrease thrombus propagation, without undermining haemostasis, is by targeting signalling processes that promote biomechanical platelet activation. The signalling enzyme that has been most thoroughly investigated in this context is the type I PI 3-kinase isoform p110β (49, 79). PI3K p110β plays an important role in modulating integrin $\alpha_{IIb}\beta_3$ adhesive function under shear, by transducing signals downstream of GPIbα, $\alpha_{IIb}\beta_3$ and P2Y$_{12}$ (49, 80). Preclinical studies have demonstrated that pharmacological inhibitors against PI3K p110β are effective at preventing thrombotic occlusion of arteries without increasing bleeding (49). Similarly, phase I clinical studies on the PI3Kβ isoform-selective inhibitor AZD6482 (79) has demonstrated that PI3Kβ is important for ADP and shear-induced platelet activation in humans without increasing skin bleeding time. Nonetheless, PI3Kβ is widely expressed therefore to minimise systemic side-effects with chronic therapy, irreversible inhibitors of platelet PI3Kβ, i.e. an aspirin-like drug, may be required.

Clinical perspective

The oral antithrombotic drugs that are currently used in the clinic primarily inhibit pathways associated with agonist-induced platelet activation (3), and therefore target processes that are important for both haemostasis and thrombosis. With the increasing use of dual antiplatelet therapy for coronary artery disease, as well as through the use of 'triple therapy' – anticoagulation in combination with dual antiplatelet therapy – in patients with multiple cardiac pathologies, bleeding has become an increasingly important clinical problem. Whether targeting processes associated with thrombus propagation or stabilisation will lead to less bleeding complications, whilst affording the same level of thrombotic protection remains to be established. Nonetheless, preclinical studies on factor XIIa inhibitors and early clinical studies on PI3K p110β inhibitors, suggest that these approaches may cause less bleeding

than conventional approaches. Time will tell whether this translates into improved safety with combination antithrombotic therapies.

Conclusions

Progress in elucidating the molecular mechanisms promoting thrombus propagation has raised the possibility of developing new approaches to inhibit arterial thrombosis without substantially increasing bleeding risk. With improvements in the understanding of the molecular events enhancing thrombus propagation other therapeutic targets are likely to emerge. Hopefully we are on the cusp of an exciting new era in antithrombotic drug development that can lead to more efficacious, safer and individualised antithrombotic therapies.

Conflicts of interest
None declared.

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