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INVITED REVIEW

Platelet adhesion and activation mechanisms in arterial thrombosis and ischaemic stroke

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Summary. Platelet adhesion, activation and aggregation on the exposed subendothelial extracellular matrix (ECM) are essential for haemostasis, but may also lead to occlusion of diseased vessels. Binding of the glycoprotein (GP)Ib-V-IX complex to immobilised von Willebrand factor (VWF) initiates adhesion of flowing platelets to the ECM, and thereby enables the collagen receptor GPVI to interact with its ligand and to mediate platelet activation. This process is reinforced by locally produced thrombin and platelet-derived secondary mediators, such as adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2). Together, these events promote a shift of β1 and β3 integrins from a low to a high affinity state for their ligands through 'inside-out' signalling allowing firm platelet adhesion and aggregation. Formed platelet aggregates are stabilised by fibrin formation and signalling events between adjacent platelets involving multiple platelet receptors, such as the newly discovered C-type lectin-like receptor 2 (CLEC-2). While occlusive thrombus formation is the principal pathogenic event in myocardial infarction, the situation is more complex in ischaemic stroke where infarct development often progresses despite sustained early reperfusion of previously occluded major intracranial arteries, a process referred to as 'reperfusion injury'. Increasing experimental evidence now suggests that early platelet adhesion and activation events, orchestrate a 'thrombo-inflammatory' cascade in this setting, whereas platelet aggregation and thrombus formation are not required. This review summarises recent developments in understanding the principal platelet adhesion receptor systems with a focus on their involvement in arterial thrombosis and ischaemic stroke models.

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Introduction

Platelets are anucleated blood cells released from bone marrow megakaryocytes through a complex process that is not fully understood [1,2]. At sites of vascular injury, platelets come into contact with exposed subendothelial components such as collagens or laminins, and form a plug to prevent excessive blood loss. However, if this process occurs in an uncontrolled manner it may also lead to thrombotic events causing life-threatening disease states such as myocardial infarction or ischaemic stroke [3,4]. During the last years, substantial progress has been made in understanding the molecular function of platelet receptors and associated signalling machineries, and in dissecting their involvement in thrombotic disease. In many cases, genetic mouse models have been instrumental in this research.

Platelet adhesion and activation is a multistep process involving multiple platelet receptor-ligand interactions. Upon vessel wall injury, circulating platelets are rapidly decelerated by transient interactions between the glycoprotein (GP) Ib-V-IX complex and von Willebrand factor (VWF) bound to collagen [5]. This interaction retains platelets close to the vessel wall and facilitates the contact between GPVI and collagen [6]. The GPVI-collagen interaction induces intracellular signalling leading to cellular activation and subsequent synthesis and release of secondary platelet agonists, most importantly thromboxane A_2 (TxA_2) and adenosine diphosphate (ADP). These soluble agonists together with locally produced thrombin further contribute to platelet activation through G protein coupled receptors (G_i, G_q, $G_{12/13}$) (reviewed in Ref. [7]). All these signalling pathways synergise to induce complex cellular responses, such as activation of integrins, release of granule contents and coagulant activity [8,9].

A detailed molecular understanding of platelet function in haemostasis and thrombosis *in vivo* is critical for the development of better antithrombotic agents to prevent or treat ischaemic disease. Mice are the most frequently used species in thrombosis research, and genetic methods that allow targeted

manipulations in the mouse genome have been exceptionally successful in unravelling protein function in platelets. In parallel, a large number of experimental models have been established to allow *in vivo* observation and kinetic analysis of thrombus formation. Arterial lesions can be induced in different branches of the vascular system and by different methods (reviewed by Day *et al*. [10]), most notably by (i) application of a high energy laser pulse [11], (ii) mechanical injury of the endothelial layer by vessel constriction/compression [12,13] or via insertion and removal of a wire [14,15], and (iii) chemical injury via systemic injection of the photoreactive substance Rose Bengal and transillumination of green light [16,17], or by topical application of ferric chloride [18]. The latter method allows variable levels of injury in different vascular beds and thrombus formation can be monitored by microscopic visualisation and blood flow measurements. However, high variability in the experimental protocols has caused controversial discussions on the pathogenic significance of certain platelet receptors and signalling pathways in this model.

Another major thromboembolic disease is ischaemic stroke, the second leading cause of death worldwide [19]. Whereas approximately 20% of strokes represent intracerebral haemorrhages, about 80% are caused by cerebral ischaemia. In the latter case, embolism to the brain mostly derives from extracranial artery stenoses or from the heart, especially in atrial fibrillation [20]. Thromboembolic occlusion of major or multiple smaller intracerebral arteries leads to focal impairment or cessation of the downstream blood flow, and to secondary thrombus formation within the cerebral microvasculature. Therefore, re-establishment of blood flow is a primary goal in stroke treatment. However, although early reperfusion of occluded major intracranial arteries is achieved in more than 80% of patients, progressive stroke may develop in a process termed 'reperfusion injury' [21,22]. Different experimental stroke models have been established among which the transient middle cerebral artery occlusion (tMCAO) model is considered most relevant [23]. In this rodent model, a thread occludes the middle cerebral artery for a certain time (typically 30–60 min) before reperfusion is allowed. Despite this recanalisation, the animals develop large infarcts within 24 h, reproducing the reperfusion injury seen in human stroke patients. Recent studies have revealed that after tMCAO early, but not late, platelet adhesion and activation mechanisms critically contribute to infarct growth, apparently by orchestrating inflammatory processes rather than thrombus formation. These observations led to the hypothesis that acute ischaemic stroke may in fact represent a 'thrombo-inflammatory' disorder [22].

In the following, we will give an overview of the considerable progress that has been made in understanding platelet adhesion/activation receptors and their involvement in arterial thrombus formation and acute ischaemic stroke. We will mainly focus on the prominent platelet adhesion (GPIb-V-IX and integrins) and activation (GPVI and CLEC-2) molecules. The role of G protein coupled receptors (GPCRs) in platelet activation has recently been reviewed [7] and is not discussed here.

Platelet adhesion and activation

The GPIb-V-IX complex

The GPIb-V-IX receptor complex is highly expressed on the platelet surface in the ratio 2:2:2:1 (approximately 25 000 copies of GPIbα, GPIbβ, and GPIX, and 12 500 of GPV per cell [24]) and belongs to the leucine-rich repeat protein family. Its subunit GPIb binds many ligands, including VWF, P-selectin, macrophage antigen 1 (Mac-1), and the coagulation factors XI, XII and thrombin. In humans, lack or dysfunction of GPIb-V-IX is associated with a rare autosomal recessive disorder, the Bernard-Soulier syndrome (BSS) [25] which is characterised by a bleeding phenotype, thrombocytopenia and giant platelets [26]. A similar phenotype is also seen in mice genetically engineered to lack GPIbα [27] or GPIbβ [28]. Notably, no mutations within the *Gp5* gene have been described in BSS patients and GPV deficiency in mice did not cause a BSS-like phenotype [29].

At high shear flow rates, as found in arteries or moderately stenosed vessels, the initial capture of circulating platelets on the ECM is mediated by the interaction of GPIbα and collagen-bound VWF [5]. This interaction is, however, transient and does not allow firm adhesion of the cells, as demonstrated by rolling of platelets on a VWF layer [5]. Efficient firm attachment under these rheological conditions requires cellular activation through other receptors, most notably GPVI and GPCRs which trigger full cellular activation allowing integrin-mediated platelet adhesion and aggregation [9,30]. However, recent reports have suggested that at extremely high shear rates ($> 10\,000\ s^{-1}$), platelet adhesion and aggregation is exclusively dependent on the GPIb-VWF interaction without obvious requirement for integrin activation [31–33]. This concept is supported by the observation that platelets lacking the 45 kDa N-terminal domain of GPIbα failed to incorporate into growing arterial thrombi in wild type mice. In contrast, platelets lacking β3 integrins were still able to incorporate into thrombi under these experimental conditions [34].

GPIb-VWF interaction induces weak intracellular signalling events that eventually lead to integrin activation (for review see Ref. [35,36]), but the underlying mechanisms are only beginning to be understood. During the last few years, a number of new molecules have been proposed to be involved in GPIb-induced signalling [35–37]. Among these was phospholipase (PL) D1 which may represent a potential linker between GPIb ligation and integrin αIIbβ3 activation [38]. Despite these advances, the exact nature of the GPIb-triggered signalling cascade in platelets is still elusive and will remain an area of intense research.

The essential role of GPIb in arterial thrombus formation was revealed by *in vivo* studies with an antibody (p0p/B) that blocks the VWF binding site of the murine receptor and in

GPIb mutant mice. Fab fragments of p0p/B virtually abolished platelet adhesion to the injured carotid artery and protected the animals from occlusive thrombus formation *in vivo* [15]. Similarly, mice in which the extracellular domain was replaced by the human interleukin 4 (IL-4) receptor (IL4Rα/GPIbα) [39] were profoundly protected from occlusion following chemical injury of the carotid artery [40] and of mesenteric arterioles [34]. This effect was more profound than that seen in *VWF*$^{-/-}$ mice suggesting that also other ligands of GPIb may be involved in thrombus formation [34]. In another study, mice in which the GPIbβ subunit was ablated reproduced the BSS phenotype and showed significantly decreased thrombotic activity in different arterial thrombosis models [41]. The antithrombotic potential of targeting the VWF-GPIb axis was also demonstrated in non-human primates. Treatment of baboons with Fab fragments of the blocking anti-GPIb antibody, 6B4, significantly reduced platelet deposition onto a collagen-coated surface *ex vivo* [42]. Interestingly, blockade of the VWF-collagen interaction by an anti-VWF antibody (82D6A3) prevented arterial thrombus formation *in vivo* in baboons, whereas the bleeding time was not significantly prolonged, indicating that inhibition of VWF-collagen binding might be a promising pharmacological target for the prevention of arterial thrombosis [43].

An alternative strategy to passivate GPIb function could be the controlled downregulation of the receptor from the platelet surface. Platelets can downregulate GPIbα through proteolytic cleavage of the approximately 130 kDa extracellular domain of the receptor (glycocalicin, GC) and a disintegrin and metalloproteinase 17 (ADAM17) has been identified as the major sheddase to mediate this proteolysis *in vitro* and *in vivo*. Mice lacking functional ADAM17 were unable to cleave GPIbα after incubation with different shedding-inducing reagents and the animals displayed significantly reduced plasma GC levels [44,45]. Based on these results, one may speculate that the direct selective activation of ADAM17 in platelets might have strong antithrombotic potential.

GPVI

The central activating platelet collagen receptor, GPVI, is a platelet-specific transmembrane type I receptor that non-covalently associates with the immunoreceptor tyrosine-based activation motif (ITAM) containing Fc receptor (FcR) γ-chain in the plasma membrane [6,46]. The FcRγ-chain becomes tyrosine phosphorylated upon ligand-induced GPVI clustering and initiates a series of tyrosine phosphorylation events finally resulting in PLCγ2 activation [47,48], Ca^{2+} mobilisation and entry [49–53], integrin activation, granule secretion, and coagulant activity [9,48,54]. In addition, results by Inoue *et al.* [55] indicated that murine and human integrin α6β1 are able to facilitate activation of GPVI upon binding to laminin, thereby contributing to platelet activation.

Only a few patients suffering from a mild bleeding disorder have been reported with GPVI-related defects mainly being caused by autoantibody-induced downregulation of the receptor in circulating platelets [56]. However, recently two groups independently described patients with proven mutations in the *Gp6* gene [57,58]. The overall normal haemostatic function in those patients and the unresponsiveness of their platelets to collagen are phenocopied in GPVI-deficient mice [59–61]. Importantly, the phenomenon that autoantibodies cause a GPVI deficiency in humans could be reproduced in mice by injection of anti-GPVI antibodies. This treatment induced the downregulation of the receptor from the platelet surface resulting in a sustained 'GPVI knockout-like' phenotype and long-term antithrombotic protection *in vivo* [12,62,63]. Similarly, the antibody-induced loss of GPVI was demonstrated in human platelets circulating in NOD/SCID mice [64], suggesting that anti-GPVI treatment could be a powerful strategy to specifically passivate the collagen-GPVI pathway in platelets.

GPVI downregulation can occur through internalisation or metalloproteinase-dependent ectodomain shedding [65] and the latter has been shown to be mediated by ADAM10 and 17 under certain experimental conditions *in vitro* [44,65,66]. However, the antibody-induced GPVI loss *in vivo* appears to involve at least one additional, yet unidentified, platelet-expressed proteinase [44]. In a very recent study, Al-Tamimi *et al.* [67] demonstrated that GPVI shedding can also be triggered by activated coagulation factor X (FXa), which directly or indirectly activates ADAM10 (and possibly other metalloproteinases) in platelets, thereby providing a novel negative feedback mechanism in the thrombotic cascade [68].

Different mouse models of GPVI deficiency (FcRγ-chain-deficient [61], GPVI-depleted, *Gp6*$^{-/-}$) have been utilised to assess the function of the receptor in platelet activation in haemostasis and thrombotic disease. Platelets lacking GPVI fail to firmly attach and form aggregates on collagen *in vitro* [59,60,69] or to build occlusive arterial thrombi *in vivo* as revealed in different experimental thrombosis models, such as transluminal wire-induced injury of the femoral artery [14] or after different injuries of the carotid artery (ligation, wire-induced arterial denudation) [15]. Also in models of ferric chloride-induced vascular injury, GPVI deficiency provided strong antithrombotic protection [15,40,70,71], although one group could not identify a role for GPVI in their ferric chloride injury model [72]. It is worth mentioning that thrombin plays a very important role in this model and the presence of high concentrations of thrombin can overcome the effect evoked by the lack of GPVI [73]. Moreover, it has been described that GPVI has no [70,74] or only a minor [73,75] role in thrombus formation after laser-induced heat injury where the process is also suggested to be largely dependent on thrombin generation. In contrast, one group could not confirm these results and reported a role of GPVI in this model [76].

Thus, although minor controversies exist concerning the functional role of GPVI in certain thrombosis models, the receptor is now widely accepted as a promising antithrombotic target. In line with this, the first study with Fab fragments of anti-GPVI antibodies in cynomolgus monkeys demonstrated a strong inhibition of collagen-induced thrombus formation *ex vivo* with only mildly prolonged bleeding times [77,78].

CLEC-2

CLEC-2 is a C-type lectin-like type II transmembrane receptor that was originally identified in immune cells [79,80] and only recently revealed to be expressed in platelets where it serves as the receptor for rhodocytin, a very potent platelet activating protein isolated from the Malayan pit viper *Calloselasma rhodostoma* [81]. Based on the observation that rhodocytin-induced platelet activation shares some similarities with the responses elicited by collagen, integrin α2β1 and GPIbα were initially proposed to be targets of the protein [82–84] but this was shortly later disproven by the finding that rhodocytin fully activated platelets in the absence of these receptors [85].

CLEC-2 uses a similar signalling pathway as the GPVI/FcRγ-chain complex but in contrast to the latter it involves tyrosine phosphorylation of only a single cytoplasmic YXXL motif (hemITAM) [79,81,86]. CLEC-2 forms monomers and dimers on resting platelets [87], and is active upon dimerisation or oligomerisation [87,88]. Upon CLEC-2 engagement, hemITAM phosphorylation of CLEC-2 is mediated by the tyrosine kinase Syk which is absolutely required for signalling and downstream phosphorylation of effector proteins, including PLCγ2 [89]. The sequential activation of (i) Syk and (ii) Src [89] and the only partial dependence of signalling on the cytoplasmic adaptor proteins SLP-76/BLNK [81,86] are different from the signalling events evoked by GPVI ligation. However, all other tested downstream events involved in CLEC-2 and GPVI signalling seem to be very similar [90].

A developmental role for CLEC-2 has been described as the constitutive CLEC-2 knockout led to embryonic/neonatal lethality in mice caused by blood-lymphatic misconnection and severe oedema [91,92], a phenotype similar to the one observed in mice lacking different proteins such as Syk [93,94], PLCγ2 [95], SLP-76 [96] or podoplanin [97,98]. How platelets mediate vessel separation is at present unclear, but it is speculated that it involves rather platelet degranulation than aggregation as mice with aggregation-incompetent platelets do not develop blood-lymphatic vessel connections [99].

Due to its impressive platelet activating potential, it was early speculated that CLEC-2 might become a target for antithrombotic agents [100], but the lethality of CLEC-2 knockout mice has made studies on the function of the receptor in haemostasis and thrombosis difficult. The first experiments that directly addressed this question were performed with mice in which CLEC-2 was immunodepleted in circulating platelets by antibody treatment [101]. Intravenous injection of the monoclonal anti-CLEC-2 IgG antibody INU1 led to a downregulation of the receptor from the platelet surface for more than 6 days [101]. Similar to antibody (JAQ1)-mediated downregulation of GPVI [62], the platelet count recovered after 4 days and the newly produced platelets lacked CLEC-2. The mechanism of receptor downregulation is currently not known, but the Fc-part of the antibody or dimerisation of the receptor are not essential since Fab fragments of INU1 were equally efficient [101]. Both, ectodomain shedding and receptor internalisation, might be involved because it was demonstrated in cell lines that CLEC-2 can be cleaved [102] and internally degraded via ubiquitination [103]. CLEC-2 depleted platelets were unresponsive to rhodocytin and this defect was highly specific as platelet activation and aggregation in response to all other tested agonists was unaltered [101]. Shortly after, two groups reported the same defined activation defect in chimeric mice lacking CLEC-2 in the hematopoietic system (*Clec2*$^{-/-}$) [92,104].

Further studies revealed an important role of CLEC-2 for aggregate stabilisation. In a whole blood perfusion assay, CLEC-2 depleted platelets were able to adhere normally to a collagen-coated surface, but subsequent stable aggregate formation was strongly impaired at intermediate to high shear rates (1000, 1700 s^{-1}) [101]. The defect in thrombus stability was also observed *in vivo* in ferric chloride-injured mesenteric arterioles, evident as normal platelet adhesion to the injured vessel wall but abolished occlusive thrombus formation [101]. In line with this, Suzuki-Inoue *et al*. [92] described that adhesion of *Clec2*$^{-/-}$ platelets to different matrices was normal whereas aggregate stabilisation under flow (2000 s^{-1}) *in vitro*, as well as upon laser injury *in vivo* was affected. Based on these results one may speculate that CLEC-2 contributes to thrombus growth by 'taking over' the function of GPVI in platelets recruited to a growing thrombus that will not get in contact with collagen (Fig. 1). However, in contrast to the described studies, others have reported normal aggregate formation of *Clec2*$^{-/-}$ platelets under flow *in vitro* [104]. The reason for this discrepancy is currently unclear.

CLEC-2 seems to play only a minor role in haemostasis because either no [92,104] or only a moderate [101] increase in bleeding time was observed in mice lacking the receptor in platelets. However, previously it was speculated [81] that the (hem)ITAM receptors in platelets, GPVI and CLEC-2, may compensate for each other in preventing blood loss because, surprisingly, patients [105–107] and mice [59–61] lacking GPVI displayed only mild bleeding tendencies. This issue could be important concerning the treatment of patients with defects in one of the (hem)ITAM signalling pathways that might cause uncontrolled bleeding when targeting the other respective (hem)ITAM receptor.

The physiological ligand of CLEC-2 involved in platelet–platelet interaction is unknown at present. It has been proposed that it could be expressed or immobilised on activated platelets [101]. One group proposed homophilic interaction of CLEC-2 to play a role here [92], but this has been questioned by others [104].

Integrins

Integrins comprise a large family of heterodimeric adhesion receptors that are formed by non-covalent association of different α and β chains and mediate cell adhesion to the ECM, as well as cell-cell interactions [108]. In platelets, integrins are absolutely essential for firm adhesion and aggregation *in vitro* and *in vivo*. Platelets express three β1 integrins: α2β1 (collagen receptor), α5β1 (fibronectin

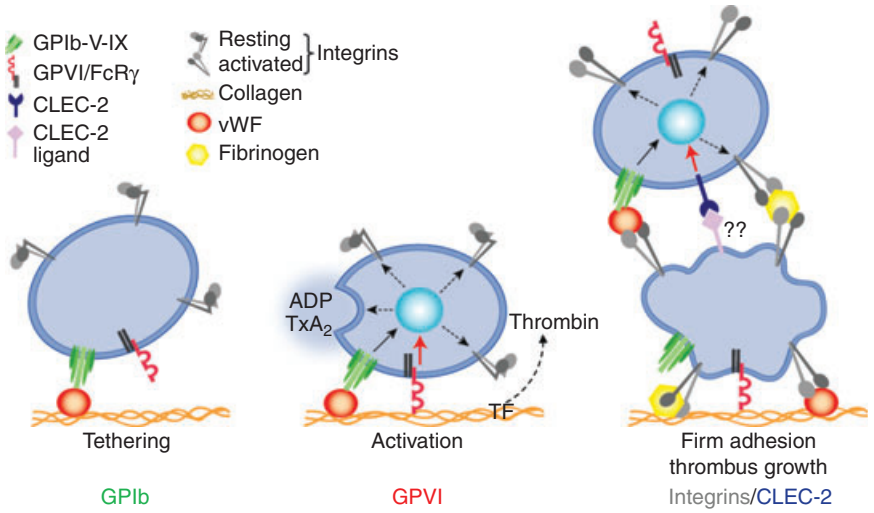

Fig. 1. Simplified model of adhesion receptor involvement in thrombus formation. Platelet tethering is mediated by the GPIb-VWF interaction which enables binding of GPVI to collagen. Cellular activation and inside-out upregulation of β1- and β3 integrin affinity is essential for firm platelet adhesion. Released ADP and TxA_2 amplify integrin activation on adherent platelets and mediate thrombus growth by activating additional platelets. Exposed tissue factor (TF) locally triggers thrombin generation that also contributes to platelet activation. The growing thrombus is stabilised by signalling through CLEC-2, whose ligand/counter-receptor remains to be identified. This model only includes adhesion/activation receptors discussed in this review and does not exclude the involvement of other receptors.

receptor), and α6β1 (laminin receptor). Integrin α2β1 was for a long time considered to be the major platelet collagen receptor [109]. However, studies with mice lacking integrin α2β1, all β1 integrins or GPVI revealed that α2β1, as well as the other β1 integrins, play a supportive rather than essential role for firm platelet adhesion and established GPVI as the major activating collagen receptor on the platelet surface (see above) [30,69,110,111].

Platelets express two β3 integrins, αIIbβ3 and αVβ3. While αIIbβ3 is present in high copy numbers (up to 100 000 per platelet) and its function is well-characterised (see below), αVβ3 (binding to vitronectin, fibronectin and osteopontin) is expressed only at very low levels (approximately 500 per platelet) and its physiological significance is largely unclear [112]. Integrin αIIbβ3 binds several ligands each containing an arginine-glycine-aspartic acid (RGD) sequence, such as fibrinogen, fibrin, VWF, fibronectin, thrombospondin and vitronectin. The integrin plays an essential role for platelet aggregation and thrombus formation by bridging of adjacent platelets via its main ligand fibrinogen, or, at high shear rates, VWF [113,114]. In addition, the αIIbβ3 integrin is of major importance for platelet adhesion on the ECM mediated by binding of the aforementioned ligands, as well as fibronectin and vitronectin [5,30,69].

Inherited deficiency or dysfunction of integrin αIIbβ3 in humans results in a disorder called Glanzmann Thrombasthenia, characterised by defective platelet aggregation and a severe bleeding diathesis (for review see Ref. [115]). Mice lacking either the αIIb (GPIIb) or β3 (GPIIIa) subunit display a similar phenotype, namely defective platelet aggregation and clot retraction *in vitro*, as well as prolonged bleeding times and cutaneous and gastrointestinal bleeding *in vivo* [116,117].

Due to the importance of αIIbβ3 integrin in platelet aggregation it has become an attractive pharmacological target for the prevention of ischaemic cardiovascular events. Strategies to inhibit its function include antibodies (abciximab), cyclic peptides adapted from a snake venom disintegrin (eptifibitide) and nonpeptide analogues of an RGD peptide (tirofiban and lamifiban) that inhibit ligand binding [118]. Although these inhibitors are beneficial for patients undergoing percutaneous coronary intervention, they do not have widespread clinical use due to their side effect of unwanted bleeding [118]. Interestingly, a recent study showed that a human single-chain fragment (scFv) directed against GPIIIa is able to specifically bind to and dissolve platelet aggregates *in vitro* and *in vivo* without affecting the function of the integrin [119]. This approach may open new avenues for the development of novel αIIbβ3 (GPIIbIIIa) inhibitors that do not cause bleeding complications (for review see Ref. [120]).

To ensure firm platelet adhesion exclusively at sites of injury, integrins are present on the surface of resting platelets in a low-affinity state. In response to cellular activation through other receptors (GPCR, ITAM or GPIb), they shift to a high affinity state through 'inside-out' signalling, thus enabling tight ligand binding. Ligand-occupied integrins in turn transduce 'outside-in' signals that orchestrate cellular responses, such as platelet spreading and clot retraction [121]. This 'outside-in' signalling results in activation of Src family kinases (SFK) through a process that involves binding of the $G\alpha_{13}$ subunit [122] and is followed by activation of Syk and PLCγ2, thereby promoting sustained platelet activation [121,123].

A large number of intracellular proteins have been identified to be involved in integrin regulation, among which Talin-1 and Kindlin-3 have central roles. Both proteins bind to integrin β

tails and are indispensable for 'inside-out' activation of β1 and β3 integrins in platelets [124–126]. Platelets of Talin-1- and Kindlin-3-deficient mice were unable to activate αIIbβ3 and α2β1 integrins in response to any tested agonist *in vitro* and this defect translated into abolished thrombus formation in $FeCl_3$-injured vessels and infinite bleeding times *in vivo*. Several mutations in *KINDLIN-3* have been identified in humans that give rise to a rare disorder called leukocyte adhesion deficiency type III (LAD-III) [127–129]. Similar to the phenotype observed in Kindlin-3-deficient mice, hematopoietic cells from patients suffering from LAD-III display defective 'inside-out' activation of β1, β2 and β3 integrins causing immune deficiency and severe bleeding.

Multiple further proteins have been identified as critical regulators of 'inside-out' activation of platelet integrins, including Rap1b [130], CalDAG-GEF [131], phospholipase (PL) D1 (see above) [38] and RIAM [132], although the latter has not been confirmed by a knockout approach.

In contrast to a large number of studies addressing the propagation of integrin 'inside-out' and 'outside-in' signalling, little is known about its negative regulation. C-terminal cleavage of β3 by the cysteinyl proteinase calpain in subpopulations of activated platelets has been demonstrated, which may serve to inhibit 'outside-in' signalling in the late phase of activation [133–135]. However, whereas regulation of other platelet receptors, such as GPIb and GPVI, by proteolytic cleavage is well established, the possibility that ectodomain shedding is also involved in platelet integrin regulation remains largely uninvestigated. Nonetheless, there are indications of integrin cleavage by ADAM sheddases in other blood cells, such as neutrophils, giving rise to speculations on a possible role for ADAM metalloproteinases also in platelet integrin shedding (reviewed in Ref. [136]).

Thrombus growth and stability

The stabilisation of a newly formed thrombus is essential to arrest bleeding at sites of vascular injury. The final thrombus is embedded in a fibrin network to withstand the shear forces generated by the flowing blood. In addition, outside-in signalling through ligand-occupied integrin αIIbβ3 plays a central role in thrombus stabilisation and beside CLEC-2, whose involvement in thrombus perpetuation is currently under discussion (see above) [92,101,104], other proteins including CD40L [137], SLAM (signalling lymphocyte activation molecule) [138], Gas6 (growth arrest-specific gene 6) [139] and semaphorin 4D [140] have been established to participate in platelet–platelet interaction. It has also been reported that blockade of Eph kinases/ephrins interactions resulted in a decreased thrombus volume *in vitro* under flow conditions, suggesting that this interaction contributes to the stabilisation of platelet–platelet contacts [141]. It can be anticipated that other platelet receptors will be identified to be involved in promoting platelet plug stability and possible candidates include JAM-A, JAM-C, CD226 and CD84 [142,143]. However, molecules that limit thrombus growth were also identified. Mice lacking the endothelial cell specific adhesion molecule (ESAM) [144] or platelet endothelial cell adhesion molecule-1 (PECAM-1) [145] developed larger thrombi *in vivo* as compared to control mice, indicating that these molecules serve as negative regulators of thrombus formation, although the effects may be limited [146].

Ischaemic stroke – a thrombo-inflammatory disorder?

Stroke is the second leading cause of death and the most frequent cause of permanent disability worldwide [19]. In patients, extracranial artery stenoses are major sources for thromboembolism in the brain and therefore anti-platelet drugs and anticoagulants are in clinical use for stroke prevention. In contrast, the complex cellular interactions leading from thromboembolic vessel occlusion to infarct development within the brain parenchyma in the setting of acute stroke are poorly understood. Thus, the only approved and effective treatment in acute ischaemic stroke is immediate thrombolysis (within 4.5 h after onset), which is, however, associated with potentially harmful bleeding complications. Importantly, a significant number of patients develop progressive stroke despite reperfusion of previously occluded major intracranial arteries, a process referred to as 'reperfusion injury' [147].

Considerable progress in understanding the pathophysiology of ischaemic brain infarction has been made by using animal models of experimental stroke. In particular rodent models play a central role in stroke research, although due to different variables a direct extrapolation of the data to the human situation may be difficult (e.g. species-specific differences, gender, sex, age) (reviewed in Ref. [23]).

Experimental studies revealed that reperfusion injury is characterised by dramatic molecular alterations of the microvascular bed including dysfunction of the cerebral endothelium and a rapid onset of neuroinflammation that potentially affects neuronal function and survival [148,149]. The underlying pathomechanisms are not understood but it is becoming increasingly clear that T cells [150,151] and other compartments of the immune system trigger inflammatory processes that significantly contribute to infarct progression in acute stroke (reviewed in Ref. [22]). This is in accordance with early findings from human patients showing the presence of monocytes and leukocytes in infarcted brain areas [152].

Studies on the involvement of platelet receptor-ligand interactions in acute stroke using the tMCAO model in mice revealed that interference with early steps of platelet adhesion and activation, but surprisingly not aggregation, limits infarct progression without increasing the risk of intracranial bleeding [153]. Thus, it appears likely that platelets may contribute to stroke progression also in patients by mechanisms that at least partially differ from those involved in thrombus formation.

There is compelling evidence that GPIb might represent a valuable pharmacological target to prevent or treat acute ischaemic stroke [154]. In the mouse tMCAO model, inhibition of the VWF binding site on GPIb via prophylactic and

therapeutic administration of anti-GPIbα Fab fragments (p0p/B) resulted in a marked protection from stroke progression (Fig. 2) and consequently the mice had significantly better neurological scores. Remarkably, this protection was not associated with increased incidence of intracranial haemorrhage, although tail bleeding times were prolonged in these animals [153]. The central role of the GPIb-VWF axis in the development of ischaemic stroke was further supported by the analysis of $VWF^{-/-}$ mice revealing significantly reduced infarct volumes and better neurological scores compared to controls [155,156]. Importantly, and in line with the above described results, studies on mice expressing mutant forms of VWF revealed that interaction of VWF with GPIb and collagen, but not αIIbβ3, is crucial for progression of acute stroke [157]. Conversely, mice lacking the VWF-cleaving enzyme ADAMTS13 developed larger infarction after tMCAO, while infusion of recombinant ADAMTS13 into wild-type mice was stroke-protective [158].

Further studies also revealed a major role for GPVI in acute experimental stroke. In the tMCAO model, GPVI-depleted mice developed significantly reduced brain infarct volumes (Fig. 2) and this was not accompanied by an increase in bleeding complications. This demonstrates that after loss of the microvascular integrity [159] the interaction between GPVI and exposed collagen is involved in stroke development [153]. Although the reduction in brain infarct sizes was less than after GPIb blockade, GPVI might nevertheless be a suitable target in the treatment of acute stroke [153,160]. Importantly, in patients, elevated GPVI expression levels in platelets have been shown to be associated with an increased risk of stroke development [161], and indirect evidence for increased GPVI activation in acute ischaemic stroke has been provided [162].

Strikingly, besides the possible importance of GPIb and GPVI in the regulation of microvascular thrombus formation in the setting of acute stroke, these molecules also exhibit pro-inflammatory properties that may at least in part explain their role in infarct growth (Fig. 3). GPIbα binds to Mac-1, an integrin expressed on neutrophils and monocytes [163], and Mac-1-deficient mice were shown to be less susceptible to cerebral ischaemia and reperfusion injury than controls in experimental stroke [164]. Thus, GPIb-Mac-1 interaction might mediate the formation of platelet-leukocyte complexes and thereby promote inflammation in cerebral ischaemia. Furthermore, GPIb binds coagulation factor XII (FXII), thereby linking platelet activation to the intrinsic pathway of coagulation, but also inflammation, since FXII is the starting point for the kallikrein-kinin system leading to the generation of the pro-inflammatory peptide hormone bradykinin (reviewed in Ref. [165]). In line with this notion, deficiency or inhibition of FXII protected mice from experimental ischaemic stroke [166,167], but also bradykinin receptor-deficient mice showed reduced infarct sizes in the tMCAO model [168]. Less is known about the potential pro-inflammatory properties of GPVI. However, studies with mice recently demonstrated that GPVI-induced platelet microparticle formation promoted inflammation in experimental rheumatoid arthritis independently of

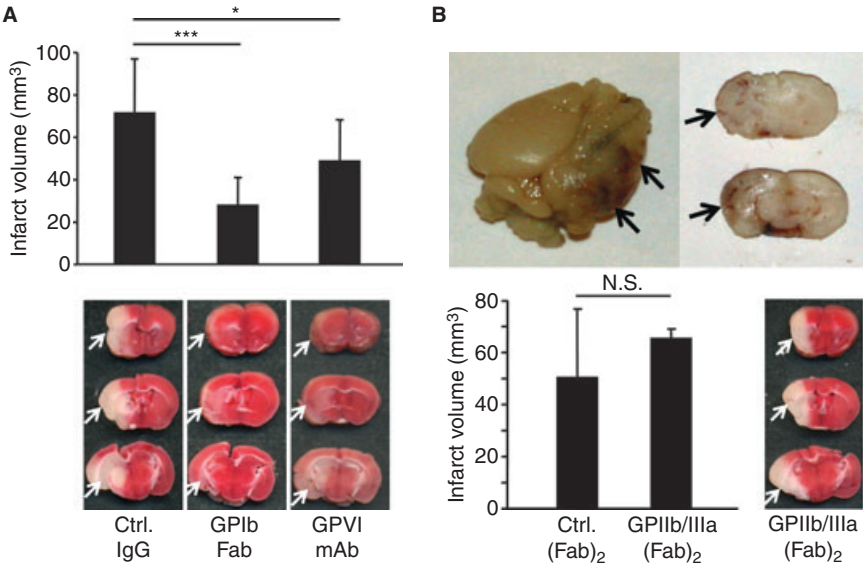

Fig. 2. Effects of platelet receptor inhibition on ischaemic stroke development. Mice were treated with the indicated platelet receptor blocking antibodies and subjected to tMCAO (60 min). Infarcts were analysed after 24 h. (A, top) Brain infarct volumes in mice treated with control IgG, anti-GPIb Fab, or anti-GPVI mAbs. $*P < 0.05$, $***P < 0.001$. (A, bottom) Representative TTC stains of three corresponding coronal brain sections of mice. Red areas represent vital brain tissue; white areas represent cerebral infarctions (indicated by arrows). (B) Representative images of brain (upper left) and coronal brain sections (upper right) from a mouse which was subjected to tMCAO after treatment with 100 μg anti-GPIIb/IIIa $F(ab)_2$, and died due to intracerebral haemorrhage. Note the massive haemorrhagic transformation (black arrows) within the infarcted brain area. Lower left: Brain infarct volumes in mice treated with control $(Fab)_2$ and anti-GPIIbIIIa $(Fab)_2$. No significant difference between the two groups was found. Lower right: Representative TTC stains of three corresponding coronal brain sections of anti-GPIIb/IIIa $F(ab)_2$ treated mice that had survived for 24 h after tMCAO. Results of all experiments are presented as mean ± SD. Figure was modified from Kleinschnitz *et al.* [153].

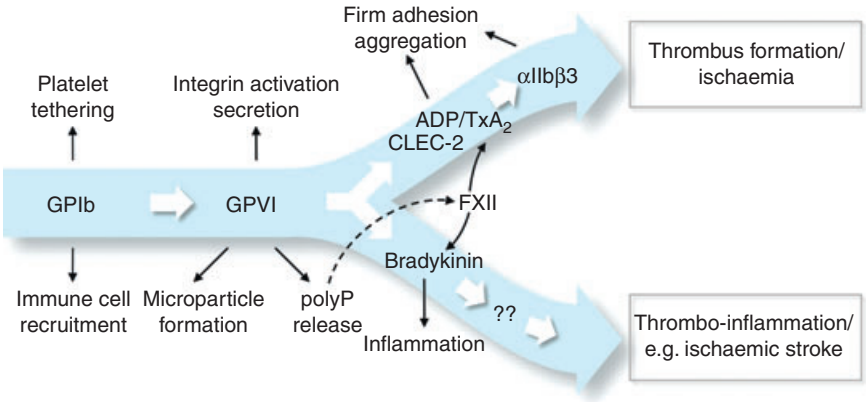

Fig. 3. Diverging pathomechanisms involved in thrombus formation and thrombo-inflammatory disease (e.g. acute stroke). GPIb initiates haemostasis and thrombosis by recruiting platelets to the injured vessel wall, but is also involved in immune cell recruitment under inflammatory conditions. GPVI is the central activating platelet collagen receptor required for thrombus formation on the ECM but may also promote inflammation through platelet microparticle formation and release of inorganic polyphosphates (PolyP). It is proposed that subsequent events may be different between thrombotic and thrombo-inflammatory disease states. While CLEC-2, integrins and secondary mediators are critical for thrombus formation, but not important for thrombo-inflammatory processes, FXII-dependent bradykinin formation and other, yet undefined platelet-mediated processes promote inflammatory reactions independently of thrombus formation and the molecules involved herein.

thrombus formation [169]. Furthermore, GPVI stimulated platelets release inorganic polyphosphates (PolyP) which are potent triggers of the intrinsic coagulation pathway and inflammation via FXII [170].

In contrast to GPIb and GPVI, interference with platelet receptors important for aggregation did not positively affect stroke outcome (Fig. 2). The clinical utility of αIIbβ3 inhibitors in acute ischaemic stroke is controversially discussed and a large phase III trial testing abciximab in acute stroke patients was prematurely terminated due to lack of efficacy and increased rates of intracranial haemorrhage and mortality [171,172]. This is in line with tMCAO studies in mice and baboons in which overall no positive outcome on infarct size but increased intracranial bleeding compared to controls was observed following treatment with higher doses of αIIbβ3 antagonists [153,173–175]. In contrast to inhibitor studies, GPIIb-deficient mice undergoing tMCAO were found to display moderately reduced brain infarction sizes compared to wild-type, however the effect of GPIIb deficiency on intracranial bleeding was not analysed [176]. Finally, although inhibition of CLEC-2 in platelets abrogates occlusive arterial thrombus formation at sites of vascular injury (see above), it did not reduce infarct growth in the tMCAO model supporting the notion that thrombus growth may not be a central pathomechanism in infarct progression [B. Nieswandt, F. May, S Bräuninger and Stoll G unpublished data].

Taken together, the currently available experimental and clinical data support the hypothesis that acute ischaemic stroke/reperfusion injury is a thrombo-inflammatory disease with platelet activation contributing not only to thrombotic vessel occlusion, but also to inflammatory processes [22]. Since patients reach the clinic with a highly variable delay after stroke onset it will be important to identify pathomechanisms during lesion development that can be targeted at subacute stages outside the time window for thrombolysis.

Conclusion

In summary, both *in vitro* and *in vivo* studies on the function of platelet adhesion receptors have provided new insights into how platelets interact with the injured vessel wall and thereby initiate haemostasis and thrombosis. In addition, it is increasingly recognised that platelets may also act as initiators and/or amplifiers of inflammatory processes and that the contributing receptors and signalling pathways are partially different from those mediating thrombus formation. A better understanding of the mechanistic interplay between platelet receptors and inflammatory signalling pathways might therefore provide the basis for more effective and safer therapies for thrombotic and thrombo-inflammatory diseases.

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Disclosure of Conflict of Interests

The authors state that they have no conflicts of interests.

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