SYMPOSIUM REVIEW

Ischaemic stroke: a thrombo-inflammatory disease?

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Abstract Ischaemic stroke is a leading cause of death and disability worldwide. The complex cellular interactions leading from thromboembolic vessel occlusion to infarct development within the brain parenchyma in acute stroke are poorly understood, which translates into only one approved effective treatment, thrombolysis. Importantly, however, patients can develop progressive stroke despite reperfusion of previously occluded major intracranial arteries, a process referred to as 'reperfusion injury' which can be reproduced in the mouse model of transient middle cerebral artery occlusion (tMCAO). Although pathological platelet and coagulant activity have long been recognized to be involved in the initiation of ischaemic stroke, their contribution to infarct maturation remained elusive. Experimental evidence now suggests that early platelet adhesion/activation mechanisms involving the von Willebrand factor (vWF) receptor glycoprotein (GP) Ib, its ligand vWF, and the collagen receptor GPVI are critical pathogenic factors in infarct development following tMCAO, whereas platelet aggregation through GPIIb/IIIa is not. Further experimental work indicates that these pathways in conjunction with coagulation factor XII (FXII)-driven processes orchestrate a 'thrombo-inflammatory' cascade in acute stroke that results in infarct growth. This review summarizes these recent developments and briefly discusses their potential clinical impact.

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Abbreviations ASA, acetylsalicylic acid; ECM, extracellular matrix; FX, coagulation factor X; FXI, coagulation factor XI; FXII, coagulation factor XII; GP, glycoprotein; KKS, kallikrein–kinin system; MCA, middle cerebral artery; PolyP, inorganic polyphosphates; tMCAO, transient middle cerebral artery occlusion; tPA, tissue plasminogen activator; vWF, von Willebrand factor.

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Introduction

Cerebral ischaemia accounts for about 80% of all strokes, the other 20% are due to intracranial haemorrhage (Feigin *et al.* 2003). Atrial fibrillation and symptomatic extracranial artery stenoses represent the main sources of thromboembolism to the brain. Seconds after an embolus has occluded an intracranial vessel the lack of oxygen and glucose supply to the respective brain territory leads to acute neurological deficits such as hemiparesis or aphasia, a situation which is regarded a medical emergency. In the (rare) case of spontaneous thrombus resolution, symptoms are fully reversible and the event is termed *transient ischaemic attack* (TIA). Otherwise, a definite stroke evolves. In either circumstance patients require a complete cardiological and neurological examination due to the high risk of recurrent ischaemic events. Thus, one clinical aim is to identify the source of thromboembolism and to instigate either anticoagulation or anti-platelet treatment for secondary stroke prevention. Anticoagulation with warfarin, mainly prescribed in patients with atrial fibrillation, however, carries a considerable risk of bleeding complications which has led to the recent development of specific thrombin and factor Xa-inhibitors. Platelet inhibitors such as acetylsalicylic acid (ASA) or clopidogrel are more effective in patients with extracranial artery stenoses.

There is only one treatment option for acute stroke when persistent neurological deficit presents on arrival at the emergency room, namely thrombolysis with the 'clot-buster' agent tissue plasminogen activator (tPA; Saver *et al.* 2009). Unfortunately, tPA application is restricted to the first 4.5 h after the onset of stroke (Hacke *et al.* 2008). Beyond this 'window of opportunity', tPA-induced bleeding complications may outweigh the benefit of dissolving the vessel-occluding thrombus. Moreover, the efficacy of tPA is only moderate at best: for one patient to have a favourable outcome (a score of 0 or 1 on the modified Rankin scale), the number needed to treat (NNT) with tPA is 9 within the first 3 h post stroke. With the extended time window between 3 and 4.5 h, the NNT even raises to 14 (Hacke *et al.* 2008). Persistent vessel occlusion is a frequent cause of tPA treatment failure, but a considerable number of patients show secondary infarct growth despite successful vessel recanalization, a phenomenon referred to as 'reperfusion injury'. The molecular mechanisms involved in secondary infarct growth are still poorly understood, but targeting 'brain injury during reperfusion' represents an important therapeutic goal (Stoll *et al.* 2008). In this review we summarize recent insights into the pathophysiological role of platelet receptors and related downstream signalling pathways as well as the intrinsic coagulation system in experimental cerebral ischaemia. By illustrating a link between thrombus formation and inflammatory pathways we introduce the tempting concept of stroke being a 'thrombo-inflammatory' disease. Based on these findings, novel pharmacological targets for both acute stroke treatment and secondary stroke prevention are highlighted.

All animal studies referred to in this review used the *transient middle cerebral artery occlusion* (tMCAO) model to induce ischaemic stroke in mice. In this model a small filament is advanced from the internal carotid artery to the origin of the middle cerebral artery (MCA) to occlude the vessel and induce tissue ischaemia. Although removal of the occluding filament, e.g. after 1 h, allows reperfusion, a complete MCA infarct, comprising the ipsilateral parietal neocortex as well as the basal ganglia, evolves within 24 h. Infarction can then be visualized by histological techniques or *in vivo* by magnetic resonance imaging (MRI) (Kleinschnitz *et al.* 2007; Pham *et al.* 2010).

Basic mechanisms of thrombus formation

At sites of vascular injury, the subendothelial extracellular matrix (ECM) is exposed to the flowing blood, which triggers adhesion and activation of blood platelets (primary haemostasis), followed by the activation of the coagulation system (secondary haemostasis). Primary and secondary haemostasis synergize to form a fibrin-rich thrombus that seals the wound and initiates wound healing (Ruggeri, 2002) (Fig. 1). The ECM contains multiple adhesive macromolecules, such as laminins, fibronectins and collagens. Platelets can bind these macromolecules via specific receptors that have distinct functions in the haemostatic cascade (Varga-Szabo *et al.* 2008*b*). The mechanisms of platelet adhesion at sites of injury are to a large extent determined by the prevailing rheological conditions. Shear forces generated between adjacent layers of the flowing blood, which are maximal at the wall, produce a drag that opposes platelet adhesion and aggregation. The initial tethering of platelets to the ECM of the injured vessel wall under conditions of high shear rates ($>500\,s^{-1}$), found for instance in small arteries and arterioles, is strictly dependent on the interaction between the platelet receptor glycoprotein (GP)Ib and von Willebrand factor (vWF) (Savage *et al.* 1998). The GPIb-vWF binding has a fast off-rate and does not produce stable platelet adhesion but rather reduces the velocity of the flowing platelets thereby allowing their interaction with highly thrombogenic collagens through the immunoglobulin superfamily receptor GPVI. GPVI is a powerful signal-transducing receptor exclusively found in platelets and their precursors, the megakaryocytes. GPVI ligation leads to full platelet activation, characterized by a rise in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), cytoskeletal rearrangements resulting in a change from discoid to spheric shape, fusion of intracellular α- and

dense granules with the plasma membrane and release of secondary platelet agonists, most notably adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin and thromboxane A_2. These agonists, together with locally produced thrombin, act on platelet-expressed G-protein-coupled receptors thereby enhancing cellular activation and recruitment of additional platelets from the bloodstream to the site of injury. This fine-tuned interplay of extra- and intracellular signalling events leads to the functional upregulation of integrin adhesion receptors, most notably integrin αIIbβ3 (also called GPIIb/IIIa), resulting in the establishment of stable platelet–ECM contacts and platelet aggregation, through the binding of plasma fibrinogen. These basic mechanisms of thrombus formation may in principle also apply to cerebral blood vessels during the course of ischaemic stroke (del Zoppo & Mabuchi, 2003), but it is not established that this is a major pathomechanism in stroke development.

Anti-thrombotic treatment in clinical stroke

Excessive platelet activity and continuous platelet adherence to the brain microvasculature during reperfusion has long been recognized to be involved in experimental stroke (Choudhri *et al.* 1998; del Zoppo, 1998). The pathophysiological significance of these findings in human stroke patients, however, remained poorly understood as did the underlying mechanisms. Accordingly, it is still a matter of debate whether the modest clinical benefit of applying platelet inhibitors such as ASA or clopidogrel within the early phase of stroke (48 h) is due to prevention of recurrent thromboembolism originating from outside the brain (e.g. heart, atherosclerotic arteries) or local anti-thrombotic effects within the brain vasculature (Adams *et al.* 2007). An attempt to more effectively block platelet aggregation by antibodies against GPIIb/IIIa receptors failed: a clinical trial testing the monoclonal antibody abciximab had to be stopped prematurely due to excess bleeding-related mortality (Adams *et al.* 2008) and in an experimental study in which the JON/A $F(ab)_2$ was applied intravenously 1 h before tCMAO more than 60% of mice died from intracranial haemorrhages (Kleinschnitz *et al.* 2007). Most importantly, surviving animals exhibited large cerebral infarcts similar to control mice, which can be taken as a first hint that final platelet aggregation via GPIIb/IIIa is not a mandatory step in stroke development in the reperfusion phase. A completely different picture emerged when early platelet adhesion and activation events rather than terminal platelet aggregation were targeted in the tMCAO model.

Platelet inhibition and experimental stroke: the GPIb–vWF–GPVI axis

Platelet tethering at sites of vascular injury must occur through a receptor that functions independently of cellular activation and allows rapid interactions that resist shear

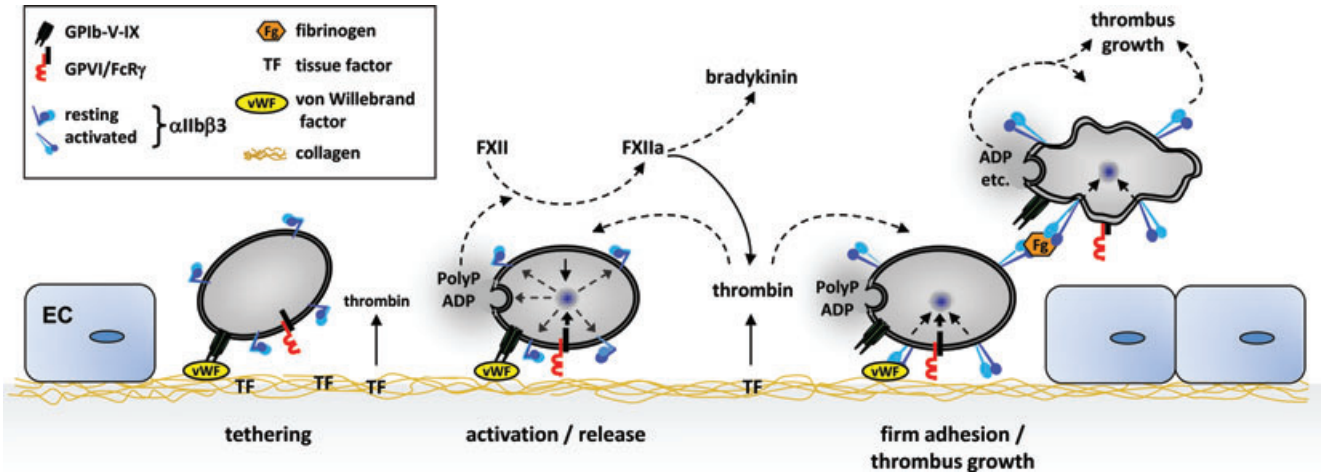

Figure 1. Simplified model of thrombus formation
At sites of vascular injury the ECM becomes exposed to the flowing blood, allowing platelet adhesion and aggregation as well as coagulation. GPIb initiates haemostasis and thrombosis by recruiting platelets to the injured vessel wall, but it may also be 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 by triggering the release of PolyP. PolyP activate FXII leading to coagulation (via thrombin) and inflammation (via bradykinin). In parallel, thrombin generation is triggered by exposed tissue factor (TF, extrinsic pathway) also leading to coagulation and further platelet activation. Cellular activation and inside-out upregulation of GPIIb/IIIa (integrin αIIbβ3) affinity is essential for firm platelet adhesion and aggregation, the latter through binding of fibrinogen (Fg). EC, endothelial cell.

forces. This is achieved by GPIb-V-IX, a platelet-specific receptor complex encoded by four different genes, the α- and β-subunits of GPIb, GPIX and GPV (Bergmeier *et al.* 2000; Berndt *et al.* 2001). Lack or dysfunction of GPIb or GPIX gives rise to the Bernard Soulier syndrome, a congenital bleeding disorder characterized by mild thrombocytopenia and giant platelets (Berndt *et al.* 2001). The observed bleeding phenotype in GPIb-V-IX-deficient humans or mice is attributed to the loss of the extracellular domain of GPIbα which contains the binding sites for several ligands including vWF, thrombin, coagulation factor XI (FXI), FXII, P-selectin or Mac-1 (Berndt *et al.* 2001). The critical role of GPIb-IX-V in thrombus formation has been demonstrated in a model of permanent femoral arteriovenous shunt implantation in baboons by the use of F′(ab) fragments of antibodies directed against the vWF binding site on GPIbα (Cauwenberghs *et al.* 2000) and in mice lacking functional GPIbα (Bergmeier *et al.* 2006).

Recent studies have provided compelling evidence that GPIb might represent a novel target to efficiently prevent or treat acute ischaemic stroke. Both prophylactic (1 h before tMCAO) and therapeutic (1 h after tMCAO) blockade of the vWF binding site on GPIbα by intravenous injection of F′(ab) fragments of the anti-GPIbα antibody, p0p/B, dramatically protected mice from stroke progression following tMCAO (Fig. 2). This was associated with significantly better functional outcomes on day 1 (Kleinschnitz *et al.* 2007). Importantly, no increased incidence of intracranial haemorrhage was noted in these animals, although GPIbα inhibition resulted in increased tail bleeding times (Kleinschnitz *et al.* 2007) thereby confirming that there is no clear correlation between bleeding time and bleeding risk (Rodgers & Levin, 1990). Ultrahigh field MRI studies at 17.6 T employing perfusion-weighted imaging revealed that blockade of GPIb 1 h after tMCAO maintained blood flow during the reperfusion phase, while blood flow continuously decreased in control animals leading to large infarcts of the MCA territory after 24 h (Pham *et al.* 2011). In line with these findings, it was found that mice lacking phospholipase D1, which transduces activation signals downstream of vWF-occupied GPIb and thereby critically contributes to GPIb-dependent integrin αIIbβ3 activation and platelet adhesion under high shear, are markedly protected from stroke progression following tMCAO again without displaying increased bleeding (Elvers *et al.* 2010).

Given the disappointing results obtained with GPIIb/IIIa inhibitors (Kleinschnitz *et al.* 2007; Adams *et al.* 2008), the profound and apparently safe protection from infarct progression following tMACO achieved by inhibition of GPIbα or downstream signalling events was very unexpected and raised the question of whether the pathogenetic significance of GPIb is based on its central role in thrombus formation or other activities. Therefore, the role of the principal GPIb ligand vWF in stroke development was assessed. vWF is a multimeric glycoprotein that becomes immobilized on exposed

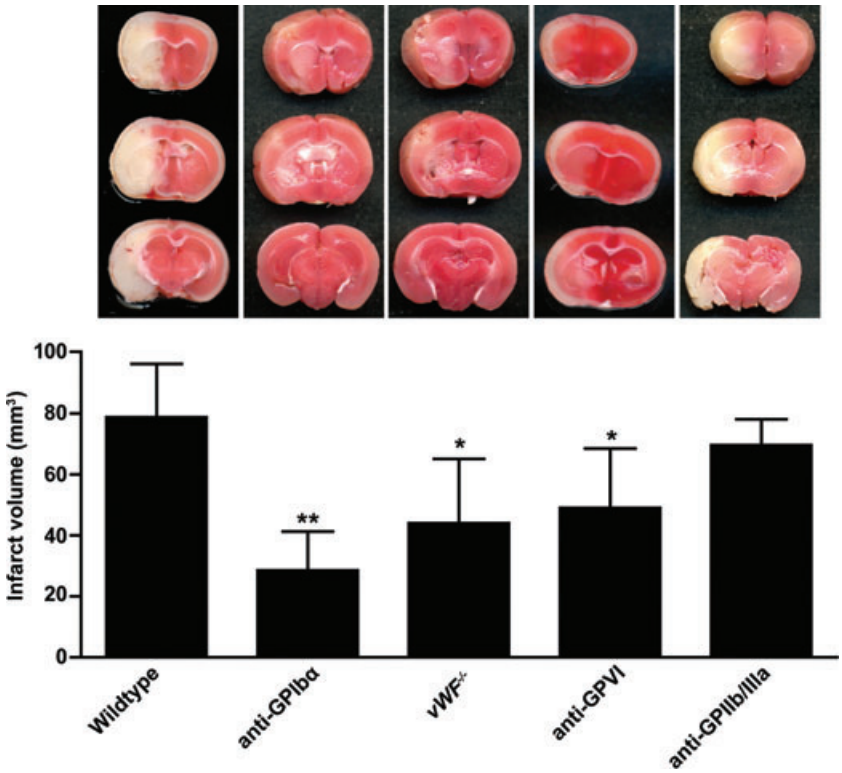

Figure 2. Blocking of early platelet adhesion and activation protects mice from acute ischaemic stroke
Upper panel, representative 2,3,5-triphenyltetrazoliumchloride (TTC)-stained coronal brain sections on day 1 after tMCAO. Ischaemic infarctions (white areas) appear smaller in anti-GPIbα-treated mice, $vWF^{-/-}$ mice and anti-GPVI-treated mice compared with wild-type controls and this was confirmed by infarct volumetry (lower panel). In contrast, blocking of the final common pathway of platelet aggregation using anti-GPIIb/IIIa F′(ab)$_2$ could not reduce stroke size indicating that mechanisms beyond platelet-derived thrombus formation are operative during infarct evolution. $^*P < 0.05$; $^{**}P < 0.01$ compared with wild-type mice (n = 8–10/group); one-way ANOVA, Bonferroni's multiple comparisons post test. (Adapted from Kleinschnitz *et al.* 2007, 2009.)

collagens at sites of vessel injury and facilitates platelet recruitment under high shear by interacting with GPIbα. In addition, vWF contains a RGD (amino acid sequence arginine, glycine, aspartic acid) motif that serves as an adhesion site for platelet GPIIb/IIIa which contributes to firm platelet attachment and thrombus growth (Ruggeri, 2003). Lack of vWF impairs haemostasis in humans and mice and provides protection from experimental thrombosis in the latter (Denis *et al.* 1998; Ruggeri, 2003). Studies in $vWF^{-/-}$ mice revealed a central role of this adhesive protein in infarct progression following tMCAO (Kleinschnitz *et al.* 2009). Very similar to p0p/B-F′(ab)-treated mice, these animals were protected from stroke and showed significantly better neurological scores 24 h after ischaemia and this was, again, not associated with increased intracranial bleeding (Kleinschnitz *et al.* 2009). In a series of studies, De Meyer *et al.* could show that the observed pathogenic function of vWF requires its binding sites for GPIbα and collagen, but not the one for GPIIb/IIIa, thereby further supporting the hypothesis that thrombus growth may not be the only pathomechanism underlying infarct progression after tMCAO (De Meyer *et al.* 2010). In line with these experimental observations several prospective clinical studies identified high plasma levels of vWF as a strong and independent risk factor for stroke (Roldan *et al.* 2005; Wieberdink *et al.* 2010).

In further support of a decisive role of vWF–GPIb interactions, two additional groups reported independently that mice lacking *a disintegrin and metalloproteinase with thrombospondin motifs* (ADAMTS) 13, which cleaves highly thrombogenic ultra-large vWF (>20 million kDa) to less active vWF, are more susceptible to infarct progression following tMCAO (Zhao *et al.* 2009; Fujioka *et al.* 2010; Nieswandt & Stoll, 2010). This was accompanied by increased accumulation of immune cells and thrombi in the ischaemic brain. Conversely, infusion of recombinant ADAMTS13 into wild-type mice was stroke-protective (Zhao *et al.* 2009). Autoantibodies against ADAMTS13 are frequently found in patients with thrombotic thrombocytopenic purpura which carry a high risk of ischaemic strokes (Sadler, 2008).

Although GPIb–vWF interaction triggers platelet activation, this stimulus is generally considered very weak (Jackson *et al.* 2003). Among the numerous constituents of the ECM, collagens are the most thrombogenic as they provide an adhesion substrate for platelets and directly activate the cells. This activation is mediated by GPVI, a type I transmembrane receptor belonging to the immunoglobulin superfamily that is exclusively expressed in platelets and megakaryocytes (Nieswandt & Watson, 2003). GPVI may be a promising anti-thrombotic target as the receptor can be depleted from the surface of circulating platelets through antibodies in humans (Boylan *et al.* 2003) and mice (Nieswandt *et al.* 2001), resulting in a 'GPVI knockout-like' phenotype and long-term anti-thrombotic protection in the latter (Massberg *et al.* 2003). Such GPVI-depleted mice displayed significantly reduced brain infarct volumes at day 1 after tMCAO (Kleinschnitz *et al.* 2007). Further studies revealed that mice lacking components of the machinery that mediates store-operated Ca^{2+} entry are likewise protected from stroke progression in the tMCAO model. Platelets isolated from these mice show a selective functional defect in response to GPVI agonists whereas other pathways are apparently not affected (Varga-Szabo *et al.* 2008*a*; Braun *et al.* 2009) thereby indirectly supporting the hypothesis that GPVI-mediated activation processes are important during stroke development. This is further confirmed by studies showing that elevated GPVI expression levels in platelets are associated with an increased risk of stroke occurrence in humans (Bigalke *et al.* 2009), and elevated levels of the soluble GPVI ectodomain have been detected in acute ischaemic stroke suggesting increased GPVI activation in this setting (Al-Tamimi *et al.* 2011).

Coagulation factor XII

Activated platelets facilitate the local activation of the coagulation cascade through two major pathways. Firstly, expression of procoagulant activity characterized by the exposure of negatively charged phosphatidylserine (PS). PS provides high-affinity binding sites for coagulation factors and, hence, facilitates the assembly of tenase and prothrombinase complexes, which generate factor Xa and thrombin, respectively (Heemskerk *et al.* 2002). Secondly, activated platelets release negatively charged inorganic polyphosphates (PolyP) that activate coagulation factor XII (FXII, Hageman factor) (Muller *et al.* 2009), which represents the starting point of the so-called intrinsic coagulation pathway. For more than 50 years FXII was believed to play no role in blood clotting *in vivo*, based on the fact that FXII-deficient humans do not show any bleeding abnormality. In line with this FXII-deficient mice display normal tail bleeding times and no signs of spontaneous bleeding. Surprisingly, however, $FXII^{-/-}$ mice are consistently unable to form occlusive thrombi in different thrombosis models, indicating that pathological thrombus formation and haemostasis may be driven by at least partially distinct pathways (Renne *et al.* 2005). In line with this novel concept, genetic disruption or pharmacological inhibition of FXII using the highly specific FXIIa inhibitor rHA-infestin-4 immediately before tMCAO profoundly reduced infarct size and this protection was not associated with an increased risk of intracranial bleeding (Kleinschnitz *et al.* 2006; Hagedorn *et al.* 2010). Perfusion-weighted neuroimaging revealed that stroke protection in $FXII^{-/-}$ mice was due to sustained patency of

intracerebral vessels during the reperfusion phase (Pham *et al.* 2010). Accordingly, immunohistochemical analyses showed reduced fibrin formation in the infarct area in FXII-deficient mice compared to wild-type controls indicating that FXII-dependent thrombin generation occurs to a considerable extent during cerebral ischaemia (Kleinschnitz *et al.* 2006).

Thrombo-inflammation: linking the thrombotic cascade to innate immunity

As outlined above, cumulating evidence suggests that platelet attachment and activation through the GPIb–vWF–GPVI axis and subsequent FXII activation constitute a critical pathomechanism in acute cerebral ischaemia. However, although these events will eventually result in intravascular thrombus formation (Stoll *et al.* 2008; Varga-Szabo *et al.* 2008*b*), this appears not to be the key step in ischaemic lesion development during the reperfusion phase as strong platelet aggregation inhibitors, such as anti-GPIIb/IIIa antibodies, do not afford protection from stroke (Kleinschnitz *et al.* 2007; Adams *et al.* 2008). A number of recent observations rather support the idea that GPIb–vWF–GPVI–FXII-triggered pathways may promote detrimental inflammation in acute cerebral ischaemia.

It is well established that ischaemic stroke elicits a strong inflammatory response involving T-cells, monocytes/macrophages and neutrophils (Stoll *et al.* 1998; Dirnagl *et al.* 1999), which is time-restricted and occurs in 'waves'. Early during cerebral ischaemia leukocytes are attracted by cell adhesion molecules expressed on the luminal endothelial surface and act within the endovascular space while infiltration into the parenchyma occurs at later stages.

There is recent evidence that platelet receptors GPIb and GPVI guide inflammation thereby providing a possible link between thrombotic activity and inflammation in ischaemic brain damage. GPIbα harbours a binding site for Mac-1 (an integrin expressed on neutrophils and monocytes (CD11b/CD18)) and mice deficient in Mac-1 are less susceptible to cerebral ischaemia/reperfusion injury (Soriano *et al.* 1999). An important role of GPIb (and vWF) in the recruitment of immune cells has recently been demonstrated in a model of acute peritonitis, but the underlying mechanisms have not been fully elucidated (Petri *et al.* 2010). Similarly, GPVI probably contributes to pro-inflammatory processes as well. Boilard *et al.* showed that GPVI-induced platelet microparticle formation promoted inflammation in experimental rheumatoid arthritis independently of thrombus formation (Boilard *et al.* 2010). Upon activation via GPVI, platelets are also a source of the proinflammatory cytokine interleukin-1α through which they can contribute to inflammation-mediated brain injury (Thornton *et al.* 2010). Moreover, platelets release PolyP as potent FXII activators (Muller *et al.* 2009). Activated FXII (FXIIa) in turn not only initiates coagulation, but also triggers

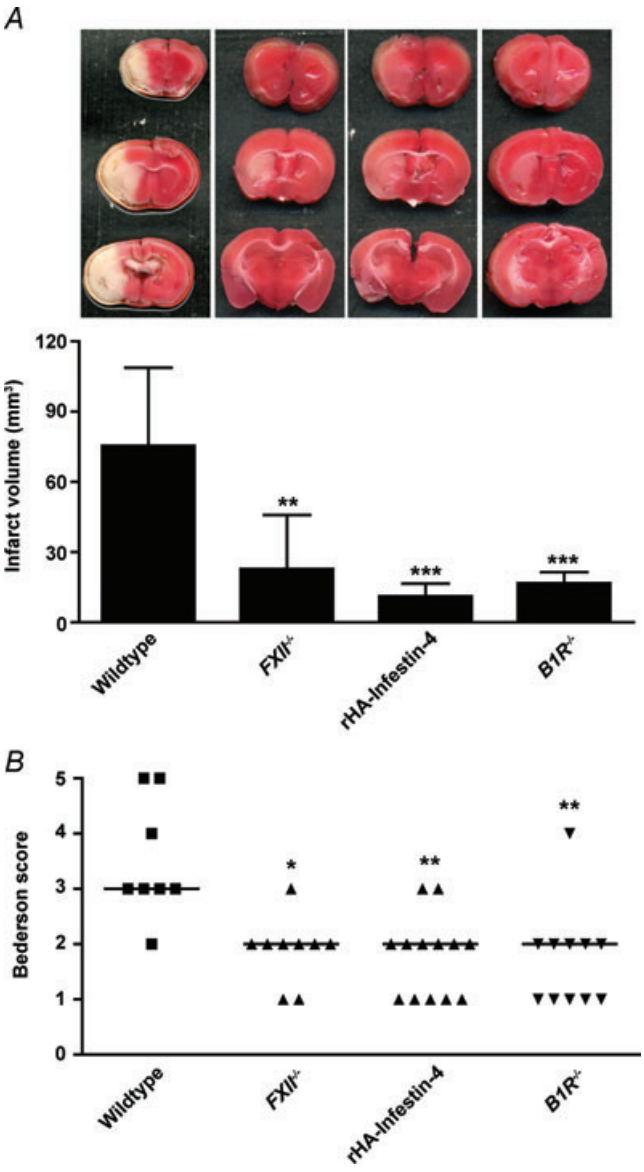

Figure 3. FXII plays a central role in stroke progression in mice
Infarct volumes as measured from TTC-stained coronal brain sections (*A*) and neurological Bederson score (*B*) on day 1 after tMCAO. Genetic ablation (*FXII*$^{-/-}$ mice) or pharmacological blockade (rHA-Infestin-4) of FXII immediately before stroke markedly protects mice from acute ischaemic brain damage (white areas) as does disruption of the bradykinin receptor B1 (*B1R*$^{-/-}$). B1R belongs to the proinflammatory kallikrein–kinin system that is also activated by FXII. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$ compared with wild-type mice ($n = 8$–10/group): one-way ANOVA, Bonferroni's multiple comparisons post test (*A*); Kruskal–Wallis test, Dunn's multiple comparison post test (*B*). Horizontal lines indicate median. (Adapted from Kleinschnitz *et al.* 2006; Austinat *et al.* 2009; Hagedorn *et al.* 2010.)

inflammation through activation of the kallikrein–kinin system (KKS). The end product of the KKS is the potent proinflammatory mediator bradykinin (Muller & Renne, 2008). The hypothesis that FXII activation links thrombotic activity with inflammation (via the KKS) was further strengthened by the analysis of bradykinin receptor B1 (B1R) knockout mice (Fig. 3). These animals developed smaller infarctions and displayed markedly reduced brain oedema formation compared with wild-type controls after tMCAO (Austinat *et al.* 2009).

In conclusion, there is increasing evidence that thrombus formation and immune-mediated processes are strongly interrelated during cerebral ischaemia and significantly contribute to brain damage. The considerable impact of immune cells on stroke outcome is underlined by the fact that immunodeficient mice lacking T-cells are remarkably protected against focal brain ischaemia (Yilmaz *et al.* 2006; Kleinschnitz *et al.* 2010). We are currently only beginning to understand the cellular and molecular interactions linking thrombus formation with neuroinflammation. GPIb, GPVI and FXII are 'hot' candidates orchestrating 'thrombo-inflammation' and provide potential novel targets for stroke treatment and prevention.

References

Adams HP Jr, del Zoppo G, Alberts MJ, Bhatt DL, Brass L, Furlan A, Grubb RL, Higashida RT, Jauch EC, Kidwell C, Lyden PD, Morgenstern LB, Qureshi AI, Rosenwasser RH, Scott PA & Wijdicks EF (2007). Guidelines for the early management of adults with ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council, Clinical Cardiology Council, Cardiovascular Radiology and Intervention Council, and the Atherosclerotic Peripheral Vascular Disease and Quality of Care Outcomes in Research Interdisciplinary Working Groups: the American Academy of Neurology affirms the value of this guideline as an educational tool for neurologists. *Stroke* **38**, 1655–1711.

Adams HP Jr, Effron MB, Torner J, Davalos A, Frayne J, Teal P, Leclerc J, Oemar B, Padgett L, Barnathan ES & Hacke W (2008). Emergency administration of abciximab for treatment of patients with acute ischemic stroke: Results of an international phase III trial. Abciximab in Emergency Treatment of Stroke Trial (AbESTT-II). *Stroke* **39**, 87–99.

Al-Tamimi M, Gardiner EE, Thom JY, Shen Y, Cooper MN, Hankey GJ, Berndt MC, Baker RI & Andrews RK (2011). Soluble glycoprotein VI is raised in the plasma of patients with acute ischemic stroke. *Stroke* **42**, 498–500.

Austinat M, Braeuninger S, Pesquero JB, Brede M, Bader M, Stoll G, Renne T & Kleinschnitz C (2009). Blockade of bradykinin receptor B1 but not bradykinin receptor B2 provides protection from cerebral infarction and brain edema. *Stroke* **40**, 285–293.

Bergmeier W, Piffath CL, Goerge T, Cifuni SM, Ruggeri ZM, Ware J & Wagner DD (2006). The role of platelet adhesion receptor GPIbα far exceeds that of its main ligand, von Willebrand factor, in arterial thrombosis. *Proc Natl Acad Sci U S A* **103**, 16900–16905.

Bergmeier W, Rackebrandt K, Schroder W, Zirngibl H & Nieswandt B (2000). Structural and functional characterization of the mouse von Willebrand factor receptor GPIb-IX with novel monoclonal antibodies. *Blood* **95**, 886–893.

Berndt MC, Shen Y, Dopheide SM, Gardiner EE & Andrews RK (2001). The vascular biology of the glycoprotein Ib-IX-V complex. *Thromb Haemost* **86**, 178–188.

Bigalke B, Stellos K, Weig HJ, Geisler T, Seizer P, Kremmer E, Potz O, Joos T, May AE, Lindemann S & Gawaz M (2009). Regulation of platelet glycoprotein VI (GPVI) surface expression and of soluble GPVI in patients with atrial fibrillation (AF) and acute coronary syndrome (ACS). *Basic Res Cardiol* **104**, 352–357.

Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, Massarotti EM, Remold-O'Donnell E, Farndale RW, Ware J & Lee DM (2010). Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science* **327**, 580–583.

Boylan B, Rathore V, Paddock C, Friedman K, Curtis B, Stapleton M, Newman DK, Chen H, Kahn M & Newman PJ (2003). JAQ-2/3-like ITP – a newly recognized clinical syndrome caused by autoantibody-mediated clearance of the GPVI/FcRγ-chain complex from the human platelet surface. *Blood* **102**, 12A.

Braun A, Varga-Szabo D, Kleinschnitz C, Pleines I, Bender M, Austinat M, Bosl M, Stoll G & Nieswandt B (2009). Orai1 (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation. *Blood* **113**, 2056–2063.

Cauwenberghs N, Meiring M, Vauterin S, van Wyk V, Lamprecht S, Roodt JP, Novák L, Harsfalvi J, Deckmyn H & Kotzé HF (2000). Antithrombotic effect of platelet glycoprotein Ib-blocking monoclonal antibody Fab fragments in nonhuman primates. *Arterioscler Thromb Vasc Biol* **20**, 1347–1353.

Choudhri TF, Hoh BL, Zerwes HG, Prestigiacomo CJ, Kim SC, Connolly ESJ, Kottirsch G & Pinsky DJ (1998). Reduced microvascular thrombosis and improved outcome in acute murine stroke by inhibiting GP IIb/IIIa receptor-mediated platelet aggregation. *J Clin Invest* **102**, 1301–1310.

del Zoppo GJ (1998). The role of platelets in ischemic stroke. *Neurology* **51**, S9–14.

del Zoppo GJ & Mabuchi T (2003). Cerebral microvessel responses to focal ischemia. *J Cereb Blood Flow Metab* **23**, 879–894.

De Meyer SF, Schwarz T, Deckmyn H, Denis CV, Nieswandt B, Stoll G, Vanhoorelbeke K & Kleinschnitz C (2010). Binding of von Willebrand factor to collagen and glycoprotein Ibα, but not to glycoprotein IIb/IIIa, contributes to ischemic stroke in mice – Brief report. *Arterioscler Thromb Vasc Biol* **30**, 1949–1951.

Denis C, Methia N, Frenette PS, Rayburn H, Ullman-Cullere M, Hynes RO & Wagner DD (1998). A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. *Proc Natl Acad Sci U S A* **95**, 9524–9529.

Dirnagl U, Iadecola C & Moskowitz MA (1999). Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* **22**, 391–397.

Elvers M, Stegner D, Hagedorn I, Kleinschnitz C, Braun A, Kuijpers ME, Boesl M, Chen Q, Heemskerk JW, Stoll G, Frohman MA & Nieswandt B (2010). Impaired $\alpha_{IIb}\beta_3$ integrin activation and shear-dependent thrombus formation in mice lacking phospholipase D1. *Sci Signal* **3**, ra1.

Feigin VL, Lawes CM, Bennett DA & Anderson CS (2003). Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. *Lancet Neurol* **2**, 43–53.

Fujioka M, Hayakawa K, Mishima K, Kunizawa A, Irie K, Higuchi S, Nakano T, Muroi C, Fukushima H, Sugimoto M, Banno F, Kokame K, Miyata T, Fujiwara M, Okuchi K & Nishio K (2010). ADAMTS13 gene deletion aggravates ischemic brain damage: a possible neuroprotective role of ADAMTS13 by ameliorating postischemic hypoperfusion. *Blood* **115**, 1650–1653.

Hacke W, Kaste M, Bluhmki E, Brozman M, Dávalos A, Guidetti D, Larrue V, Lees KR, Medeghri Z, Machnig T, Schneider D, von Kummer R, Wahlgren N & Toni D (2008). Thrombolysis with alteplase 3 to 4.5 h after acute ischemic stroke. *N Engl J Med* **359**, 1317–1329.

Hagedorn I, Schmidbauer S, Pleines I, Kleinschnitz C, Kronthaler U, Stoll G, Dickneite G & Nieswandt B (2010). Factor XIIa inhibitor recombinant human albumin Infestin-4 abolishes occlusive arterial thrombus formation without affecting bleeding. *Circulation* **121**, 1510–1517.

Heemskerk JW, Bevers EM & Lindhout T (2002). Platelet activation and blood coagulation. *Thromb Haemost* **88**, 186–193.

Jackson SP, Nesbitt WS & Kulkarni S (2003). Signaling events underlying thrombus formation. *J Thromb Haemost* **1**, 1602–1612.

Kleinschnitz C, De Meyer SF, Schwarz T, Austinat M, Vanhoorelbeke K, Nieswandt B, Deckmyn H & Stoll G (2009). Deficiency of von Willebrand factor protects mice from ischemic stroke. *Blood* **113**, 3600–3603.

Kleinschnitz C, Pozgajova M, Pham M, Bendszus M, Nieswandt B & Stoll G (2007). Targeting platelets in acute experimental stroke: impact of glycoprotein Ib, VI, and IIb/IIIa blockade on infarct size, functional outcome, and intracranial bleeding. *Circulation* **115**, 2323–2330.

Kleinschnitz C, Schwab N, Kraft P, Hagedorn I, Dreykluft A, Schwarz T, Austinat M, Nieswandt B, Wiendl H & Stoll G (2010). Early detrimental T cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood* **115**, 3835–3842.

Kleinschnitz C, Stoll G, Bendszus M, Schuh K, Pauer HU, Burfeind P, Renne C, Gailani D, Nieswandt B & Renne T (2006). Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. *J Exp Med* **203**, 513–518.

Massberg S, Gawaz M, Gruner S, Schulte V, Konrad I, Zohlnhofer D, Heinzmann U & Nieswandt B (2003). A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. *J Exp Med* **197**, 41–49.

Muller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM, Schmidbauer S, Gahl WA, Morrissey JH & Renne T (2009). Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell* **139**, 1143–1156.

Muller F & Renne T (2008). Novel roles for factor XII-driven plasma contact activation system. *Curr Opin Hematol* **15**, 516–521.

Nieswandt B, Schulte V, Bergmeier W, Mokhtari-Nejad R, Rackebrandt K, Cazenave JP, Ohlmann P, Gachet C & Zirngibl H (2001). Long-term antithrombotic protection by in vivo depletion of platelet glycoprotein VI in mice. *J Exp Med* **193**, 459–470.

Nieswandt B & Stoll G (2010). The smaller, the better: VWF in stroke. *Blood* **115**, 1477–1478.

Nieswandt B & Watson SP (2003). Platelet-collagen interaction: is GPVI the central receptor? *Blood* **102**, 449–461.

Petri B, Broermann A, Li H, Khandoga AG, Zarbock A, Krombach F, Goerge T, Schneider SW, Jones C, Nieswandt B, Wild MK & Vestweber D (2010). von Willebrand factor promotes leukocyte extravasation. *Blood* **116**, 4712–4719.

Pham M, Helluy X, Kleinschnitz C, Kraft P, Bartsch AJ, Jakob P, Nieswandt B, Bendszus M & Stoll G (2011). Sustained reperfusion after blockade of glycoprotein-receptor-Ib in focal cerebral ischemia: an MRI study at 17.6 Tesla. *PLoS One* **6**, e18386.

Pham M, Kleinschnitz C, Helluy X, Bartsch AJ, Austinat M, Behr VC, Renne T, Nieswandt B, Stoll G & Bendszus M (2010). Enhanced cortical reperfusion protects coagulation factor XII-deficient mice from ischemic stroke as revealed by high-field MRI. *Neuroimage* **49**, 2907–2914.

Renne T, Pozgajova M, Gruner S, Schuh K, Pauer HU, Burfeind P, Gailani D & Nieswandt B (2005). Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med* **202**, 271–281.

Rodgers RP & Levin J (1990). A critical reappraisal of the bleeding time. *Semin Thromb Hemost* **16**, 1–20.

Roldan V, Marin F, Garcia-Herola A & Lip GY (2005). Correlation of plasma von Willebrand factor levels, an index of endothelial damage/dysfunction, with two point-based stroke risk stratification scores in atrial fibrillation. *Thromb Res* **116**, 321–325.

Ruggeri ZM (2002). Platelets in atherothrombosis. *Nat Med* **8**, 1227–1234.

Ruggeri ZM (2003). Von Willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost* **1**, 1335–1342.

Sadler JE (2008). Von Willebrand factor, ADAMTS13, and thrombotic thrombocytopenic purpura. *Blood* **112**, 11–18.

Savage B, Almus-Jacobs F & Ruggeri ZM (1998). Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell* **94**, 657–666.

Saver JL, Gornbein J, Grotta J, Liebeskind D, Lutsep H, Schwamm L, Scott P & Starkman S (2009). Number needed to treat to benefit and to harm for intravenous tissue plasminogen activator therapy in the 3- to 4.5-h window: joint outcome table analysis of the ECASS 3 trial. *Stroke* **40**, 2433–2437.

Soriano SG, Coxon A, Wang YF, Frosch MP, Lipton SA, Hickey PR & Mayadas TN (1999). Mice deficient in Mac-1 (CD11b/CD18) are less susceptible to cerebral ischemia/reperfusion injury. *Stroke* **30**, 134–139.

Stoll G, Jander S & Schroeter M (1998). Inflammation and glial responses in ischemic brain lesions. *Prog Neurobiol* **56**, 149–171.

Stoll G, Kleinschnitz C & Nieswandt B (2008). Molecular mechanisms of thrombus formation in ischemic stroke: novel insights and targets for treatment. *Blood* **112**, 3555–3562.

Thornton P, McColl BW, Greenhalgh A, Denes A, Allan SM & Rothwell NJ (2010). Platelet interleukin-1α drives cerebrovascular inflammation. *Blood* **115**, 3632–3639.

Varga-Szabo D, Braun A, Kleinschnitz C, Bender M, Pleines I, Pham M, Renne T, Stoll G & Nieswandt B (2008*a*). The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction. *J Exp Med* **205**, 1583–1591.

Varga-Szabo D, Pleines I & Nieswandt B (2008*b*). Cell adhesion mechanisms in platelets. *Arterioscler Thromb Vasc Biol* **28**, 403–412.

Wieberdink RG, van Schie MC, Koudstaal PJ, Hofman A, Witteman JC, de Maat MP, Leebeek FW & Breteler MM (2010). High von Willebrand factor levels increase the risk of stroke: the Rotterdam study. *Stroke* **41**, 2151–2156.

Yilmaz G, Arumugam TV, Stokes KY & Granger DN (2006). Role of T lymphocytes and interferon-γ in ischemic stroke. *Circulation* **113**, 2105–2112.

Zhao BQ, Chauhan AK, Canault M, Patten IS, Yang JJ, Dockal M, Scheiflinger F & Wagner DD (2009). von Willebrand factor-cleaving protease ADAMTS13 reduces ischemic brain injury in experimental stroke. *Blood* **114**, 3329–3334.

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