Platelets in Acute Ischemic Stroke

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Abstract

Ischemic stroke and myocardial infarction are the major causes of death and disability worldwide. Rapid restoration of blood flow by pharmacological thrombolysis and/or mechanical thrombectomy is the mainstay of acute stroke treatment, but does not guarantee a favorable outcome. Reperfusion injury denotes the acute, paradoxically harmful aspect of blood flow return in the ischemic brain which involves platelet activation and, surprisingly, immune cell recruitment. In experimental stroke, glycoprotein (GP)Ibα facilitated tethering of platelets to the postischemic brain endothelium by binding to von Willebrand factor (VWF), while firm adhesion and platelet activation were mediated by GPVI signaling. Accordingly, blocking of platelet GPIbα or GPVI, as well as reducing circulating VWF, dramatically improved stroke outcome by protecting the microvasculature during reperfusion and in addition accelerated recanalization during thrombolysis. Despite interfering with platelet function, no bleeding complications occurred, in contrast to devastating intracranial hemorrhages observed after blocking platelet aggregation via $\alpha_{IIb}\beta_3$. It will be essential to further dissect pathological platelet functions and activation pathways involved in reperfusion injury from those indispensable as gatekeepers of hemostasis in the stroke-injured brain. The pathophysiology of acute stroke is even more complex since it involves concerted detrimental actions of platelets and T-cells, referred to as "thrombo-inflammation," which await further elucidation.

Introduction

Stroke is a leading cause of death worldwide and significantly contributes to permanent disability in the aging world population Global Burden of Disease Study 2013 collaborators 2015). Cerebral ischemia accounts for roughly two thirds of strokes, while the remaining cases are caused by primary intracerebral hemorrhages (ICH) (Krishnamurthi et al. 2013) which are not covered in this chapter. Atrial fibrillation (AF) and high-grade internal carotid artery (ICA) stenosis represent the major sources of cerebral thromboembolism (Lip and Lane 2015; Stoll and Bendszus 2006). Thromboembolic occlusion of major or multiple smaller intracerebral arteries leads to impairment or cessation of the downstream blood flow (Fig. 1a, b) which triggers a plethora of consecutive pathological events in the brain leading to ischemic brain injury (Stoll et al. 1998; Dirnagl et al. 1999). For decades, prevention has been the mainstay of stroke treatment. Anticoagulation is the treatment of choice for prevention of cardiogenic thromboembolism in AF patients (Lip and Lane 2015), while thromboembolism emerging from atherosclerotic plaques of extracranial

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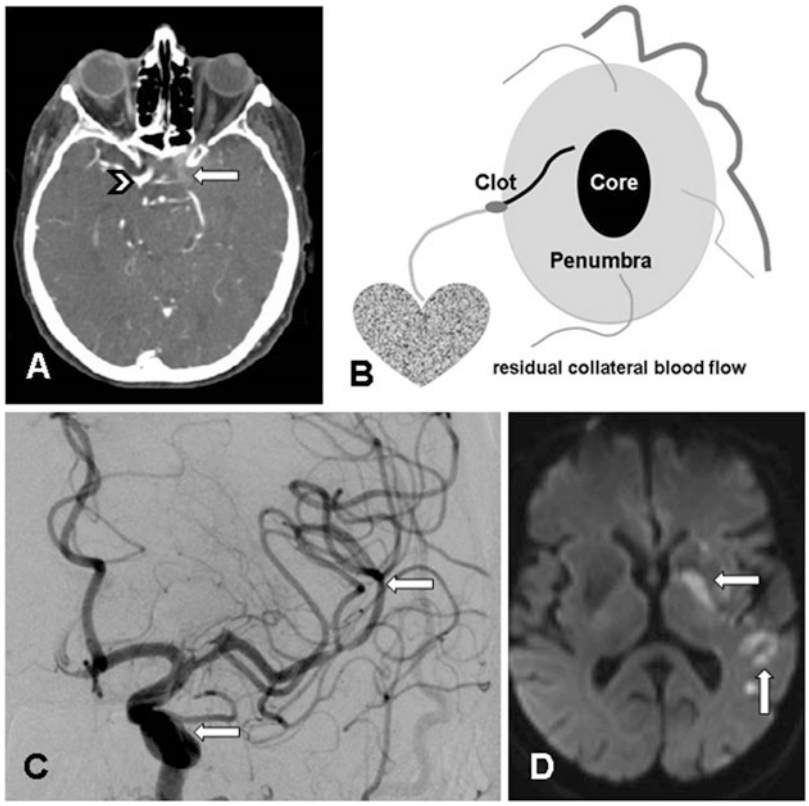

Fig. 1 Successful recanalization and favorable clinical outcome in a patient with acute internal carotid artery (ICA) occlusion. (**a**) CT angiogram on admission showing a patent distal ICA on the right side (*arrowhead*) and a missing contrast enhancement due to occlusion of the left ICA (*arrow*). Clinically, the patient exhibited hemiplegia on the right side and was aphasic. (**b**) Depicts the penumbra concept: After occlusion of a major cerebral artery (like in this patient), severe hypoxia occurs in the center of the corresponding vascular territory (core), while in the surround, called penumbra, residual blood flow is maintained by collaterals. Thereby, the penumbra contains salvageable brain tissue upon successful reperfusion. (**c**) Shows a conventional digital subtraction angiogram after successful removal of the vessel-occluding clot by mechanical thrombectomy. Note recanalization of the ICA and the branches of the MCA (*arrows* in **c**). According to a good clinical outcome with a residual hemiparesis and mild aphasia, diffusion-weighted magnetic resonance imaging, the most sensitive sequence for detection of ischemic brain lesions, showed only small embolic lesion areas, which appear white (*arrows* in **d**)

vessels is amenable to carotid endarterectomy/stenting (Stoll and Bendszus 2006) and/or conventional antiplatelet therapies (Sandercock et al. 2014). The clinical aspects of antiplatelet therapy in primary and secondary stroke prevention are discussed in chapter by Spokoysy and Albers (2017 in this volume); we here focus on the pathophysiological role of platelets in the acute phase of stroke, e.g., within 24 h of symptom onset, with emphasis on reperfusion injury of the brain. While immune cell recruitment has been thoroughly analyzed up to weeks after stroke onset (Stoll et al. 1998; Gelderblom et al. 2009) to the best of our knowledge, no similar studies on secondary recruitment of platelets and their role within the brain parenchyma in the subacute and chronic stages of infarct maturation are available. There is evidence that lesion-induced accumulation of platelets promotes survival of adult neural stem cells in the brain (Kazanis et al. 2015), and, moreover, platelets play a decisive role in wound healing (Nurden 2011). Whether platelets are involved in lesion maturation and tissue remodeling after brain ischemia, and if late outcome could be modified by antiplatelet treatment, is completely unknown and therefore not covered in this chapter. Prompt application of antiplatelet drugs in stroke patients is currently based solely on their effects on secondary prevention of recurrent thromboembolic events (Sandercock et al. 2014).

Acute Stroke Treatment: Thrombolysis and Mechanical Thrombectomy

The main goal of treatment in acute ischemic stroke is recanalization of occluded extra- and intracranial vessels (Fig. 1). Intravenous thrombolysis by recombinant tissue-type plasminogen activator (rt-PA) within 4.5 h after symptom onset introduced in 1995 was the only proven effective treatment for acute stroke (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group

1995). Broad clinical experience showed that recanalization was the strongest predictor of good clinical outcome, but it also became obvious that only a low rate of recanalization could be achieved by intravenous thrombolysis alone in proximal vessel occlusion, e.g., occlusion of the ICA or the M1 segment of the middle cerebral artery (MCA) (Bhatia et al. 2010). This prompted further clinical trials evaluating intra-arterial thrombolysis and mechanical thrombectomy using stent retriever systems which at the beginning revealed equivocal results. The recent MR CLEAN trial (Berkhemer et al. 2015) was the first to document superiority of mechanical thrombectomy over pharmacological thrombolysis alone and has changed stroke care worldwide (Grotta and Hacke 2015). Five hundred patients with acute stroke were randomized to standard thrombolysis with and without intra-arterial thrombectomy within 6 h of symptom onset. Although the reperfusion rate of 58.7 % in MR CLEAN was relatively low as compared with previous,

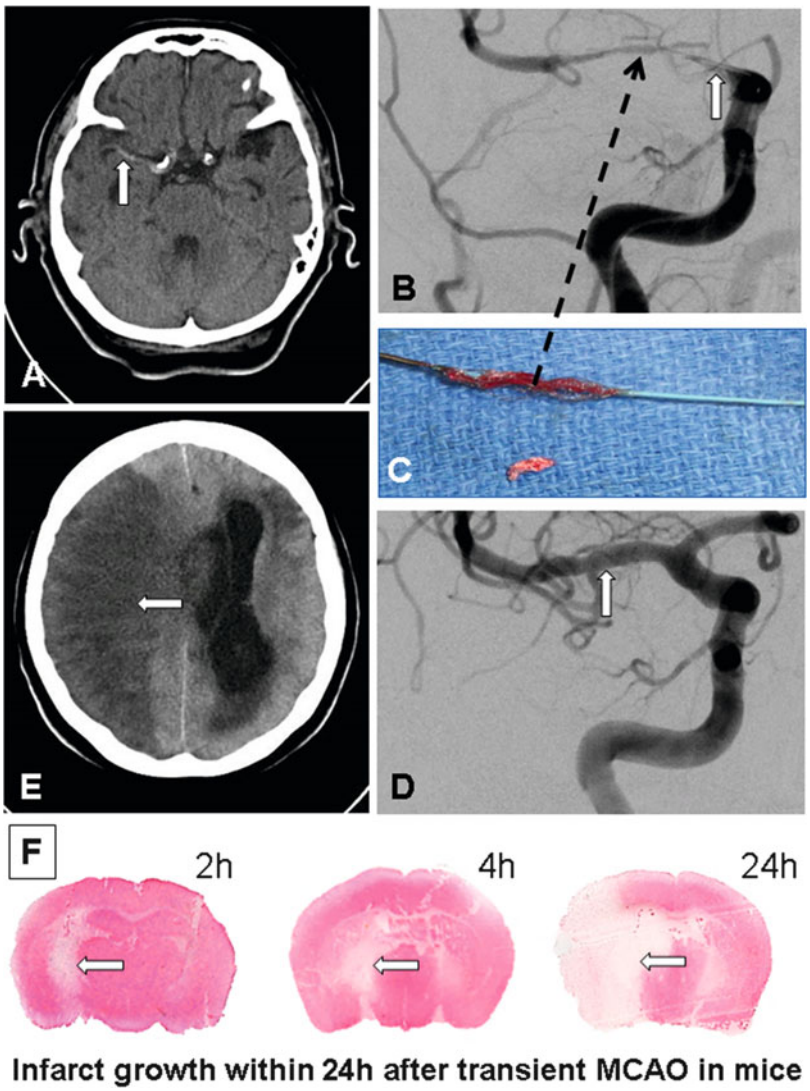

Fig. 2 Infarct development despite successful recanalization. A patient with acute left-sided hemiparesis due to occlusion of the right middle cerebral artery (MCA). The intravascular clot appears white on the native CT scan (*arrow* in **a**). A catheter device (stent retriever) is advanced through the femoral artery into the internal carotid artery and further upward to the proximal end of the intravascular clot within the MCA; the fresh clot is penetrated (*white arrow* in **b** denotes proximal site of occlusion), captured by the devise, and a stent then extended which facilitates immediate restoration of blood flow. The stent retriever system containing the former vessel-occluding clot (shown in **c**) is then removed and the patency of the MCA completely restored (*arrow* in **d** depicts the reperfused MCA segment). Despite successful recanalization, the patient unfortunately developed a large MCA infarction due to reperfusion injury (**e**). This common clinical situation can be mimicked by the transient MCA occlusion model in mice: (**f**) show infarct development in mice after 1 h of MCAO by TTC staining, an indicator for vital tissue. Dark areas show vital brain tissue, while pale areas are necrotic marked by arrows. Note that 2 h and 4 h after reperfusion, cortical areas are still spared from ischemic brain damage, but despite recanalization a full-blown MCA infarct inevitably develops within 24 h

clinically equivocal trials which were 80 % or higher, for the first time there was an absolute difference in the rate of functional independence (modified Rankin score 0–2) in favor of the intervention (32.6 % vs. 19.1 %) (Berkhemer et al. 2015). After publication of MR CLEAN, a number of concurrent clinical studies were prematurely halted after interim analyses confirming these encouraging results (Grotta and Hacke 2015). Mechanical thrombectomy increases the number of acute stroke patients with successful restoration of blood flow and favorable outcome (Fig. 1), but a considerable number of patients still develops severe neurological deficits or die, despite recanalization (Fig. 2). The number needed to treat for a good functional outcome in one patient is around four to six over all thrombectomy trials. The mechanisms underlying infarct growth despite recanalization are largely unknown, but there is increasing evidence from animal studies that platelets are critically involved.

The Concept of Reperfusion Injury After Brain Ischemia and Early Observations Regarding the Function of Platelets Herein

As discussed, revascularization therapies in stroke patients aim to rescue brain tissue at risk, the so-called ischemic penumbra (Jung et al. 2013), by restoring the patency of an occluded major supplying artery (recanalization) (Fig. 1) and the downstream capillary blood flow (reperfusion) (Soares et al. 2010). Importantly, recanalization does not necessarily facilitate reperfusion (Nour et al. 2013). The phenomenon that "blood does not flow despite recanalization" has been termed "no-reflow" phenomenon (Ames et al. 1968), and the paradoxically harmful aspect of blood flow return in transiently ischemic organs has been termed "reperfusion injury" (Hallenbeck and Dutka 1990; del Zoppo and Mabuchi 2003). In the brain, conversion of the luminal surface of the postischemic endothelium within the microvasculature from an anticoagulant to a procoagulant membrane has been proposed as one key mechanism of these harmful events (Hallenbeck and Dutka 1990). In support of this notion, Del Zoppo and colleagues could show that ^{111}In-labeled platelets are deposited in the ischemic basal ganglia early during reperfusion in a primate model of transient middle cerebral artery occlusion (tMCAO) (Del Zoppo et al. 1986). Electron microscopic examination of the microvasculature within the ischemic region further demonstrated aggregates of degranulated platelets together with fibrin and leukocytes and provided direct evidence that platelet activation occurs in the ischemic zone (Okada et al. 1994). Baboons treated with ticlopidine, a first generation P_2Y_{12}-ADP receptor inhibitor, in combination with heparin displayed a significant reduction in platelet deposition and in the number of microvascular occlusions in the ischemic basal ganglia (Del Zoppo et al. 1986). In clinical practice, platelet aggregation inhibitors, most notably acetylsalicylic acid (ASA), are routinely given to ischemic stroke patients not amenable to recanalization treatments within 48 h of symptom onset (Sandercock et al. 2014). This recommendation is based on the results of two major clinical trials involving more than 40,000 participants, in which treated patients had a moderate, but statistically significant, benefit on stroke outcome (CAST (Chinese Acute Stroke Trial) Collaborative Group 1997; International Stroke Trial Collaborative Group 1997). It is widely held, however, that the primary effect of ASA was due to reduction of early recurrent stroke rather than halting ongoing subacute lesion development in the brain (Jauch et al. 2013). A recent retrospective, case-control study in 3025 patients with first-ever ischemic stroke, however, provided class II evidence that patients with pre-stroke antiplatelet agent use were partly protected in the acute phase as they showed decreased stroke severity (Jung et al. 2015). On the other hand, treatment of stroke patients with ASA immediately after intravenous thrombolysis provided no evidence for a beneficial antithrombotic effect, but was associated with early deterioration caused by an increased rate of ICH (Zinkstok et al. 2014) (Fig. 4). Thus, although the protective role of platelet aggregation inhibitors in primary and secondary stroke prevention is well established (CAPRIE Steering Committee 1996; Bhatt et al. 2006) (see Spokoyny and Albers (2017)), the functional role of platelets during the acute stroke phase is less well established and awaits further clarification (Sandercock et al. 2014). The task is to dissect detrimental platelet effects in reperfusion injury from their essential function as gatekeepers of hemostasis in acute stroke. The tremendous progress made in our understanding of the mechanisms of platelet activation, as well as the receptors and signaling molecules involved (as described in detail in other chapters of this book), allows translational research to better define the role of platelets during cerebral ischemia. Thereby the transient middle cerebral artery occlusion model (tMCAO) of focal cerebral ischemia is widely used (Braeuninger et al. 2012). Usually a filament is inserted into the ICA of rodents and further advanced intracranially to occlude the MCA for variable time periods. In most studies the final stroke volume is assessed around 24 h after onset of ischemia. The extent of ischemic brain damage depends on the occlusion time until reperfusion is allowed by removal of the filament. It is important to note that despite reperfusion, infarcts further develop and maturate within the following 24 h. In mice a MCA occlusion time of 1 h finally leads to complete infarction of the MCA territory, but infarcts gradually grow during reperfusion as shown in Fig. 2f [for further details see Braeuninger et al. (2012)]. Thus, the tMCAO model partly mimics the clinical situation in which patients with a thrombotic MCAO undergo thrombolysis or mechanical thrombectomy and, in

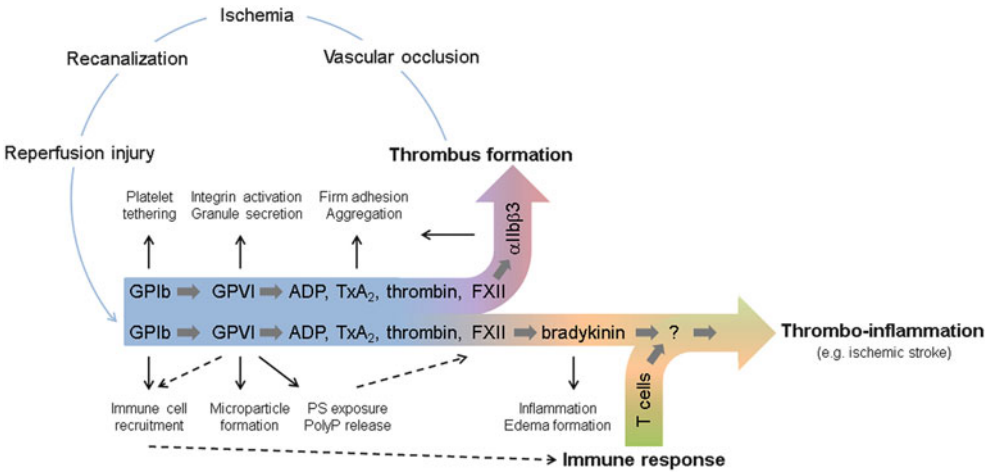

Fig. 3 Distinct but overlapping mechanisms drive thrombus formation and thrombo-inflammatory responses. GPIb mediates platelet tethering at the site of vascular injury, but also participates in immune cell recruitment during inflammatory responses. The central activating collagen receptor on the platelet surface, GPVI, supports platelet activation and triggers granule release which is essential for the secretion of ADP and TxA_2 that together with locally produced thrombin potentiate platelet activation. In parallel, strong cellular activation via GPVI culminates in the exposure of procoagulant phosphatidylserine (PS) and the release of inorganic polyphosphates (PolyP) which fuels coagulation and potently activates FXII, respectively. Additionally, GPVI signaling has been implicated in immune cell recruitment and inflammation via microparticle formation. Integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) is essential for stable thrombus formation which may lead to occlusion of diseased vessels and ischemia. Recanalization of a previously occluded vessel is often accompanied by reperfusion injury associated with damage to the affected tissue which triggers platelet recruitment to the site of injury. However, downstream signaling events may diverge from those leading to thrombus formation. Here, activation of FXII may also trigger formation of pro-inflammatory bradykinin which, in synergy with T-cells, contributes to a profound thrombo-inflammatory response, such as found in ischemic stroke. The mechanisms of T-cell recruitment and downstream effector molecules in this process are still elusive

significant numbers, develop ischemic infarcts, despite successful recanalization.

Detrimental Role of Platelets in Reperfusion Injury of the Brain

Platelet Tethering: GPIbα/VWF

To be able to adhere to the arterial wall under high shear flow conditions ($>$ ~500 s^{-1}), platelets must be slowed down. This process, named tethering, depends on GPIb-V-IX, a structurally unique receptor complex exclusively expressed in platelets and megakaryocytes (Berndt et al. 2001) (Fig. 3). Platelet GPIbα binds to the A1 domain of immobilized von Willebrand factor (VWF) exposed on the surface of the vessel wall (Savage et al. 1996). VWF can be released from Weibel-Palade bodies of endothelial cells or α-granules of platelets (Kanaji et al. 2012). Although sufficient to support platelet binding, this adhesive interaction is characterized by a rapid dissociation rate. Firm platelet adhesion depends on cellular activation which is induced by other receptors such as the collagen receptor GPVI which is enabled to bind its ligand once the platelet is decelerated and in contact with the injury site (Nieswandt and Watson 2003) (Fig. 3). The VWF-binding site on GPIbα can be blocked in mice by Fab fragments of the antibody p0p/B which abrogates platelet tethering and adhesion under high shear flow conditions (Massberg et al. 2003). Treatment of mice with anti-GPIbα Fab prevented infarct development in ischemic stroke when given preventively before or therapeutically shortly after tMCAO during reperfusion (Kleinschnitz et al. 2007). Perfusion- and diffusion-weighted imaging employing ultrahigh-field magnetic resonance imaging (MRI) revealed that anti-GPIbα treatment secured permanent reperfusion after removal of the vessel-occluding filament, while in untreated mice blood flow steadily decreased leading to cerebral infarctions (Pham et al. 2011). Although tail bleeding times were strongly elevated in anti-GPIbα Fab-treated mice, no increase in ICH was detected histologically and on MRI scans, which represents the main obstacle for anti-thrombotic therapy during the acute stroke phase in clinical practice (Zinkstok et al. 2014). Moreover, the therapeutic effects were durable for at least 1-week follow-up after tMCAO (Kleinschnitz et al. 2007). Recently, these findings could be confirmed in aged and comorbid mice (Kraft et al. 2015). Atherosclerotic $Ldlr^{-/-}$, streptozotocin-treated diabetic, as well as hypertensive mice were likewise protected against reperfusion injury upon treatment with anti-GPIbα Fab after tMCAO. This is important because it has often been criticized that experimental findings in healthy laboratory animals do not reflect the real world situation in which stroke patients display multiple vascular risk factors that could modify or diminish treatment responses.

Phospholipase D (PLD) isoforms become activated downstream of major platelet signaling pathways including GPIbα, and PLD1 has been shown to be required for the formation of stable thrombi. Platelets from *Pld1*$^{-/-}$ mice displayed impaired $\alpha_{IIb}\beta_3$ integrin activation in response to major agonists and defective GPIbα-dependent aggregate formation under high shear flow conditions (Elvers et al. 2010). *Pld1*$^{-/-}$ mice were protected from ischemic stroke in the tMCAO model, whereas the animals showed normal tail bleeding times and, importantly, no ICH after stroke induction (Elvers et al. 2010). Pharmacological inhibition of PLD was similarly effective, opening a new and safe antithrombotic strategy in reperfusion injury of the brain downstream of GPIbα (Stegner et al. 2013b).

The critical role of GPIbα/VWF interactions in stroke development after tMCAO could further be substantiated in *Vwf*$^{-/-}$ mice. Infarct volumes were reduced by 60 % in these mice compared to wild-type control (Kleinschnitz et al. 2009), and reconstitution of plasma VWF by hydrodynamic gene transfer restored the susceptibility of mutant mice to cerebral ischemia (De Meyer et al. 2010). Further experiments using bone-marrow chimeric mice revealed that not only endothelial-derived VWF but also VWF stored in α-granules of platelets contributes to infarct development after tMCAO (Verhenne et al. 2015). In support of a major role of VWF/GPIbα interactions in cerebral ischemia, increased serum VWF concentrations have been found in the acute phase in patients (Kraft et al. 2014), and, moreover, VWF levels could be established as an independent stroke risk factor (Bongers et al. 2006).

Ultra-large VWF released from endothelial Weibel-Palade bodies into the circulation is rapidly cleaved by the enzyme "a disintegrin-like and metalloproteinase with thrombospondin repeats-13" (ADAMTS13) to reduce its high thrombotic activity (Sadler 2008). Thereby surplus VWF remote from a vascular lesion is cleared to avoid uncontrolled clotting and to limit thrombus growth. After 30 min of tMCAO, mice usually develop small infarcts restricted to the basal ganglia in contrast to an occlusion time of 1 h which leads to a complete MCA infarct within 24 h (Braeuninger et al. 2012). Thus, 30 min of tMCAO is a stroke model suitable for testing the role of factors expected to accelerate infarct development. In further support of an important pathophysiological role of VWF in stroke development, *Adamts13*$^{-/-}$ mice showed a dramatic increase in infarct volumes when compared to wild-type mice after 30-min tMCAO (Zhao et al. 2009; Fujioka et al. 2010). Vice versa, infusion of recombinant human ADAMTS13 into wild-type mice immediately before reperfusion reduced infarct volumes and improved neurological outcome in the 1-h tMCAO model (Zhao et al. 2009). Thus, blocking of GPIbα/VWF interactions or enhancing VWF clearance by ADAMTS13 consistently mitigated reperfusion injury after tMCAO and, remarkably, was not associated with bleeding complications despite increased tail bleeding times in these mice.

Momi and colleagues took this approach one step further toward a clinical application (Momi et al. 2013). They induced a complete MCA occlusion in guinea pigs by intravascular photothrombosis and treated the animals immediately with nanobodies directed against the A1 domain of VWF named ALX-0081. Surprisingly, ALX-0081, when given within 15 min after tMCAO in guinea pigs, facilitated thrombus dissolution and rapid, almost complete reperfusion similar to the fibrinolytic agent rt-PA which served as an active control substance and is commonly used in acute stroke patients. Independent from its thrombolytic effect at the site of vessel occlusion, ALX-0081 treatment in addition improved microvascular patency in the ischemic hemisphere and neurological outcome (Momi et al. 2013). Accordingly, disruption of platelet cross-linking by GPIbα/VWF inhibitors disaggregated the external layer of occlusive thrombi and restored vessel patency even at a stage when thrombi were resistant to fibrinolysis or traditional antithrombotic drugs (Le Behot et al. 2014). Collectively these studies suggest that GPIbα/VWF inhibitors may be suitable to accelerate recanalization during thrombolysis or mechanical thrombectomy and further protect the microvasculature during reperfusion as shown by ultrahigh-field MRI (Pham et al. 2011).

Platelet Adhesion/Activation: GPVI

GPIbα/VWF binding facilitates tethering of platelets to the vessel wall, but does not allow firm adhesion which requires cellular activation. At sites of endothelial damage, this is mainly mediated by GPVI, the principal activating platelet collagen receptor (Nieswandt and Watson 2003; Stegner et al. 2014) (Fig. 3). Upon activation GPVI non-covalently associates with the Fc receptor (FcR)γ-chain, and this complex signal through tyrosine phosphorylation cascades downstream of the FcRγ-chain-associated immunoreceptor tyrosine-based activation motif (ITAM) (Berlanga et al. 2002). Platelets in which GPVI has been depleted by antibody treatment in vivo do not respond to collagen (Nieswandt et al. 2001; Nieswandt and Watson 2003). To elucidate a putative role of platelet-collagen interactions during reperfusion injury of the brain, GPVI was either depleted in platelets by the anti-GPVI antibody JAQ1 (Kleinschnitz et al. 2007) or binding of platelets to the vessel wall was blocked by Revacept, a recombinant soluble dimeric GPVI-Fc, which occupies subendothelial collagen-binding sites for the receptor (Goebel et al. 2013). Both treatments led to a significant reduction of microvascular thrombus formation and reduced infarct volumes after tMCAO (Kleinschnitz et al. 2007; Goebel et al. 2013). These findings indicate that firm platelet adhesion is an

important step in the pathophysiology of reperfusion injury in the brain. In further support of this notion, elevated plasma levels of soluble GPVI were detected in patients with acute thrombotic stroke, but not in patients with transient ischemic attacks in which vessel-occluding thrombi rapidly and spontaneously dissolve (Wurster et al. 2013). Blocking of GPVI function can also be achieved by targeting tyrosine kinases and adaptor proteins downstream of GPVI (Stegner et al. 2014). The spleen tyrosine kinase (Syk) is such an essential signaling mediator downstream of the ITAM receptor GPVI (Poole et al. 1997). Accordingly, *Syk*$^{-/-}$ mice were protected from ischemic stroke and pharmacological blockade of Syk likewise ameliorated infarct development and clinical outcome (van Eeuwijk et al. 2016).

Platelet Aggregation and Formation of Occlusive Thrombi

Following initial adhesion of platelets to the extracellular collagen matrix, extension of the thrombus requires amplification of the initial platelet response, further recruitment of circulating platelets, and platelet aggregation which is mediated by GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) (Shattil and Ginsberg 1997) (Fig. 3) and other secretory platelet components (Nurden 2011). Earlier studies already described that partial pharmacological blockade of $\alpha_{IIb}\beta_3$ receptors in the tMCAO model reduced platelet and fibrin accumulation in the microvasculature as well as infarct volumes after tMCAO (Choudhri et al. 1998; Abumiya et al. 2000; Ishikawa et al. 2004). It became apparent, however, that the therapeutic window of $\alpha_{IIb}\beta_3$ inhibition was very narrow since near complete inhibition of platelet aggregation regularly led to large ICH. We reassessed efficacy and safety of anti-$\alpha_{IIb}\beta_3$ treatment in ischemic stroke by using $(Fab)_2$ fragments of the mouse $\alpha_{IIb}\beta_3$-blocking mAb, JON/A (Bergmeier et al. 2002) in young (Kleinschnitz et al. 2007) and aged, comorbid mice (Kraft et al. 2015). JON/A-$F(ab)_2$ treatment had no significant effect on peripheral platelet counts, but completely inhibited ex vivo platelet aggregation in response to different stimuli resulting in dramatically prolonged tail bleeding times (Bergmeier et al. 2002). In confirmation of previous studies, most mice with a virtually complete receptor blockade died mainly due to ICH, but, in contrast, the few surviving animals exhibited comparable infarct volumes as controls (Kleinschnitz et al. 2007). Reduction of $\alpha_{IIb}\beta_3$ blockade to 80 % or 70 % decreased bleeding-related mortality, but infarct volumes and neurological outcomes were not different from vehicle-treated mice. Similar results have been obtained in a clinical stroke trial that was prematurely stopped due to a dramatic increase in ICH in the anti-$\alpha_{IIb}\beta_3$-treated group (Adams et al. 2008). In transgenic mice lacking the GPIIb (and thus GPIIb/IIIa), cerebral infarct size was reduced at 24 h after tMCAO (Massberg et al. 2005). The reasons for these discrepant results are currently unclear, but it is conceivable that pharmacological blockade of $\alpha_{IIb}\beta_3$ could have different effects on reperfusion injury in the brain than $\alpha_{IIb}\beta_3$ deficiency. Further studies are necessary to clarify whether $\alpha_{IIb}\beta_3$-mediated platelet aggregation as the final step of platelet activation is involved in reperfusion injury of the brain or dispensable as hypothesized recently by us (Stoll et al. 2010; Nieswandt et al. 2011) (see "Platelet-Immune Interactions in Reperfusion Injury of the Brain").

Thrombus stability not only depends on platelet aggregation via $\alpha_{IIb}\beta_3$ but also on secretory platelet products. Platelets contain α- and dense granules which are released following adhesion to collagen or other matrix components as well as in response to agonists such as ADP and thrombin (Nurden 2011). Alpha-granules contain more than 300 proteins which are required for the propagation and stabilization of platelet-rich thrombi, while dense granules contain small, nonprotein molecules such as calcium, serotonin, ADP, and ATP. *Nbeal2*$^{-/-}$ mice, which lack α-granules and thereby reproduce the gray platelet syndrome, exhibit reduced adhesion on collagen and impaired thrombus growth at high shear rates and could not form occlusive thrombi in vascular injury models (Deppermann et al. 2013). Munc13-4 is a limiting factor for platelet granules release (Ren et al. 2010). Mice carrying an inactivating point mutation are not able to release dense granules, and α-granule release is diminished (Ren et al. 2010). In support of a critical role of granule release in reperfusion injury in the brain, both *Nbeal2*$^{-/-}$ and Munc13-4 mice were protected in the tMCAO model (Deppermann et al. 2013; Stegner et al. 2013a). An important component of the dense granule releasate is inorganic polyphosphate (PolyP), which acts as a potent activator of coagulation factor XII (FXII), the starting point of the intrinsic pathway of coagulation as well as inflammatory pathways through the formation of bradykinin (Muller et al. 2009). FXII deficiency or blockade was shown to markedly reduce cerebral infarct growth after tMCAO (Kleinschnitz et al. 2006; Hagedorn et al. 2010), but it remained unclear whether this protective effect was primarily based on reduced pro-coagulatory or pro-inflammatory activity in the acutely ischemic brain, or both (Fig. 3).

Platelets Are Gatekeepers of Hemostasis in the Ischemic Brain

Hemorrhagic transformation (HT) is a frequent complication of acute ischemic stroke, in particular after thrombolysis (Jickling et al. 2014) (Fig. 4a). In contrast to other organ systems, the brain is escluded from the circulation by the blood-brain barrier (BBB) which controls and limits the access of fluids and cells. As an early consequence of

cerebral ischemia, the BBB breaks down (Engelhardt et al. 2014; Knowland et al. 2014), mainly due to activation of matrix metalloproteinases which degrade the basal lamina around brain vessels (Hamann et al. 1996). The rate of HT is higher after transient MCAO with reperfusion than after permanent MCAO and further increases when reperfusion is delayed (Lu et al. 2009). Thus, there is a dilemma when blocking platelet functions during reperfusion in the brain: prevention of reperfusion injury may increase the risk of HT or overt ICH? As described above blockade of $\alpha_{IIb}\beta_3$ is associated with an extremely high risk of ICH in experimental (Kleinschnitz et al. 2007; Kraft et al. 2015) as well as clinical stroke (Adams et al. 2008; Kellert et al. 2013) and, even after ASA treatment in conjunction with thrombolysis, additional protective effects were offset by an unacceptable increase in ICH (Zinkstok et al. 2014). Accordingly, baseline antiplatelet use is associated with an increased risk of post-rt-PA symptomatic ICH in acute stroke (Cucchiara et al. 2009). This also applies to the intact, non-ischemic brain: improved efficacy of platelet inhibition in cardio- or cerebrovascular disorders, e.g., arterial stenoses and stenting, is often neutralized by a higher incidence of ICH (Gachet 2015) (Fig. 4b). Recently, it was reported in patients that a subset of procoagulant platelets, so-called COAT platelets, were increased in non-lacunar strokes, and lower levels were associated with acute hemorrhagic complications (Prodan et al. 2015) indicating a putative role in stroke hemostasis. It is therefore important to understand how platelets act to prevent ICH especially in the stroke-injured brain.

The most simple question one could imagine is how many platelets are needed for hemostasis. We induced thrombocytopenia of variable degree in mice by injection of polyclonal anti-GPIbα antibodies which deplete circulating platelets in mice without involving immune effector mechanisms (Nieswandt et al. 2000). Mice with significantly reduced platelet counts down to a range of 99–25 platelets/nL were still able to arrest bleeding in the tail bleeding test, and only after reduction to <2.5 % of control, bleeding no longer stopped spontaneously (Morowski et al. 2013). When thrombocytopenic mice were challenged by tMCAO, full-blown MCA infarcts developed unless platelet counts were reduced <10 % of normal (Morowski et al. 2013). Importantly, no HT or ICH was observed in these stroke-protected mice despite severe thrombocytopenia. These findings indicate that significant thrombocytopenia does not protect from reperfusion injury in the brain and that a very low count of intact platelets is sufficient to maintain hemostasis similar to clinical observations in idiopathic thrombocytopenic purpura (Neunert et al. 2013). Goerge and coworkers further showed that the propensity of thrombocytopenic mice to bleed depends on the local tissue environment (Goerge et al. 2008). Thrombocytopenic mice did not bleed spontaneously, but major hemorrhages occurred in the skin and lung when an additional local inflammatory stimulus was set. As outlined in detail below, ischemic stroke exhibits a significant extrinsic inflammatory component (Stoll et al. 1998). In support of the concept that inflammation predisposes to HT, thrombocytopenic mice (<2.5 % of normal) undergoing tMCAO exhibited multiple hemorrhagic foci (Goerge et al. 2008). In the skin, the presence of platelets prevented bleeding during inflammation, and this protective effect was unexpectedly also seen in mice lacking functional GPIbα, VWF, or GPVI indicating that tethering and firm adhesion of platelets via classical adhesion receptors were not required for hemostasis. This is in accordance with studies in the tMCAO model, in which mice lacking GPIb, VWF, or GPVI were stroke protected, but did not develop ICH (Kleinschnitz et al. 2007, 2009). Thus, it appears that the mechanisms by which platelets contribute to the pathogenesis of ischemic brain injury are different from those required to maintain hemostasis following ischemia/reperfusion which raises the intriguing possibility that strong platelet inhibition can be achieved without dramatically increasing the risk of (spontaneous) intracranial

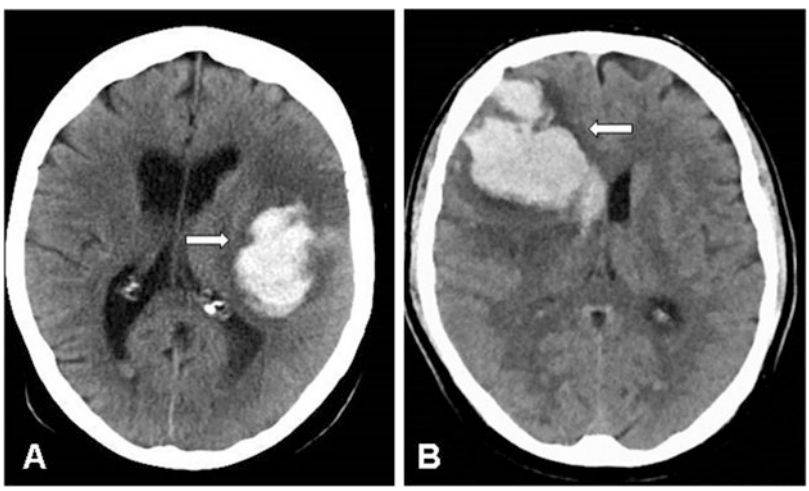

Fig. 4 The brain is vulnerable to intracranial hemorrhage (ICH). (**a**) CT scan of an ICH in the left hemisphere (*white* area marked by *arrow*) occurring spontaneously after thrombolysis using rt-PA. (**b**) Shows a frontally located ICH (*white* area marked by *arrow*) which occurred after arterial stenting under double platelet inhibition by ASA and ticagrelor

bleeding. Studies in the skin and lung moreover showed that platelets lacking G protein-coupled receptor signaling, thereby not responding to thrombin, ADP, and thromboxane A_2, were still protected against inflammation-induced bleedings, while blocking of the ITAM signaling pathway led to massive hemorrhages (Boulaftali et al. 2013). It is unclear whether this also applies to ischemic stroke. Interestingly, lack of Munc13-4 resulted in severely defective platelet aggregate formation (Savage et al. 2013), but mice did not develop increased ICH after tMCAO, in sharp contrast to mice with massive ICH in a parallel group in which $\alpha_{IIb}\beta_3$ was blocked (Stegner et al. 2013a). To improve stroke outcome and safety during recanalization procedures, it is mandatory to understand which platelet functions secure hemostasis during acute brain ischemia, a so far widely neglected field in experimental stroke research. At subacute and late stroke stages beyond 24 h, additional factors such as proteases, vascular remodeling (Jickling et al. 2014), and macrophage responses (Gliem et al. 2012) contribute to HT or prevent it, but at this stage the use of antiplatelet drugs is less critical and of proven value for secondary stroke prevention (Sandercock et al. 2014).

Platelet-Immune Interactions in Reperfusion Injury of the Brain

Brain ischemia has traditionally been regarded as a pure thrombotic disorder, but already more than 20 years ago, experimental and human studies revealed that immune cells infiltrate the brain after cerebral ischemia, and cytokines which orchestrate immune responses are upregulated within the ischemic brain parenchyma (Stoll et al. 1998; Lambertsen et al. 2012). The complex immune cell-brain interactions during the subacute and chronic phases of ischemic stroke have been reviewed elsewhere (Iadecola and Anrather 2011; Chamorro et al. 2012); here we focus on the role of T-cells and platelets during reperfusion early after cerebral ischemia (Fig. 3). In a seminal study, Yilmaz and colleagues showed that immune-deficient *Rag1*$^{-/-}$ mice which lack B- and T-cells are protected against cerebral ischemia in the tMCAO model (Yilmaz et al. 2006). These surprising data were soon confirmed thereafter (Kleinschnitz et al. 2010). Both studies revealed that adoptive transfer of T-cells into *Rag1*$^{-/-}$ mice restored susceptibility to ischemic brain damage, while B-cell transfer had no effect. A detailed immunological analysis showed that the detrimental T-cell effects in reperfusion injury of the brain largely were antigen independent and conferred by all major subclasses of T-cells, e.g., $CD4^+$, $CD8^+$, natural killer T-cells, etc. Surprisingly, also regulatory T-cells were deleterious (Kleinschnitz et al. 2013), and boosting of regulatory T-cells by CD28 agonist further increased ischemic brain damage (Schuhmann et al. 2015). FTY720 is an immunomodulator clinically approved for treatment of multiple sclerosis patients which rapidly reduces peripheral lymphocyte counts by blocking the egress of lymphocytes from lymphoid organs. FTY720, when given immediately before reperfusion in the tMCAO model, improved outcome (Kraft et al. 2013), a finding that could also be reproduced in a pilot trial of acute stroke patients who were treated by thrombolysis and in addition received FTY720 (Zhu et al. 2015). Thus, although it is too early for firm conclusions, it appears that T-cell responses are also critically involved in acute human stroke. The profound detrimental effect of T-cells is not unique for brain ischemia but rather represents a general pathophysiological phenomenon during organ reperfusion (Eltzschig and Eckle 2011). Importantly, thrombus formation itself was not altered in stroke-protected *Rag1*$^{-/-}$ mice as assessed in two standardized in vitro and in vivo thrombus formation models. Platelets from *Rag1*$^{-/-}$ mice normally adhered to collagen fibers and formed aggregates on collagen-coated surfaces in a whole-blood perfusion system under high shear conditions. In addition, vessel occlusion times were identical compared to immune-competent mice after $FeCl_3$-induced injury on mesenteric arterioles (Kleinschnitz et al. 2013). Taken together, these findings showed that brain damage after ischemia cannot merely be explained by a thrombotic event in the microcirculation. Taking into account the unequivocal contribution of T-cells to reperfusion injury, we coined the term "thrombo-inflammation" (Stoll et al. 2010; Nieswandt et al. 2011) (Fig. 3), a concept supported by the fact that detrimental T-cell effects are platelet dependent (Kleinschnitz et al. 2013). We could show that *Rag1*$^{-/-}$ mice were still protected from ischemic brain injury when platelets had been removed before adoptive transfer of detrimental T-cells (Kleinschnitz et al. 2013). Platelets can influence lymphocyte function and vice versa (Li 2008). In the liver $CD4^+$ T-cells have been shown to interact with platelets in postischemic sinusoids during reperfusion via platelet CD62P (P-selectin) (Khandoga et al. 2006). The molecular mechanisms underlying the concerted detrimental actions of T-cells and platelets in the brain are still elusive, but the encouraging results obtained by both experimental studies and a recent clinical pilot trial warrant further investigations to disclose the mystery of reperfusion injury and the paradoxically harmful restoration of blood flow in the brain.

Take-Home Messages

- Successful restoration of cerebral blood flow by thrombolysis and/or mechanical thrombectomy is the mainstay of acute stroke treatment, but recanalization alone does not guarantee a favorable outcome.

(continued)

- Reperfusion injury denotes the acute, paradoxically harmful aspect of blood flow return in the ischemic brain which involves platelet activation and immune cell recruitment.
- In experimental animals, blocking of platelet GPIbα or GPVI, as well as reducing circulating VWF, dramatically improved stroke outcome by protecting the microvasculature during reperfusion and further accelerated recanalization during thrombolysis without bleeding complications.
- Contrastingly, interfering with platelet aggregation by blocking $\alpha_{IIb}\beta_3$ led to devastating intracranial hemorrhages in acute experimental and human stroke.
- Before clinical application it is essential to further dissect pathological platelet functions and activation pathways involved in reperfusion injury from those indispensable as gatekeepers of hemostasis in the stroke-injured brain.
- The pathophysiology of reperfusion injury in acute stroke is even more complex and involves concerted detrimental actions of platelets and T-cells, referred to as "thrombo-inflammation," which awaits further elucidation.

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