Molecular mechanisms of thrombus formation in ischemic stroke:
Novel insights and targets for treatment

Short title: Thrombus formation in ischemic stroke

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Abstract

In ischemic stroke treatment options are limited. Therapeutic thrombolysis is restricted to the first few hours after stroke, and the utility of current platelet aggregation inhibitors including GPIIb/IIIa receptor antagonists, and anticoagulants is counterbalanced by the risk of intracerebral bleeding complications. Numerous attempts to establish neuroprotection in ischemic stroke have been unfruitful. Thus, there is strong demand for novel treatment strategies. Major advances have been made in understanding the molecular functions of platelet receptors such as glycoprotein (GP)Ib and GPVI and their downstream signaling pathways which allows interference with their function. Inhibition of these receptors in the mouse stroke model of transient middle cerebral artery occlusion prevented infarctions in vivo without increasing the risk of intracerebral bleeding. Similarly, it is now clear that the intrinsic coagulation factors (F)XII and FXI play a functional role in thrombus formation and stabilization during stroke: their deficiency or blockade protects from cerebral ischemia without overtly affecting hemostasis. Based on the accumulating evidence that thrombus formation and hemostasis are not inevitably linked, new concepts for prevention and treatment of ischemic stroke may eventually emerge without the hazard of severe bleeding complications. This review discusses recent advances related to antithrombotic strategies in experimental stroke research.
**Introduction**

Stroke is the second leading cause of death worldwide \(^1\)\(^,\)\(^2\). About 80% of strokes are caused by focal cerebral ischemia due to arterial occlusion, while up to 20% are caused by intracerebral hemorrhages \(^3\)\(^,\)\(^4\). Extracranial artery stenoses are prone to destabilization and plaque rupture leading to cerebral thromboembolism \(^5\). In about one third of ischemic stroke patients embolism to the brain originates from the heart, especially in atrial fibrillation \(^6\). Thrombembolic occlusion of major or multiple smaller intracerebral arteries leads to focal impairment of the downstream blood flow, and to secondary thrombus formation within the cerebral microvasculature.

In the center of the ischemic territory oxygen and glucose deprivation, neuronal depolarization, and Ca\(^{2+}\)-mediated excitotoxicity induce necrotic and apoptotic cell death. In the penumbra region surrounding the infarct core, however, tissue is preserved for a certain time span depending on whether or not blood flow is restored \(^7\). Since numerous agents that proved neuroprotective in experimental stroke failed in subsequent clinical trials \(^8\), the only effective treatment option in acute ischemic stroke remains immediate thrombolysis. In this review we will focus on the initiating event of stroke development, namely intravascular thrombus formation, and highlight promising novel molecular targets for its prevention and treatment.

**Current treatment options in ischemic stroke**

**Thrombolytic therapy**

In acute thrombembolic stroke the principal treatment goal is to rapidly achieve recanalization of occluded intracerebral vessels. In case of a permanent vessel occlusion a complete infarct will inevitably develop. At present, early intravenous or intraarterial thrombolysis are the only established therapeutic options \(^9\)\(^,\)\(^10\). Less than 10% of patients are amenable to this treatment due to the limited time window of up
to 3-6 hours after symptoms onset because of the risk of severe intracerebral hemorrhage with later application. A trial to extend the therapeutic window up to 9 hrs by use of recombinant desmoteplase, a novel plasminogen activator, failed. For unknown reasons, thrombolytic treatment leads to the dissolution of the vessel occluding clots in some cases, but not in others. Moreover, secondary arterial re-occlusion may follow a previously successful re-canalization. Most importantly, patients may develop progressive stroke despite sustained early reperfusion of previously occluded major intracranial arteries, a process referred to as “reperfusion injury”. These observations suggest that reperfusion of occluded major arterial branches is a prerequisite for salvage of tissue, but does not inevitably guarantee prevention of infarct growth and clinical recovery.

**Platelet inhibitors**

There have been numerous attempts to improve stroke outcome by use of platelet aggregation inhibitors and anticoagulants. The antithrombotic effect of acetylsalicylic acid (ASA) is based on the irreversible inhibition of platelet cyclooxygenases 1 and 2 leading to reduced prostaglandin and thromboxane A₂ synthesis. ASA has been evaluated within 48 hours of stroke onset in two large trials. There was a moderate, but statistically significant benefit on stroke outcome. It was assumed, yet not based on solid data, that the primary effect of ASA might be due to prevention of early stroke recurrence rather than limiting the neurological consequences of the initial stroke per se. Although the beneficial role of platelet aggregation inhibitors including ASA, ASA in combination with dipyridamole, and the platelet P2Y12 receptor inhibitor clopidogrel in stroke prevention is well established, the multifaceted role of platelets in acute stroke development is unclear. This limited
understanding extends to the mechanisms by which anti-platelet agents may act in preventing thrombus growth within the brain microvasculature\textsuperscript{16,19}.

The formation of a thrombus requires functional glycoprotein (GP) IIb/IIIa, a heterodimeric receptor of the integrin family expressed at high density (50,000-80,000 copies/cell) on the platelet membrane\textsuperscript{20}. In resting platelets, GPIIb/IIIa exists in a low-affinity state and does not bind its ligands. During platelet activation, intracellular signals are generated that are integrated at defined checkpoints such as CalDAG-GEFI\textsuperscript{21} and culminate in the activation of talin-1\textsuperscript{22,23} and kindlin-3\textsuperscript{24}. These bind to the intracellular tails of the integrin β3-subunit (GPIIIa) and induce a conformational change that involves both subunits of the complex. This "final common pathway" of platelet activation results in the exposure of the binding site(s) for a variety of ligands, most notably fibrinogen, von Willebrand factor (vWF) and fibronectin (inside-out signaling) which allows firm adhesion to the extracellular matrix and aggregation. Current strategies to inhibit GPIIb/IIIa include antibodies (abciximab), cyclic peptides adapted from a snake venom disintegrin (eptifibatide) and nonpeptide analogues of an RGD peptide (tirofiban and lamifiban) all of which directly inhibit ligand binding. While the utility of intravenous GPIIb/IIIa inhibitors in acute coronary syndromes is well established\textsuperscript{25}, a recent phase III trial applying abciximab in acute ischemic stroke was prematurely stopped due to an increased intracranial hemorrhage rate and mortality, as well as lack of efficacy\textsuperscript{26}. A number of studies also evaluated GPIIb/IIIa inhibitors in conjunction with thrombolytic therapy and described some benefit\textsuperscript{27-29}. Based on the available data, the use of GPIIb/IIIa inhibitors in acute stroke patients cannot be recommended at present\textsuperscript{26,30}. 
Anticoagulants

Anticoagulation with warfarin targets the synthesis of coagulation factors II, VII, IX and X and is effective in primary and secondary prophylaxis of thromboembolism to the brain in patients with atrial fibrillation. Although other anticoagulants, namely unfractionated heparin, low-molecular-weight heparin, or heparinoids which block FXa activity have frequently been used for acute stroke therapy within 48 hours, several randomised studies have been negative. A most recent trial did not find a significant advantage of low-molecular-weight heparin over ASA. The few studies that addressed the potential benefit of anticoagulation immediately within the first hours after cerebral ischemia gave inconsistent results regarding clinical outcome and stroke recurrence but mostly found a significant increase in intracranial bleeding. Consequently, the recently updated American Heart Association (AHA) guidelines state that “urgent anticoagulation with the goal of preventing early recurrent stroke, halting neurological worsening, or improving outcomes is not recommended.” Since anticoagulation carries a significant risk for intracerebral bleedings in the setting of acute stroke, it remains a major challenge to develop novel anticoagulants and/or antiplatelet agents with a more favourable safety profile and better efficacy.

Studies in experimental stroke

Animal models for focal ischemic stroke

For the study of ischemic stroke several animal models have been developed. Most frequently, occlusion of major extra- and intracranial arteries is applied in rodents or higher mammals leading to focal cerebral ischemia. Permanent occlusion of the middle cerebral artery (MCAO) at proximal sites, either by a suture or by an intraluminal thread causes complete infarctions of the middle cerebral artery brain...
territory involving neocortex and basal ganglia. The clinical situation with vessel occlusion followed by resolution of clots and reperfusion can be mimicked in the MCAO paradigm by withdrawing of the intraluminal thread (so-called “transient” MCAO model) (Fig. 1). It has been firmly established that final infarct size depends on the prior occlusion time: Ischemic periods below 30 minutes will lead to infarctions of the caudate and putamen (basal ganglia) and only partly affect the neocortex because ischemic cortical tissue is salvaged by collateral blood supply and reperfusion. If reperfusion is further delayed, however, the size of neocortical infarctions will increase since the surrounding penumbra is subsequently involved in the definite infarct area (Fig. 1). One of the enigmas holds that sufficient reflow does not guarantee salvage of brain tissue. The observation that reperfusion of major intracerebral arteries did not completely prevent further infarct growth led to the concept of a focal “no-reflow” within the brain microvasculature. It was shown that thrombus formation continues with accumulation of platelets and fibrin deposition despite removal of the vessel occluding thread. These data indicate that ongoing thrombus formation within the brain during reperfusion is an important pathophysiological step in stroke development which acts in concert with activation of endothelial cells and adhesion of leukocytes to the vessel wall.

In contrast to focal cerebral ischemia, global ischemia refers to an interruption of the entire brain circulation typically seen during cardiac arrest. If transient, global ischemia causes delayed and selective neuronal death in hypoxia-susceptible brain areas without widespread necrosis. The underlying pathologic mechanisms are quite different between global ischemia and MCAO induced focal ischemia and therefore global ischemia is not further considered in this review. Cerebral photothrombosis has often been used as an alternative model to induce focal cerebral lesions, but recent studies have shown that the development of brain lesions after
photothrombosis does not require intravascular thrombus formation. Recently, a promising novel mouse model of thromboembolic stroke was reported based on microinjection of murine thrombin, but, as with older similar clot models in mice, no data on the effect of antiplatelet treatment or anticoagulation are available yet. Moreover, embolic models are limited by variable infarct sizes since it is difficult to anticipate which branch of the middle cerebral artery will finally be occluded by the inserted clot. All experimental studies included in this review were based on the transient MCAO (tMCAO) model in mice which is the most widely used. Here, brain infarctions are initiated by mechanical, but not thromboembolic occlusion of a major cerebral artery. Although a single model can only cover some aspects of human stroke which is a complex and heterogenous disease, the tMCAO model turned out to be useful in elucidating basic pathomechanisms of thrombus formation in the downstream microvasculature.

**The role of platelets in experimental stroke**

Pathological platelet activity and platelet receptor-ligand interactions have been linked to cerebral ischemic events. By use of ¹¹¹In-labeled platelets in a primate model of tMCAO, the group of Del Zoppo showed that platelets are deposited in the ischemic basal ganglia early during reperfusion and electron microscopic examination demonstrated aggregates of degranulated platelets together with fibrin and leukocytes. Accordingly, baboons treated with ticlopidine and heparin displayed a significant reduction in platelet deposition and microvascular occlusions in the ischemic basal ganglia. The advent of genetic methods that allow targeted manipulations in the mouse genome has paved the way for novel concepts of thrombus formation in mice that may help to identify important steps in the pathogenesis of human atherothrombosis and ischemic stroke. In the following, we
will summarize the recent experimental evidence in support of a pathophysiological role of platelet receptors GPIIb/IIla, GPIb, and GPVI (Fig. 2), and the involvement of the intrinsic coagulation cascade in focal cerebral ischemia. Novel drugs targeting these molecules may help to overcome the current limitations and hazards of conventional anticoagulation and platelet inhibition in acute ischemic stroke.

**Glycoprotein IIb/IIla (integrin αIIbβ3).** In a seminal paper, Choudhri and colleagues used pharmacological blockade of GPIIb/IIla in the tMCAO model as proof of concept that even if large arteries are recanalized after thromboembolic stroke, microvascular thrombosis continues to occur at distal sites. When the GPIIb/IIla antagonist GPI 562 was administered to mice immediately before or after 1h of MCAO, platelet and fibrin accumulation as well as cerebral infarct volumes were reduced. This effect was dose-dependent with an associated significant increase in the rate of intracerebral hemorrhages. Treatment of mice with an anti-GPIIb/IIla antibody similarly led to reduced endothelial adhesion of leukocytes and platelets during reperfusion after tMCAO. In baboons a 30% inhibition of platelet aggregation by the GPIIb/IIla antagonist TP9201 was sufficient to regain complete microvascular patency after 3h of tMCAO, while near complete inhibition of platelet aggregation again led to large intracerebral hemorrhages. In a recent study, we reassessed efficacy and safety of anti-GPIIb/IIla treatment in ischemic stroke by using (Fab)₂ fragments of the mouse GPIIb/IIla-blocking mAb, JON/A, which completely inhibits ex vivo platelet aggregation and induces prolonged tail bleeding times. Most animals which had received 100 µg anti-GPIIb/IIla F(ab)₂ leading to a virtually complete receptor blockade died due to intracerebral hemorrhage, and the few surviving animals exhibited infarct volumes of the same extension as seen in controls (Fig. 3). A 78% and 68% receptor blockade, improved survival rates, but
failed to influence infarct volumes or neurological outcome. By contrast, in GPIIb-deficient mice cerebral infarct size was reduced at 24h after tMCAO, but no information on bleeding complications is available from this study. Excessive GPIIb/IIIa blockade appears to be inevitably associated with major bleeding complications in mice and reflects similar findings in stroke patients. Thus rather than blocking this final common pathway of platelet activation, their aggregation, targeting platelet adhesion and/or early signaling events may provide a promising therapeutic alternative.

**Glycoprotein Ib-V-IX.** The initial tethering of platelets at sites of vascular injury is mediated by GPIb-V-IX, a structurally unique receptor complex exclusively expressed in platelets and megakaryocytes (Fig. 2). In humans, lack or dysfunction of this receptor has been associated with the Bernard-Soulier syndrome, a congenital bleeding disorder characterized by mild thrombocytopenia, giant platelets, a platelet inability to adhere to subendothelial matrices and a dramatically prolonged bleeding time. This phenotype has been reproduced in mice lacking functional GPIb-V-IX. The binding of GPIbα to the A1 domain of vWF is the principal interaction capable of and necessary for tethering platelets to the vessel wall at high shear flow conditions (> ~500 s⁻¹), whereas this interaction may not be relevant at lower shear rates. Although sufficient to support platelet binding, this adhesive interaction is characterized by a rapid dissociation rate and thus cannot mediate irreversible adhesion by itself. Rather, the interaction keeps platelets in close contact with the matrix, while the cells continuously translocate in the direction of blood flow. The specific requirement for GPIbα for platelet adhesion under conditions of high shear, such as found in diseased arteries, makes this receptor a potentially attractive target for pharmacological inhibition of pathological thrombus formation. Inhibition of the
vWF-binding site on GPIbα with Fab fragments of the antibody p0p/B in wild-type mice abrogated platelet tethering and adhesion in a model of mechanically induced arterial thrombosis. Such mice have prolonged tail bleeding times but do not show signs of spontaneous hemorrhage. The central role of GPIbα in arterial thrombus formation was later confirmed and extended in a study showing that transgenic mice expressing GPIbα in which the extracellular domain was replaced by that of the human interleukin-4 receptor (GPIb-TG) are completely unable to produce intravascular thrombi.

A crucial role of GPIb in stroke development has recently been elucidated in experimental focal cerebral ischemia. Complete blockade of the vWF binding site of GPIbα by i.v. injection of 100 µg Fab fragments of p0p/B into mice before tMCAO led to a reduction of stroke volumes of about 60%. Importantly, delayed application of anti-GPIb Fab 1h after MCAO was likewise effective (Fig. 3). Although tail bleeding times were strongly elevated in anti-GPIb Fab treated mice, no increase in intracerebral hemorrhages was detected. Together, this indicated that GPIbα is critically involved in the pathogenesis of ischemic stroke, but not required to prevent bleeding at sites of ischemia/reperfusion damage in the brain and supported the previous notion that there is no clear correlation between bleeding time and bleeding risk. This surprising result was shortly after confirmed by Goerge and coworkers who found that local inflammation in the brain (induced by tMCAO) and other tissues triggers bleeding in the absence of platelets. In the presence of platelets, bleeding was prevented and this protective effect was unexpectedly also seen in mice lacking functional GPIbα. Thus, it appears that the mechanisms by which platelets contribute to the pathogenesis of ischemic brain injury are different from those required to maintain vascular integrity following ischemia/reperfusion in this organ. These observations not only demonstrate that GPIb is a central player in murine
experimental stroke but also raise the intriguing possibility that strong platelet inhibition can be achieved without significantly increasing the risk of (spontaneous) intracerebral bleeding.

Besides its principal ligand vWF, GPIbα also binds thrombin, high molecular weight kininogen, Factor XII, Mac-1, a β2 integrin expressed in neutrophils and monocytes (CD11b/CD18), and P-selectin \(^{70}\). The corresponding binding sites are located in the N-terminal region of the receptor but their precise local arrangement is unknown. It is not entirely clear at present, which of the GPIbα interactions are critical for thrombus formation in stroke. However, allelic variants of platelet GPIbα causing enhanced vWF/GPIb interactions are associated with an increased risk of ischemic stroke \(^{71}\) and increased serum levels of vWF have been recognized as an independent stroke risk factor \(^{72}\). In support of this, we found in a most recent investigation that vWF deficient mice are protected against cerebral ischemia, although to a lesser extent than mice treated with anti-GPIbα antibodies (Kleinschnitz, Deckmyn, Nieswandt, Stoll, unpublished). This indicates that different ligands of GPIb may be involved in the development of infarcts in this model.

Previous studies have shown that mice deficient in Mac-1 are less susceptible to cerebral ischemia/reperfusion injury \(^{73}\). This protection was associated with reduced neutrophil infiltration after tMCAO, but the exact contribution of Mac-1 to the pathology is unclear. Therefore, it is tempting to speculate that Mac-1-GPIb interactions could mediate platelet-leukocyte adhesion promoting inflammation at sites of thrombosis after cerebral ischemia. Moreover, GPIbα binds to P-selectin \(^{74}\). During cerebral ischemia, increased surface P-selectin expression was noted on endothelial cells and platelets as early as 1 hour after reperfusion and inhibition of P-selectin improved stroke outcome indicating that this interaction is also functionally
relevant^{75}. Taken together, GPIbα plays a central role as a receptor mediating complex platelet-platelet, platelet-endothelium, and platelet-leukocyte interactions all of which may be critical in secondary infarct growth after tMCAO in rodents. Fab fragments of the monoclonal antibody 6B4, raised against human GPIbα, exhibited a powerful antithrombotic effect in baboons by blocking the GPIbα binding site for vWF without significant prolongation of the skin bleeding time^{76}. This antibody was recently humanized by variable-domain resurfacing guided by computer modeling^{77} and may provide an important tool to study the role of GPIbα in human thrombotic diseases, including stroke.

**Glycoprotein VI.** Although GPIb-vWF interactions can elicit intracellular signals^{78} these are generally considered very weak compared to other stimuli, most notably subendothelial collagens, which are exposed to the cells at sites of endothelial damage. Among the numerous collagen receptors expressed in platelets, GPVI is of central importance for cellular activation and subsequent firm arrest^{79}. GPVI, a 62 kDa type I transmembrane receptor of the Ig superfamily^{80}, is exclusively expressed in platelets and megakaryocytes^{81}. It non-covalently associates with the Fc receptor (FcR)γ-chain and the complex signals through tyrosine phosphorylation cascades leading to calcium mobilization, degranulation, activation of GPIIb/IIIa and aggregation^{79}. Platelets in which GPVI has been depleted by *in vivo* administration of antibodies against the receptor do not respond to collagen^{79,81}. Several reports have demonstrated a profound antithrombotic effect of such GPVI inhibition after arterial wall injury and collagen-induced thrombembolism^{81-83} which is associated with a very moderate increase in tail bleeding^{81}. In tMCAO, treatment of mice with the anti-GPVI antibody JAQ1 significantly reduced the brain infarct volumes at day 1 after tMCAO^{57} but did not increase the incidence of intracerebral hemorrhages. This
indicates that platelet/collagen interactions via GPVI may also be involved in stroke development in this model. However, GPVI depletion was less effective than GPIb blockade and did not affect clinical outcome variables suggesting that other platelet agonists contribute to thrombus formation in the tMCAO model. Among these, thrombin is the most powerful initiator of platelet adhesion and thrombus formation sufficient to drive these processes independently of collagen under certain experimental conditions 84.

The observation that inhibitors of GPIb or GPVI function provide significant protection from ischemic brain injury suggests that signaling pathways downstream of these receptors could be promising therapeutic targets. This has recently been confirmed by the analysis of mice lacking stromal interaction molecule 1 (STIM1) a key regulator of agonist-induced Ca\(^{2+}\) entry in immune cells and platelets 85,86. Platelets derived from these mice display selective defects in cellular activation downstream of GPVI and shear-resistant adhesion and thrombus formation in vitro and in vivo 87. In contrast, activation in response to G protein-coupled agonists, such as thrombin, is largely preserved, which indicates that STIM1-mediated signaling events are particularly important for the GPIb-GPVI-ITAM pathway in platelets. Mice with STIM1-deficient platelets showed profound reduction in secondary infarct growth following tMCAO, but no increase in intracerebral hemorrhages 87.

The intrinsic coagulation pathway as a novel target for stroke prevention

**The role of coagulation factor XI (FXI) and XII in thrombus stability and hemostasis.** Hemostasis and pathological thrombus formation occluding coronary or cerebral arteries in myocardial infarction and stroke, respectively, have long been considered to share identical molecular pathways. According to this concept,
treatment of thrombosis would only be possible at the expense of impaired hemostasis. However, this dogma has recently been challenged\textsuperscript{55,56,88}. Two distinct pathways for initiating plasmatic coagulation exist, triggered either by vessel wall (extrinsic) or blood-borne (intrinsic) factors and converge on a common pathway leading to thrombin and fibrin formation. The extrinsic pathway is initiated by exposure of subendothelial tissue factor upon vessel injury (Fig. 2). Tissue factor activates the plasma protease factor VIIa. The intrinsic pathway of coagulation is initiated when coagulation factor XII (FXII, Hageman factor) comes into contact with negatively charged surfaces (contact activation). Because individuals with hereditary FXII deficiency do not show an abnormal bleeding phenotype, FXII has been considered dispensable for proper hemostasis\textsuperscript{89}. Recent investigations, however, revealed a role of FXII in pathological thrombus formation and stability\textsuperscript{88}. FXII-deficient mice, similar to FXII-deficient patients, exhibit a prolonged activated partial thromboplastin time, but no bleeding tendency. Importantly, FXII-deficient mice showed impaired formation and stabilization of thrombi in different models of arterial thrombosis\textsuperscript{88}. These unexpected findings indicate that it is possible to target thrombus formation without ensuing bleeding complications. The molecular mechanisms that activate FXII during pathological thrombus formation in vivo await elucidation. One possible mechanism has recently been proposed by Kannemeier and coworkers who demonstrated that extracellular RNA, but not DNA, augments (auto-)activation of FXII and FXI and thereby acts as a potent trigger of coagulation in vivo\textsuperscript{90}. It is at present not clear whether this pathway is the predominant mechanism of FXII activation or whether other, presumably polyanionic, molecules also contribute to this process.
The significance of the intrinsic coagulation system for stroke development. FXII-deficient mice are also protected from cerebral ischemia. After tMCAO, infarct volumes were less than 50% in FXII-deficient compared to wildtype mice at 24h and FXII mutants developed significantly less neurological impairment. Follow-up MRI on day 3 and 7 after tMCAO revealed that this protective effect was sustained over time. Infarcts in FXII-deficient mice were restricted to the basal ganglia and fibrin deposition in the microvasculature of cortical vessels was markedly reduced. Administration of the FXIIa inhibitor D-Pro-Phe-Arg-chloromethyl ketone (PCK) to wildtype mice similarly conferred protection from stroke. Because FXI-deficient mice were similarly protected from experimental stroke as FXII-deficient mice the intrinsic coagulation pathway appears to be critically involved in infarct development. A recent epidemiological study disclosed protection against cerebrovascular events in Jewish patients with severe congenital FXI deficiency indicating that our experimental results in mice may be relevant for the human situation. Of note, FXII knockout mice and wildtype mice treated with FXII inhibitors had a prolonged activated partial thromboplastin time, but did not display excessive bleeding during surgery. Moreover, serial MRI using blood-sensitive sequences did not show an increased frequency of intracerebral hemorrhages. Importantly, pharmacological blockade of factor IX downstream of factor XII and XI was similarly protective after tMCAO. However, factor IX deficiency reflects human hemophilia type B which is associated with a significant bleeding phenotype. This can be explained by the fact that factor IX is also activated by the factor VIIa-tissue factor complex and thereby participates in the extrinsic pathway of coagulation. Intracerebral hemorrhage is the most feared complication of coumarins, the conventional anticoagulant today. It appears that FXII inhibition could be a novel target for safer anticoagulation and stroke prevention since FXII is an essential...
component of pathological thrombus formation, but not of physiological hemostasis.

**Perspectives for future stroke prevention and treatment**

Basic molecular biology has uncovered the functions of a number of platelet receptors such as GPIb and GPVI and downstream signaling pathways in thrombus formation. The fact that inhibition of these platelet receptors/signaling pathways is not associated with increased intracerebral bleeding following tMCAO may open up new avenues for stroke treatment in the future. Novel therapeutic approaches are eagerly awaited in view of the recent negative experience with GPIIb/IIIa receptor inhibition 26, and the limited access of stroke patients to thrombolytic treatment. Therefore, it is very promising that antibodies against human GPIb effectively prevented thrombus formation after peripheral vessel injury in baboons 76 and that humanized F(ab)’ fragments against GPIbα have been generated by molecular engineering 77. Before these can be clinically tested in stroke patients they await proof of safety and efficacy in a translational primate model. This is important to rule out species-specific effects restricted to rodents as seen in many other potential therapeutics in the past 8, 96.

Besides the prevention and treatment of cerebral ischemia resulting from spontaneous thromboembolism, iatrogenic stroke might become another area of application for the novel antithrombotics described. Frequently performed routine procedures such as angiography, angioplasty or heart surgery are accompanied by a significant incidence of (clinically silent) cerebral thrombemboli which above a certain threshold can cause severe cognitive dysfunction or even large brain infarction 97. Now, the role of the intrinsic pathway of coagulation in pathological thrombus formation has been unravelled 56,88 and may provide novel options for anticoagulation during these potentially pro-embolic interventions 95. In balancing risk and benefit of
any new stroke prophylaxis or treatment, the reduction of the bleeding risk is as essential as preventing thrombus formation and improving reperfusion. Highly selective FXII inhibitors are currently developed for the application in humans. They may help to control and limit thromboembolic complications with a better safety profile than coumarins or heparins in which the therapeutic benefit has often been neutralized by excess bleeding complications.

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Author Contributions:

G.S., C.K., B.N. wrote the paper.

The authors declare no conflict of interest
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Legends to figures

Figure 1

Transient middle cerebral artery occlusion model (tMCAO) in the mouse:
A,B represent three coronal sections through the brain in individual animals. (A) shows a cerebral infarct at 24h after 30 min of tMCAO and (B,C) after 1h of tMCAO as revealed on tissue sections stained for 2,3,4-Triphenyltetrazoliumchloride (TTC), a mitochondrial marker. Red areas represent vital brain tissue, white areas indicate cerebral infarctions. With short occlusion times of 30 min infarcts are restricted to the basal ganglia (arrow in A) while prolonged occlusion leads to infarction of the entire MCA territory (B). Infarct development can also be assessed in vivo by magnetic resonance imaging (MRI) as shown in D. Infarcts appear white on T2-w or diffusion-weighed MRI (D) and closely correspond to the extent of infarction seen on tissue sections (C).

Figure 2

Model of platelet-vessel wall interaction.
(A) The initial contact (tethering) of platelets to the extracellular matrix (ECM) is mediated predominantly by GPIbα-vWF interactions. The GPIbα-vWF interaction is essential at high shear rates (> 500 s⁻¹). GPIbα may also interact with P-selectin exposed on activated endothelial cells and thereby contribute to platelet recruitment to the intact vessel wall. (B) At sites of vascular injury, GPVI-collagen interactions initiate cellular activation followed by shifting of integrins to high affinity state and the release of secondarily acting agonists, most importantly ADP, ATP and TxA₂. GPIb-mediated signaling may amplify GPVI-induced activation pathways. In parallel, exposed tissue factor (TF) locally triggers the formation of thrombin (extrinsic
pathway), which in addition to GPVI mediates cellular activation. On the growing thrombus, activation of FXII and FXI also leads to thrombin formation. (C) Activated GPIIb/IIIa (integrin αIIbβ3) together with β1 integrins (not shown) mediates firm adhesion by binding to vWF, fibronectin and other ligands. Released ADP, ATP and TxA2 amplify integrin activation on adherent platelets and mediate thrombus growth by activating additional platelets and fibrinogen binding to GPIIb/IIIa. (D) Adherent platelets may recruit leukocytes to the thrombus through GPIbα-MAC1 interactions. This scheme does not exclude the involvement of other receptor-ligand interactions.

Figure 3
The influence of platelet glycoprotein receptor blockade on stroke outcome:
(A) shows coronal 2,3,4-Triphenyltetrazoliumchloride stained sections at 24h after 1h of tMCAO in a sham-treated mouse. Note the large infarction of the entire middle cerebral artery territory in (A), and the corresponding T2-w magnetic resonance image of the infarct at the bottom. Blockade with anti-GPIb Fab significantly reduced infarct size in (B). The arrow points to the small infarct within the basal ganglia, while the cerebral cortex is protected. The corresponding magnetic resonance image correctly depicts decreased infarct size (arrow at bottom panel). Surprisingly, blockade of GPIIb/IIIa had no influence on the infarct size in surviving animals (C), and was associated with lethal intracerebral hemorrhage (ICH) in many animals (not shown). Importantly, no areas with signal loss indicating bleeding complications were seen in mice after GPIb-Fab blockade (see bottom panel B; compare to ICH in fig. 2C).
Figure 1: Images showing brain tissue at different time points and conditions.

- **A** and **B**: Images of brain tissue at 30 min and 60 min, respectively, showing the effects of transient MCAO.
- **C**: Close-up view of brain tissue at 60 min, highlighting the area affected by MCAO.
- **D**: MRI scan showing brain tissue at 60 min, indicating the extent of the injury.

**30 min**

**60 min transient MCAO**
Molecular mechanisms of thrombus formation in ischemic stroke: novel insights and targets for treatment

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