

# Targeting Platelets in Acute Experimental Stroke

## Impact of Glycoprotein Ib, VI, and IIb/IIIa Blockade on Infarct Size, Functional Outcome, and Intracranial Bleeding

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**Background**—Ischemic stroke is a frequent and serious disease with limited treatment options. Platelets can adhere to hypoxic cerebral endothelial cells by binding of their glycoprotein (GP) Ib receptor to von Willebrand factor. Exposure of subendothelial matrix proteins further facilitates firm attachment of platelets to the vessel wall by binding of collagen to their GPVI receptor. In the present study, we addressed the pathogenic role of GPIb, GPVI, and the aggregation receptor GPIIb/IIIa in experimental stroke in mice.

**Methods and Results**—Complete blockade of GPIIb/IIIa was achieved by intravenous injection of 100  $\mu$ g Fab fragments of the monoclonal antibody p0p/B to mice undergoing 1 hour of transient middle cerebral artery occlusion. At 24 hours after transient middle cerebral artery occlusion, cerebral infarct volumes were assessed by 2,3,5-triphenyltetrazolium chloride staining. In mice treated with anti-GPIIb/IIIa Fab 1 hour before middle cerebral artery occlusion, ischemic lesions were reduced to  $\approx$ 40% compared with controls ( $28.5 \pm 12.7$  versus  $73.9 \pm 17.4$  mm<sup>3</sup>, respectively;  $P < 0.001$ ). Application of anti-GPIIb/IIIa Fab 1 hour after middle cerebral artery occlusion likewise reduced brain infarct volumes ( $24.5 \pm 7.7$  mm<sup>3</sup>;  $P < 0.001$ ) and improved the neurological status. Similarly, depletion of GPVI significantly diminished the infarct volume but to a lesser extent ( $49.4 \pm 19.1$  mm<sup>3</sup>;  $P < 0.05$ ). Importantly, the disruption of early steps of platelet activation was not accompanied by an increase in bleeding complications as revealed by serial magnetic resonance imaging. In contrast, blockade of the final common pathway of platelet aggregation with anti-GPIIb/IIIa F(ab)<sub>2</sub> fragments had no positive effect on stroke size and functional outcome but increased the incidence of intracerebral hemorrhage and mortality after transient middle cerebral artery occlusion in a dose-dependent manner.

**Conclusions**—Our data indicate that the selective blockade of key signaling pathways of platelet adhesion and aggregation has a different impact on stroke outcome and bleeding complications. Inhibition of early steps of platelet adhesion to the ischemic endothelium and the subendothelial matrix may offer a novel and safe treatment strategy in acute stroke. (*Circulation*. 2007;115:2323-2330.)

**Key Words:** cerebrovascular disorders ■ drug therapy ■ glycoproteins ■ intracerebral hemorrhage ■ magnetic resonance imaging ■ platelets ■ stroke

Ischemic stroke is the third-leading cause of death and permanent disability in industrialized countries.<sup>1</sup> Although the beneficial role of anticoagulation and the platelet aggregation inhibitors acetylsalicylic acid, clopidogrel, and dipyridamole in stroke prevention is well established, their use in the acute phase of cerebral ischemia is a matter of debate. A moderate benefit on stroke progression and recurrence often is outweighed by a significant increase in the rate of intracerebral hemorrhage (ICH).<sup>2-5</sup>

### Clinical Perspective p 2330

Recently, major progress has been made in the functional analysis of platelet activation and platelet-dependent throm-

bus formation. Platelets can adhere to hypoxic endothelial cells by binding of their glycoprotein (GP) Ib receptor to von Willebrand factor (vWF) on the endothelial surface.<sup>6</sup> Exposure of subendothelial matrix proteins during ischemia further facilitates firm attachment of platelets to the vessel wall by binding of collagens to their GPVI receptor.<sup>7,8</sup> These processes lead to activation of platelet GPIIb/IIIa and platelet aggregation.<sup>9</sup> Although it is well established that endothelial cells undergo activation<sup>10</sup> and that microvascular integrity is disturbed during cerebral ischemia,<sup>11</sup> less is known about the signaling cascades that lead to intravascular thrombus formation in the brain. We recently demonstrated that coagulation factor XII plays a decisive role in thrombus formation after

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**Effects of Selective Anti-Platelet Antibodies on Hemostasis in Mice**

	Rat IgG (Control)	GPIb $\alpha$ Fab	100 $\mu$ g GPIIb/IIIa F(ab) <sub>2</sub>	20 $\mu$ g GPIIb/IIIa F(ab) <sub>2</sub>	10 $\mu$ g GPIIb/IIIa F(ab) <sub>2</sub>	GPVI mAbs
Receptor occupancy, %	0	>95	>95	78.4 $\pm$ 4.8	67.8 $\pm$ 6.1	Depletion
Platelet count, $\times 10^{-6}/\mu$ L	1.00 $\pm$ 0.20	0.91 $\pm$ 0.18	1.04 $\pm$ 0.08	0.90 $\pm$ 0.14	1.02 $\pm$ 0.12	0.98 $\pm$ 0.11
Bleeding time, min	4.4 $\pm$ 3.8	9.3 $\pm$ 5.8	...	ND	ND	7.3 $\pm$ 4.6
Mice bleeding >20 min, n/N	1/16	6/14	12/12			0/12

Mice received the indicated antibodies or proteolytic fragments and were analyzed after 1 hour. Values are mean $\pm$ SD unless otherwise indicated. Treatment of mice with the anti-GPVI antibody JAQ1 leads to a loss of GPVI protein in platelets for a prolonged period of time and thus induces a "GPVI knockout-like" phenotype.<sup>17</sup> Platelet counts in antibody-treated mice were not statistically different from the rat IgG control ( $P>0.05$ , unpaired Student *t* test).

transient focal cerebral ischemia.<sup>12</sup> In the present study, we show that targeting platelet GPIb or GPVI receptors protects mice from ischemic brain injury in an experimental stroke model without an increase in bleeding complications. In contrast, blockade of the final common pathway of platelet aggregation with anti-GPIIb/IIIa antibodies had no positive effect on stroke outcome and dose-dependently raised the incidence of ICH and mortality.

**Methods****Anti-Platelet Antibodies**

All monoclonal antibodies (mAbs) were produced, characterized, and modified in our laboratories as described previously in detail. The following mAbs were used: mAbs against mouse GPIIb/IIIa (JON/A),<sup>13</sup> GPIb $\alpha$  (p0p/B),<sup>14</sup> and GPVI (JAQ1).<sup>15</sup> Fab and F(ab)<sub>2</sub> fragments were prepared as described.<sup>16</sup> Briefly, antibodies were incubated for 6 to 8 hours with immobilized papain or for 24 hours with immobilized pepsin according to the manufacturer's instructions (Pierce Biotechnology, Inc, Rockford, Ill), and the preparations were then applied to an immobilized protein A column, followed by an immobilized protein G column (Pharmacia, Freiburg, Germany) to remove Fc fragments and undigested IgG. Purity of Fab or F(ab)<sub>2</sub> fragments was tested by SDS-PAGE. For control experiments, purified rat IgG2a (Serotec, Darmstadt, Germany) and nonimmune control rat IgG Fab were used.

**Antibody Administration**

To inhibit GPIb $\alpha$ , mice received 100  $\mu$ g p0p/B Fab IV 1 hour before or 1 hour after transient middle cerebral artery occlusion (tMCAO). GPIIb/IIIa receptors were blocked by injection of 100  $\mu$ g (>95% receptor blockade; see the Table), 20  $\mu$ g (78.4% receptor blockade), or 10  $\mu$ g (67.8% receptor blockade) JON/A F(ab)<sub>2</sub> IV 1 hour before the start of the experiment. To inhibit GPVI function, mice received 100  $\mu$ g JAQ1 IP 5 days before infarct induction. Mice had at that time point no detectable GPVI in platelets for at least 5 more days.<sup>17</sup> Two different groups of control animals received either 100  $\mu$ g purified rat IgG2a or 100  $\mu$ g rat IgG Fab.

**Bleeding Time Experiments**

To determine bleeding times, mice were anesthetized, and a 3-mm segment of the tail tip was amputated with a scalpel. The tail was then blotted with filter paper every 15 seconds until the paper was no longer blood stained.<sup>18</sup> When necessary, bleeding was manually stopped after 20 minutes to prevent death.

**Animal Studies**

Animal studies were approved by the Regierung von Unterfranken and conducted according to the recently published recommendations for research in mechanism-driven basic stroke studies.<sup>19</sup> Adult male C57/BL6 mice (20 to 25 g) were purchased from Charles River (Sulzfeld, Germany). The tMCAO model was used to induce focal cerebral ischemia as described in detail elsewhere.<sup>12,20</sup> Briefly, mice were anesthetized with 2% isoflurane in a 70% N<sub>2</sub>O/30% O<sub>2</sub> mixture. A servo-controlled heating blanket was used to maintain core body

temperature close to 37°C throughout surgery. After a midline neck incision was made, a standardized silicon rubber-coated 6.0 nylon monofilament (60-1720RE, Doccol, Redlands, Calif) was inserted into the right common carotid artery and advanced via the internal carotid artery to occlude the origin of the MCA. After 1 hour, mice were reanesthetized, and the occluding filament was removed to allow reperfusion. All animals were operated on by the same operator (C.K.) to reduce infarct variability; operation time per animal did not exceed 15 minutes.

After recovery from anesthesia and again after 24 hours, neurological function was assessed by 2 blinded investigators. Global neurological status was scored according to Bederson et al.<sup>21</sup> Motor function and coordination were graded with the grip test.<sup>22</sup>

Laser Doppler flowmetry (Moor Instruments, Devon, UK) was used to monitor regional cerebral blood flow in the MCA territory in antibody-treated animals and controls before surgery (baseline), immediately after MCA occlusion, and again 5 minutes after removal of the occluding monofilament (reperfusion).<sup>12,23</sup> After the thread was advanced, regional cerebral blood was reduced by 95 $\pm$ 3% and recovered to 68 $\pm$ 5% of baseline (100%) after removal of the filament, indicating sufficient occlusion and reperfusion of the vessel beds. Values did not differ statistically between the groups at any time point (not shown).

After the right femoral artery was punctured, blood gases (PO<sub>2</sub>, PCO<sub>2</sub>, pH) were analyzed during the operation in 3 animals per group (antibody-treated mice or controls). The measured values were within the physiological range and showed no significant differences (not shown).

**Determination of Infarct Size and ICH**

Mice were killed 24 hours after tMCAO. Brains were quickly removed and cut into 2-mm-thick coronal sections using a mouse brain slice matrix. The slices were stained with 2% 2,3,5-triphenyl-tetrazolium chloride (TTC; Sigma-Aldrich, Seelze, Germany) in PBS to visualize the infarctions. Planimetric measurements (ImageJ software, National Institutes of Health, Bethesda, Md) were performed by researchers blinded to the treatment group and were used to calculate lesion volumes, which were corrected for brain edema as described.<sup>24</sup>

The occurrence of ICH was macroscopically assessed on whole brains and again after the 2-mm-thick coronal brain slices were cut (see above) before TTC staining. Brains showing ICH were excluded from the assessment of infarct volumes.

**Stroke Assessment by Magnetic Resonance Imaging**

To investigate the frequency of ICH over time after tMCAO, magnetic resonance imaging (MRI) was performed repeatedly at an early stage (24 hours) and at 7 days after stroke on a 1.5-T MR unit (Vision, Siemens, Berlin, Germany) under inhalation anesthesia as described previously.<sup>12</sup> For all measurements, a custom-made dual-channel surface coil designed for the examination of mice head was used (A063HACG, Rapid Biomedical, Würzburg, Germany). The image protocol comprised a coronal T2-weighted sequence (slice thickness, 2 mm) and a coronal 3D T2-weighted gradient-echo constructed interference in steady state (slice thickness, 1 mm) sequence. MRIs were visually assessed by researchers blinded to the

prior treatment with respect to infarct morphology and, in particular, the occurrence of ICH.

### Statistical Analysis

Results are presented as mean  $\pm$  SD. The frequency of ICH and the mortality rate at day 1 were compared between groups with the  $\chi^2$  test. Infarct volumes and functional data were tested for gaussian distribution with the D'Agostino and Pearson omnibus normality test and then analyzed by Bonferroni-corrected 1-way ANOVA. For statistical analysis, PrismGraph 4.0 software (GraphPad Software, San Diego, Calif) was used. Values of  $P < 0.05$  were considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

## Results

### GPIIb and GPVI Blockade Improves Stroke Outcome After Transient Cerebral Ischemia

To study the involvement of platelets in the development of ischemic stroke, we inhibited the function of platelet membrane receptors involved in primary adhesion and activation. Complete blockade of GPIIb $\alpha$  was achieved by intravenous injection of 100  $\mu$ g Fab fragments of the monoclonal antibody p0p/B. In contrast to the intact IgG,<sup>16</sup> the Fab is not cytotoxic and therefore had no significant influence on platelet counts. After 1 hour, receptor occupancy was  $>95\%$  as shown by flow cytometric analysis of p0p/B<sup>FITC</sup> binding (the Table). Tail bleeding times in anti-GPIIb $\alpha$ -treated mice were markedly prolonged, with 6 of 14 mice being unable to stop bleeding within the 20-minute observation period and the other 8 mice showing a strong prolongation compared with control IgG- or control Fab-treated animals. In parallel, mice were treated with the anti-GPVI antibody JAQ1 (100  $\mu$ g IP) and analyzed on day 5. As previously described,<sup>17</sup> these animals lacked detectable GPVI in circulating platelets but displayed normal platelet counts and only very moderately increased tail bleeding times (the Table).

Mice treated with anti-GPIIb $\alpha$  Fab 1 hour before or anti-GPVI mAbs 5 days before challenge were subjected to transient (60 minutes) cerebral ischemia and analyzed after 24 hours. At that time point, infarct volumes were reduced dramatically in anti-GPIIb $\alpha$ -treated mice to  $\approx 40\%$  compared with controls ( $28.5 \pm 12.7$  versus  $73.9 \pm 17.4$  mm<sup>3</sup>;  $P < 0.001$ ; Figure 1A). Similarly, depletion of GPVI significantly diminished the infarct volume but to a lesser extent ( $49.4 \pm 19.1$  mm<sup>3</sup>;  $P < 0.05$ ; Figure 1A). Reduction in infarct size after anti-GPIIb $\alpha$  treatment was functionally relevant in that the Bederson score assessing global neurological function and the grip test that specifically measures motor function and coordination were significantly better than in controls (Figure 1B) (Bederson score,  $3.7 \pm 0.6$  versus  $2.2 \pm 0.6$ , respectively;  $P < 0.001$ ; grip test,  $1.7 \pm 0.9$  versus  $3.4 \pm 0.7$ , respectively;  $P < 0.01$ ). Anti-GPVI-treated mice tended to develop less severe neurological deficits compared with controls, but the differences did not reach statistical significance (Figure 1B). To test whether GPIIb blockade also is beneficial in the acute phase after focal cerebral ischemia, anti-GPIIb $\alpha$  Fab was applied 1 hour after the induction of tMCAO. Indeed, this therapeutic approach was as effective as prophylactic anti-GPIIb $\alpha$  infusions before tMCAO because

brain infarct volumes were reduced to a comparable extent ( $24.5 \pm 7.7$  mm<sup>3</sup>;  $P < 0.001$ ; Figure 1A) and the neurological status again was improved (Bederson score,  $1.9 \pm 0.7$ ;  $P < 0.001$ ; grip test,  $3.4 \pm 1.1$ ;  $P < 0.01$ ; Figure 1B). Taken together, these results indicate that the platelet receptors GPIIb and GPVI may contribute critically to stroke development after tMCAO.

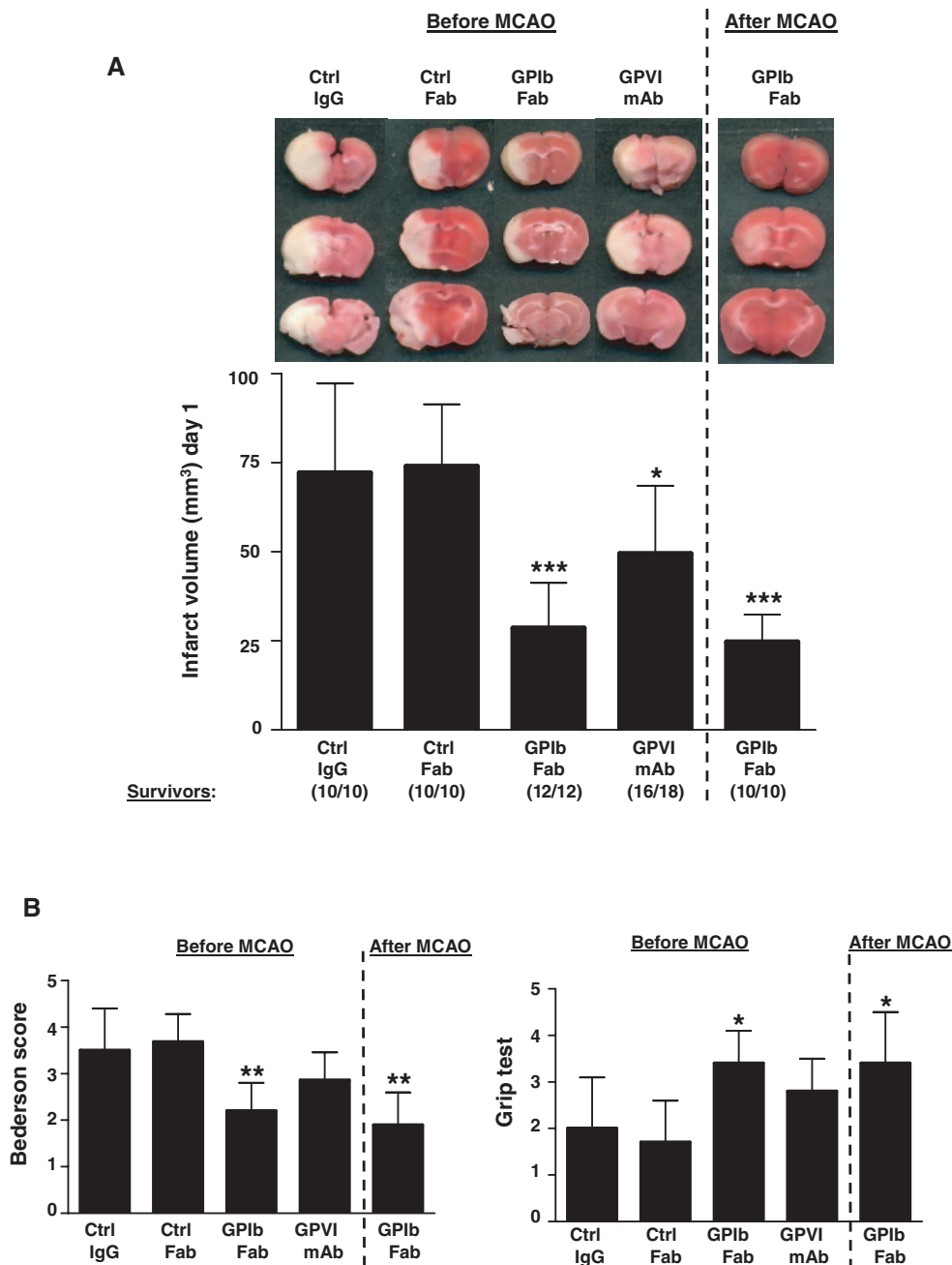
We next analyzed the impact of platelet inhibition on the occurrence of intracerebral bleeding complications after experimental cerebral ischemia. Mice receiving anti-GPIIb $\alpha$  Fab or anti-GPVI mAbs did not show hemorrhagic transformation of infarcted brain regions as assessed by morphological analysis (not shown). Accordingly, mortality rates were not increased compared with IgG or Fab controls (not shown). This important notion could be confirmed by serial MRI studies. In animals treated with anti-GPIIb $\alpha$  Fab, ischemic infarcts always appeared hyperintense on T2-weighted MRI. There were no additional hypointense areas indicating hemorrhage on days 1 and 7 after tMCAO when blood-sensitive T2-weighted gradient-echo MR sequences were used. These MRI findings exclude the occurrence of ICH (Figure 2).

### GPIIb/IIIa Blockade Is Ineffective in tMCAO and Dose-Dependently Increases the Risk of ICH

We next asked whether blockade of the final common pathway of platelet aggregation via GPIIb/IIIa would effectively reduce infarct volumes even more after tMCAO. Unexpectedly, 4 of the 7 animals that had received 100  $\mu$ g anti-GPIIb/IIIa F(ab)<sub>2</sub> leading to a virtually complete receptor blockade (see the Table) died as a result of ICH ( $P < 0.05$ ; Figure 3A and 3B), and the 3 surviving animals exhibited infarct volumes of the same extension as controls ( $65.7 \pm 3.4$  mm<sup>3</sup>;  $P > 0.05$ ; Figure 4). The high incidence of ICH was very similar to that in mice in which platelets had been depleted by  $>98\%$  by injection of cytotoxic anti-GPIIb antibodies (not shown).<sup>16</sup> To analyze whether the risk of ICH after GPIIb/IIIa blockade is dose dependent and to further evaluate the efficacy of anti-GPIIb/IIIa F(ab)<sub>2</sub> treatment in experimental stroke, additional groups of mice received 20 or 10  $\mu$ g anti-GPIIb/IIIa F(ab)<sub>2</sub>, which led to a 78.4% and 67.8% receptor blockade, respectively (see the Table). In contrast to complete GPIIb/IIIa inhibition, only 1 of 15 animals developed ICH and died (Figure 3B), but both concentrations failed to influence infarct volumes ( $58.8 \pm 6.3$  and  $61.6 \pm 12.4$  mm<sup>3</sup>;  $P > 0.05$ ; Figure 4) or neurological outcome (Bederson score,  $3.0 \pm 0.6$  and  $3.2 \pm 0.9$ ;  $P > 0.05$ ; grip test,  $2.0 \pm 0.8$  and  $2.3 \pm 1.0$ ;  $P > 0.05$ ).

## Discussion

As a principal finding, we demonstrate here that selective blockade of key pathways mediating platelet adhesion and aggregation has different impacts on stroke outcome. Our study shows for the first time that interfering with early steps of platelet-vessel wall interactions mediated by GPIIb and GPVI reduces stroke severity after tMCAO. Anti-GPIIb $\alpha$  Fab treatment had a very strong protective effect when performed both before and after tMCAO. This suggests a central role of this receptor in the pathogenesis of ischemic stroke, whereas GPVI may have a significant but less prominent function. This is in agreement with the model that GPIIb $\alpha$  is mandatory



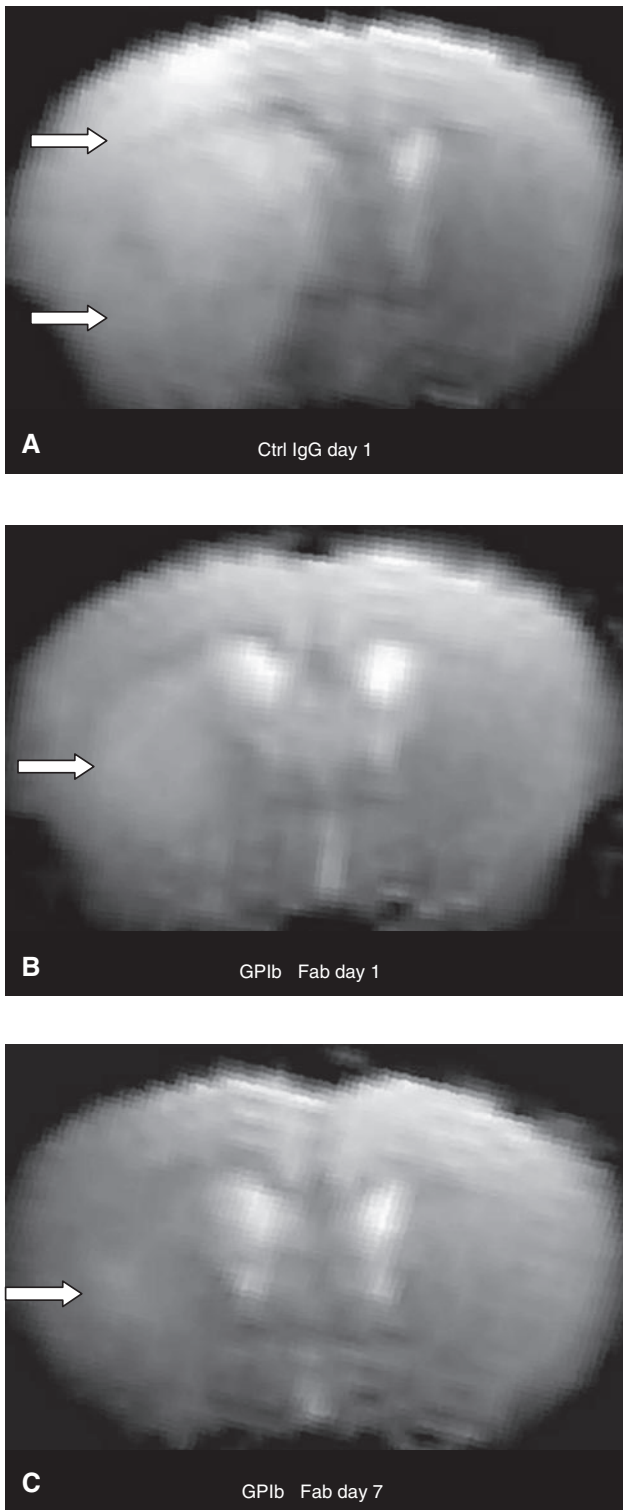
**Figure 1.** Infarct volumes and functional outcomes 24 hours after focal cerebral ischemia in mice treated with different anti-platelet antibodies. A (top), Representative TTC stains of 3 corresponding coronal brain sections of mice treated with rat IgG (controls), rat IgG Fab (controls), anti-GPIIb Fab (1 hour before or 1 hour after MCAO), and anti-GPVI mAbs. A (bottom), Brain infarct volumes in mice treated with control rat IgG (n=10), control rat IgG Fab (n=10), anti-GPIIb Fab (n=12 at 1 hour before or n=10 at 1 hour after MCAO), and anti-GPVI mAbs (n=16). B, Neurological Bederson score (left) and grip test (right) as assessed at day 1 after tMCAO for controls (rat IgG, n=10; rat IgG Fab, n=10), mice treated with anti-GPIIb Fab (n=12 at 1 hour before or n=10 at 1 hour after MCAO, respectively) and anti-GPVI mAbs (n=16). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ , Bonferroni-corrected 1-way ANOVA vs controls.

for the initial attachment of platelets to the vessel wall under conditions of elevated shear, whereas GPVI serves mainly as an activating receptor, a function that also can be fulfilled by alternative pathways, most notably G protein-coupled receptors.<sup>25,26</sup> Importantly, stroke protection after anti-GPIIb Fab or anti-GPVI mAb treatment was not accompanied by intracranial bleeding complications. In contrast, application of F(ab)<sub>2</sub> targeting the GPIIb/IIIa receptor had no positive effect on stroke

outcome but significantly increased the rate of intracerebral bleedings and mortality in a dose-dependent manner.

The cerebral microvasculature rapidly responds to brain ischemia.<sup>10</sup> Endothelial cells upregulate cell adhesion molecules, and endothelial denudation of vessels exposes subendothelial matrix proteins such as collagen to the bloodstream. Recently, 2 important pathways have been described that facilitate early adhesion of platelets to vessel walls: binding





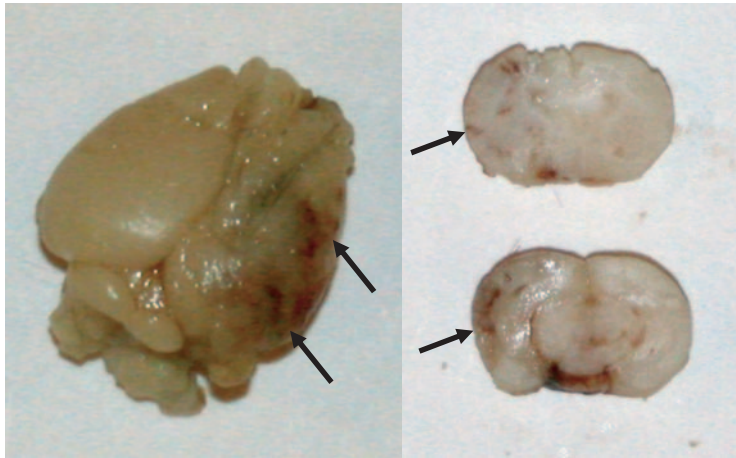
**Figure 2.** MRIs of cerebral infarcts. Serial coronal T2-weighted MR brain images show hyperintense ischemic lesions (white arrows) at days 1 (A, B) and 7 (C) after tMCAO in controls (rat IgG) (A) and anti-GPIIb $\alpha$  Fab-treated rats (B, C). Infarcts are smaller in anti-GPIIb $\alpha$  Fab-treated mice (B) vs controls (A), and T2 hyperintensity further decreases at day 7 as a result of a “fogging” effect during infarct maturation (C). Importantly, hypointense areas indicative of ICH were always absent, demonstrating that the selective blockade of the GPIIb receptor in platelets does not increase the risk of secondary hemorrhagic transformation even at more advanced stages of infarct development.

of the platelet surface receptor GPIIb to endothelial vWF<sup>6</sup> and adhesion of platelets to collagen via their GPVI receptor.<sup>27</sup> In accordance with our findings in experimental stroke, inhibition of either platelet GPIIb or vWF reversed flow reductions after experimental femoral artery stenosis.<sup>28–31</sup> Importantly, reduced stroke volumes after GPIIb inhibition in our study were accompanied by a significant reduction in neurological deficits even when anti-GPIIb $\alpha$  Fab was injected with a delay of 1 hour after the induction of tMCAO. This underlines the functional significance of this novel therapeutic approach and indicates its potential suitability for clinical application during the acute phase of ischemic stroke in humans, in whom treatment options are very limited. Interestingly, polymorphisms of platelet GPIIb $\alpha$  exist, and variants that lead to enhanced vWF/GPIIb interactions are associated with an increased risk of ischemic stroke in humans.<sup>32,33</sup> Similarly, increased serum levels of vWF represent an independent stroke risk factor.<sup>34</sup> Although tail bleeding times are strongly elevated after treatment of mice with anti-GPIIb $\alpha$  Fab fragments, no increase in ICH, which represents the main obstacle for antithrombotic therapy during the acute stroke phase in clinical practice, was detected. This surprising finding suggests that the mechanisms by which platelets prevent intracranial bleeding are different from those involved in the sealing of a tail bleeding wound. Both processes are clearly platelet dependent because platelet depletion or virtually complete inhibition of GPIIb/IIIa results in both markedly prolonged tail bleeding times and intracranial bleeding after tMCAO (Figure 3A). Thus, our results strongly confirm the previous finding that no direct correlation exists between bleeding time and bleeding risk.<sup>35</sup>

The initial loose adhesion of platelets to the damaged endothelium is followed by firm attachment, which is mediated through the platelet collagen receptors.<sup>6</sup> Among the numerous collagen receptors expressed in platelets, GPVI is of central importance for cellular activation and subsequent firm arrest.<sup>36</sup> Treatment of mice with anti-GPVI antibodies specifically and persistently depletes GPVI from platelets.<sup>17,37</sup> Several reports have demonstrated a profound antithrombotic effect of GPVI inhibition after artificial arterial wall injury and collagen-induced thromboembolism.<sup>14,17,38</sup> We now extend these previous findings by showing that treatment of mice with the anti-GPVI antibody JAQ1 significantly reduced the brain infarct volumes at day 1 after tMCAO. This indicates that platelet/collagen interactions via GPVI also may be involved in stroke development. GPVI depletion was less effective than GPIIb blockade and did not significantly affect clinical outcome variables. However, it is well established that significant reductions in stroke volumes on histological examination after MCAO often do not translate into measurable clinical improvement.<sup>39</sup> Although tail bleeding times were slightly increased by JAQ1, intracerebral bleeding frequency and mortality after 24 hours were not altered, indicating a favorable safety profile. The different extent of stroke protection in favor of GPIIb blockade may be due to a more general role of GPIIb in stroke development. GPIIb, but not GPVI, additionally mediates leukocyte adhesion to attached platelets by a Mac-1–dependent pathway.<sup>6,40</sup> Furthermore, resting platelets can bind to the

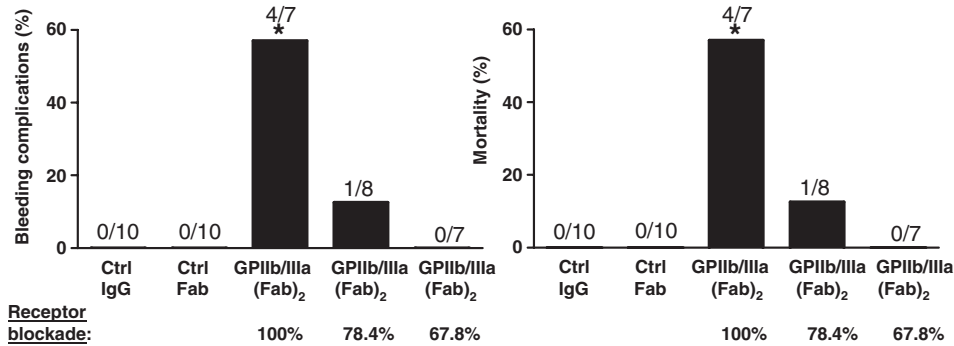
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**GPIIb/IIIa (Fab)<sub>2</sub>  
(100% receptor blockade)**



**Figure 3.** Frequency of ICH and mortality rate after tMCAO in mice treated with different doses of anti-GPIIb/IIIa F(ab)<sub>2</sub>. A, Representative images of a whole brain (left) and 2 corresponding coronal brain sections (right) from a mouse treated with 100 μg anti-GPIIb/IIIa F(ab)<sub>2</sub> (100% receptor blockade), followed by 60 minutes of tMCAO. Note the massive hemorrhagic transformation (black arrows) within the infarcted brain area. B, Percentage of ICH and mortality rate at day 1 after tMCAO in controls (rat IgG and rat IgG Fab, n=10) and mice treated with 100 μg (100% receptor blockade, n=7), 20 μg (78.4% receptor blockade, n=8), and 10 μg (67.8% receptor blockade, n=7) anti-GPIIb/IIIa F(ab)<sub>2</sub>. Note that the frequency of ICH and mortality after tMCAO are strictly dose dependent. \*P<0.05, χ<sup>2</sup> test vs controls.

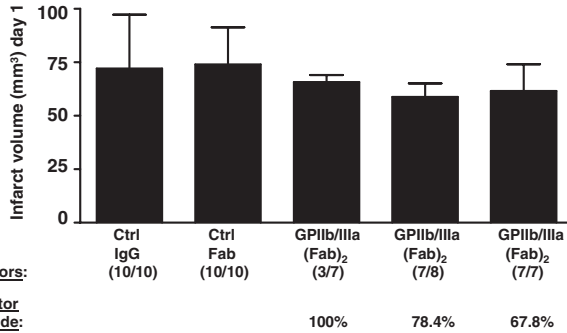
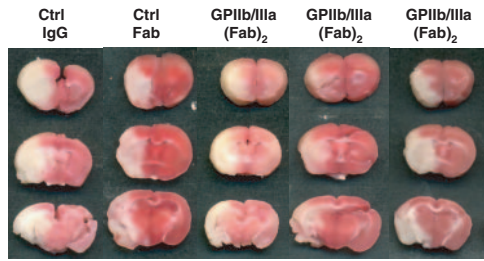
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activated endothelium via GPIb interaction with P-selectin.<sup>41</sup> Both, leukocyte adhesion and P-selectin up-regulation have been shown to contribute to stroke development, probably by impairing reperfusion of the cerebral microvasculature.<sup>10,42</sup>

GPIIb/IIIa antagonists inhibit the final common pathway of platelet aggregation regardless of the agonist that stimulates platelet activation.<sup>6</sup> In agreement with previous reports,<sup>43</sup> antibody-mediated blockade of the GPIIb/IIIa receptor had no significant effect on peripheral platelet counts but completely inhibited ex vivo platelet aggregation in response to different stimuli and resulted in tail bleeding times consistently >20 minutes in our study (the Table). Impaired hemostasis after >95% GPIIb/IIIa blockade could explain the high frequency of ICH and mortality after tMCAO in our study. A substantial risk of ICH has previously been reported after tMCAO in mice treated with the GPIIb/IIIa inhibitor SDZGPI562.<sup>44</sup> The unexpected high rate of bleeding complications is consistent with similar observations during a recent phase III, double-blind, placebo-controlled, multicenter study testing the safety and efficacy of abciximab in ischemic stroke. This clinical study was stopped prematurely because of significantly increased ICH and mortality, as well as lack of efficacy.<sup>45</sup> In the preceding phase II trial, treatment with abciximab had shown a nonsignificant shift in favorable outcomes only.<sup>46</sup> Several

experimental studies reported a beneficial effect of GPIIb/IIIa antagonists on stroke size and functional outcome.<sup>44,47,48</sup> GPIIb/IIIa antagonists also have been successfully used in experimental and clinical settings in conjunction with recombinant tissue-type plasminogen activator thrombolysis therapy without major complications reported.<sup>49,50</sup> This is in contrast to our study in which no effect on stroke volume or functional deficit was observed, regardless of the anti-GPIIb/IIIa F(ab)<sub>2</sub> dosage used. Increasing evidence exists, however, that the extent of GPIIb/IIIa inhibition may be critical, which could explain the divergent experimental and clinical observations. At peak concentrations, GPIIb/IIIa inhibitors may effectively act as platelet antagonists accompanied by increased bleeding complications, whereas subthreshold GPIIb/IIIa antagonism may lead to platelet activation and thrombus formation.<sup>51</sup> In line with this, a 67.8% or 78.4% GPIIb/IIIa receptor blockade was safe (but ineffective) in our experimental stroke model, whereas complete inhibition substantially increased ICH and mortality. Taken together, it appears that GPIIb/IIIa antagonists have a very narrow therapeutic window, limiting their clinical use at least in cerebral ischemia,<sup>51,52</sup> in contrast to their proven utility in percutaneous coronary artery interventions, which, however, is age dependent.<sup>53,54</sup> Currently, not enough evidence exists from randomized controlled trials on the efficacy or safety of GPIIb/IIIa



**Figure 4.** Infarct volumes 24 hours after focal cerebral ischemia in mice treated with different doses of anti-GPIIb/IIIa F(ab)<sub>2</sub>. Top, Representative TTC stains of 3 corresponding coronal brain sections of mice treated with rat IgG (controls), rat IgG Fab (controls), and 100 μg (100% receptor blockade), 20 μg (78.4% receptor blockade), and 10 μg (67.8% receptor blockade) anti-GPIIb/IIIa F(ab)<sub>2</sub>. Bottom, Brain infarct volumes in mice treated with control rat IgG (n=10), control rat IgG Fab (n=10), and 100 μg (100% receptor blockade, n=3), 20 μg (78.4% receptor blockade, n=7), and 10 μg (67.8% receptor blockade, n=7) anti-GPIIb/IIIa F(ab)<sub>2</sub>. Because of increased intracerebral bleeding and mortality in the group receiving 100 μg (100% receptor blockade) anti-GPIIb/IIIa F(ab)<sub>2</sub>, only 3 animals were available for analysis.

inhibitor therapy in acute stroke or its use combined with thrombolysis.<sup>55</sup> The devastating consequences of ICH in patients require a particularly high safety profile for any antiplatelet therapy or anticoagulation during stroke because, depending on location, even small bleedings can cause major neurological deficits. Our present study provides evidence that blocking of the platelet receptor GPIb involved in platelet adhesion can diminish infarct development in mice and, because of a lack of ICH, may open new avenues for acute stroke treatment in humans in the future.

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### Disclosures

None.

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### CLINICAL PERSPECTIVE

Ischemic stroke is a major cause of death and permanent disability in industrialized countries. The only approved therapy during the acute stroke phase is thrombolysis within a narrow time window of 3 hours (up to 6 hours), but the number of patients amenable to this treatment is low. Moreover, (intracerebral) hemorrhages may counterbalance the benefit of therapeutic or prophylactic use of the established antiplatelet or anticoagulant drugs in acute cerebral ischemia. In the present study, we show that targeting platelet glycoprotein Ib and glycoprotein VI receptors, which mediate initial adhesion/attachment of platelets to endothelial cells and the subendothelial matrix, protects mice from ischemic brain injury after transient occlusion of the middle cerebral artery. Importantly, this was not associated with an increase in bleeding complications as revealed by magnetic resonance imaging. Delayed treatment with anti-glycoprotein Ib Fab during reperfusion was similarly effective. In contrast, blockade of platelet aggregation with anti-glycoprotein IIb/IIIa F(ab)<sub>2</sub> had no positive effect on stroke outcome but raised the incidence of intracerebral hemorrhages in a dose-dependent manner. In conclusion, targeting initial adhesion/attachment of platelets to the endothelium rather than blocking the common pathway of platelet aggregation may open new avenues in the future for acute stroke treatment with a more favorable safety profile. It remains to be tested whether this novel therapeutic approach also holds promise in cardiovascular medicine.