Integrin $\alpha_{IIb}\beta_3$ Inhibitor Preserves Microvascular Patency in Experimental Acute Focal Cerebral Ischemia

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Background and Purpose—Platelets become activated and accumulate in brain microvessels of the ischemic microvascular bed after experimental focal cerebral ischemia. The binding of glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) on platelets to fibrinogen is the terminal step in platelet adhesion and aggregation. This study tests the hypothesis that inhibition of platelet-fibrin(ogen) interactions may prevent microvascular occlusion after experimental middle cerebral artery occlusion (MCA:O).

Methods—TP9201 is a novel Arg-Gly-Asp (RGD)-containing integrin $\alpha_{IIb}\beta_3$ inhibitor. Microvascular patency after 3-hour MCA:O and 1-hour reperfusion within the ischemic and nonischemic basal ganglia was compared in adolescent male baboons who received high-dose TP9201 (group A: IC$_{80}$ in heparin, $n=4$), low-dose TP9201 (group B: IC$_{10}$ in heparin, $n=4$), or no treatment (group C: $n=4$) before MCA:O.

Results—After MCA:O, microvascular patency decreased significantly in group C. However, in the ischemic zones of groups A and B compared with group C, patencies were significantly greater in the 4.0- to 7.5-μm-diameter (capillary) and 7.5- to 30.0-μm-diameter vessels ($2P<0.05$). A dose-dependent increase in hemorrhagic transformation was seen in group A (3 of 4 animals) compared with group B (1 of 4 animals), and no hemorrhage was visible in group C ($\chi^2$ analysis for trend, $P<0.05$).

Conclusions—Platelet activation contributes significantly to ischemic microvascular occlusion. Occlusion formation may be prevented by this RGD–integrin $\alpha_{IIb}\beta_3$ inhibitor at a dose that does not produce clinically significant parenchymal hemorrhage. The effect of microvascular patency on neuron recovery can now be tested. (Stroke. 2000;31:1402-1410.)

Key Words: cerebral ischemia, focal integrins microcirculation platelet glycoprotein GPIIb/IIIa complex

The participation of activated platelets in ischemic stroke has been deduced from the observation of refractile bodies in retinal arteries and of platelet emboli in the cerebral vasculature during carotid endarterectomy and by direct visualization within the cerebral circulation. The common observation of platelet-fibrin thrombi on carotid artery atheromas in patients with symptoms of transient cerebral ischemia and the numerous reports describing platelet activation after stroke onset provide further indirect support for the participation of platelets in cerebral ischemia arising from large-artery injuries. However, the accumulation of platelets in target cerebral microvascular beds in response to focal ischemia and their contribution to further injury have received little attention.

In situ accumulation of platelets in the cerebral microvasculature after middle cerebral artery (MCA) occlusion (MCA:O) has been observed in the nonhuman primate and by guest on May 28, 2017 http://stroke.ahajournals.org/ Downloaded from
activated upon platelet activation.\textsuperscript{11,12} Integrin $\alpha_m\beta_3$ interacts with fibrinogen in an arginine-glycine-aspartic acid (RGD)-dependent manner.\textsuperscript{11,12} Integrin $\alpha_m\beta_3$ receptor activation followed by fibrinogen ligation is the common terminal step of several identified cascades of steps leading to platelet activation,\textsuperscript{13} suggesting that inhibition of this receptor-ligand interaction may provide potent antiplatelet effects and the potential for therapeutic intervention. Plow and colleagues\textsuperscript{11,14} have demonstrated that RGD-containing peptides inhibit the binding of fibrinogen to thrombin-stimulated platelets via the integrin $\alpha_m\beta_3$. The Fab fragment of the humanized monoclonal antibody c7E3 inhibits platelet aggregation and platelet thrombus formation and reduces reocclusion after coronary artery angioplasty by binding to the integrin subunit $\beta_3$.\textsuperscript{15} Inhibition of platelet integrin $\alpha_m\beta_3$ and/or vascular integrin $\alpha_\text{IIb}$$\beta_3$–mediated events most probably underlies those responses.\textsuperscript{15}

TP9201 is a synthetic RGD-containing cyclic nonapeptide with a molecular mass of 1136 Da.\textsuperscript{16} It selectively blocks platelet integrin $\alpha_m\beta_3$ (and not $\alpha_\text{IIb}$$\beta_3$) by competitive dose-dependent inhibition of the interaction of the receptor with fibrinogen in platelets activated by a variety of agonists. In vivo, TP9201 has been shown to inhibit thrombus formation on injured carotid arteries in a canine model.\textsuperscript{17} In other animal models of acute in situ coronary artery thrombosis, TP9201 contributed to an increased frequency of recombinant tissue plasminogen activator–associated coronary artery reflow\textsuperscript{18} and inhibited reocclusion after endothelial injury.\textsuperscript{19} Furthermore, in baboons, TP9201 has been shown to inhibit platelet accumulation on a Dacron graft mounted in an exteriorized arteriovenous shunt.\textsuperscript{20} In all models, in vivo efficacy could be achieved at IC\textsubscript{80} to IC\textsubscript{90} or less as determined in heparinized plasma and, in most cases, without an increase in cutaneous bleeding time.\textsuperscript{18,20} This feature may be advantageous for any potential role of a GPIIb/IIIa inhibitor in cerebral ischemia when complications due to hemorrhage are severe.

The hypothesis tested by the present study states that platelet activation contributes substantially to the obstruction of the brain microvasculature after experimental MCA:O and that inhibiting the integrin $\alpha_m\beta_3$–fibrin(ogen) interaction can increase microvascular patency. A 3-arm, dose-sequential, placebo-controlled study of 2 dose rates of TP9201 was undertaken to test the effect of this integrin $\alpha_m\beta_3$ inhibitor on microvascular patency and the risk of intracerebral hemorrhage when begun immediately before MCA:O. TP9201 significantly inhibited microvascular occlusion, but a dose-related increase in clinically significant intracranial hemorrhage was observed.

Materials and Methods

All procedures in the present study were approved by the Institutional Animal Research Committee and were performed in accordance with the standards published by the National Research Council (The Guide for Care and Use of Laboratory Animals) and the US Department of Agriculture and Animal Welfare Act. In compliance with these standards, every effort was made to ensure that the animals were free of pain or discomfort. Before undergoing the experimental procedures, all animals were clinically normal and free of apparent infection or inflammation and showed no neurological deficits.

Integrin $\alpha_m\beta_3$, Inhibitor TP9201

TP9201 is a synthetic cyclic nonapeptide with the composition acetyl-L-cysteinyl-L-asparaginyl-L-propyl-arginylglycyl-L-aspartyl-O-methyl-L-tyrosyl-L-arginyl-L-cysteinamide, cyclic 1- to 9-sulfide. It was synthesized in an automated fashion (model 430A, Applied Biosystems) and purified by high-performance liquid chromatography as previously described.\textsuperscript{16} Purity was $\geq 98\%$. TP9201 inhibits platelet aggregation in platelet-rich plasma (PRP) or whole blood.\textsuperscript{20}

Cohorts

Twelve adolescent male baboons (\textit{Papio anubis/cynocephalus}) were used in the present study. Each animal served as his own control in the initial pharmacokinetic studies (see below), which preceded the interventional study. For the dose-sequential interventional study, the animals were distributed to 3 experimental groups: (1) animals that received TP9201, 675 $\mu$g/kg bolus +12 $\mu$g/kg per minute infusion (group A, n=4); (2) animals that received TP9201, 170 $\mu$g/kg bolus +3 $\mu$g/kg per minute infusion (group B, n=4); and (3) a control group that received saline vehicle only (group C, n=4).

Model Preparation

Preparation of the nonhuman primate model of right MCA occlusion and reperfusion and surgical implantation of the MCA occlusion device (PS Medical) have been described previously in detail.\textsuperscript{7} Anesthesia was undertaken with isoflurane by assisted ventilation (5\% induction) and maintained with the same agent (1.5\% to 2.0\%). A Silastic balloon device was placed around the MCA and secured by titanium hooks by a transorbital approach. The proximal terminus was accessible under the scalp by a local cutdown procedure. Full recovery was routinely achieved 1 to 2 hours after completion of the surgery. Subsequently, all animals were allowed a 7-day procedure-free interval before entry into the experimental protocol and displayed normal neurological function during that interval.\textsuperscript{22} Before MCA:O, the animals (n=12) had a mean hematocrit of 38.7$\pm$2.8\% and hemoglobin of 12.6$\pm$0.7 g/dL, platelet count of 427.3$\pm$72.7$\times$10\textsuperscript{3} /\mu$L, and total leukocyte count of 10.1$\pm$3.6$\times$10\textsuperscript{3} /\mu$L, which were not significantly different from their naive state.

Experimental Procedures

Occlusion of the right MCA in the awake animal was accomplished by inflation of the extrinsic MCA balloon with compression of the artery. After a 3-hour MCA:O occlusion, the MCA territory was reperfused for 1 hour after balloon deflation. The experiments were terminated 60 minutes after MCA balloon deflation by pressure-perfusion fixation with a chilled carbon tracer. Perfusion fixation consisted of an initial perfusion flush under antithrombotic and isosmotic conditions to wash out all blood elements and a subsequent isosmotic tracer-fixative perfusion. At the time of experiment termination, with the animals under thiopental sodium anesthesia (15 mg/kg infusion) and assisted ventilation, the thorax was opened, and the descending aorta and inferior vena cava were clamped. The left ventricle was rapidly cannulated and perfused with chilled flush solution containing 25 g/L BSA (Sigma Chemical Co), 2000 U/L heparin, and 6.7 $\mu$mol/L sodium nitroprusside (Fisher Scientific) in Plasmalyte (Baxter Healthcare) adjusted to 340 mOsm/L with NaCl, pH 7.4, at pressures of 180 to 210 mm Hg for 3 minutes. This was immediately followed by fixation with chilled carbon suspension/fixative solution consisting of india ink (1:1 [vol/vol], Pelikan Fount India, Pelikan AG) in Plasmalyte/paraformaldehyde (2\% final concentration)/glutaraldehyde (0.5\% final concentration) for up to 17
minutes. High mean arterial perfusion pressures were chosen to maximize vascular patency.

**Specimen Preparation**

The exposed brain was immersed in alcohol-formaldehyde-acid solution (87% ethanol, 10% formaldehyde, and 3% glacial acetic acid [vol/vol]) for 1 week. The fixed brain was sectioned in the coronal plane at 1-cm intervals and immersed for another week in alcohol-formaldehyde-acid solution to achieve complete intravascular gelation of the carbon tracer. Tissue blocks (1.0 cm×1.0 cm×0.2 cm) from stereanatomically identical sites of the left and right basal ganglia and from the left (normal) temporal lobe were embedded in glycol methacrylate (Polysciences, Inc), sectioned to 10-μm thickness, stained with basic fuchsin/methylene blue, and examined by light microscopy for the presence of india ink-filled (patent) microvascular structures. All specimens from all animals demonstrated complete continuous carbon impactions of microvessels.9,22,23

**Quantitative Analyses**

Sections were analyzed with the aid of a computerized video-imaging system consisting of an image system unit connected in-line with a Hamamatsu C2400 Newvicon NTSC video camera (Hamamatsu Photonics) staged vertically on the light microscope (VIDAS, Kontron and Carl Zeiss). The minimum transverse diameters of india ink-filled microvessels in 90 nonoverlapping 526.1-μm×491.4-μm fields at ×200 optical magnification (25 mm²) were processed in each section. The sections were taken at 30-μm intervals from stereanatomically identical sites of both basal ganglia. Identical numbers of fields were analyzed until >1000 vessels were counted in the left control basal ganglia. Reproducibility and reliability data have been reported previously.9,22 In the case of specimens with hemorrhage, regions of interest were taken at the hemorrhage border. The extent of reflow is expressed as the ratio of the number of microvessels containing carbon in the ischemic basal ganglia to those in the control basal ganglia. Microvessels are defined as vessels <100-μm minimum diameter and are represented as 4 discrete vessel size classes as previously published.22,23

**Neurological Outcomes**

Neurological function was assessed according to a well-described quantitative (100-point) scale weighted toward unilateral motor function loss.21,24

**Platelet Aggregation, Rationale, and Pharmacodynamic Studies**

To rationally test the effect of the inhibitor on microvascular thrombosis, each naive animal was screened for platelet aggregation responses at baseline and after TP9201 exposure before selecting the appropriate TP9201 dose rate for each animal for the MCA:O studies. Blood for the platelet aggregation studies was collected in tubes containing citrate (0.38%) or heparin (5 IU/mL) by separate venipunctures. In vitro platelet aggregation studies were performed in triplicate for each naive animal. PRP and platelet-poor plasma were prepared by centrifugation of 10 mL whole blood at room temperature. The platelet count was adjusted to 2×10⁵/μL with autologous platelet-poor plasma. For in vitro and ex vivo platelet aggregation studies, PRP was incubated with 10 μmol/L ADP, and the activation was followed for 3 minutes with the aid of a Lumi-aggregometer (model 400VS, Chrono-Log Corp). To derive the inhibitory concentrations (eg, IC₅₀), concentration-response curves were generated by adding different concentrations of TP9201 to adjusted PRP from each naive animal in in vitro platelet aggregation experiments. Subsequently, on the basis of the in vitro studies, each naive animal received 1 of 2 dose rates of TP9201 to determine the exact dose rate that would achieve the 2 target platelet inhibitory effects (IC₅₀ and IC₉₀). Whole-blood samples were obtained at 0, 1, 2, 3, and 4 hours of infusion for the ex vivo aggregation studies. The degree of inhibition is expressed as the percentage of control values in the absence of TP9201 determined at maximal amplitudes of activation, with the mean of 3 baseline measurements set as 100%.

**Bleeding Time Measurements**

The bleeding time was measured by use of an automated template device (Simplate II, General Diagnostics).25 Baseline bleeding times were determined at 1 hour before infusion and were repeated during the infusions at 1, 2, and 3 hours and before the end of the infusion (4 hours).

**Plasma Levels of TP9201 and Pharmacokinetic Studies**

TP9201 levels were determined to correlate ex vivo platelet inhibition with the in vitro inhibition curves. The plasma assays were performed by trifluoroacetic acid extraction of plasma followed by reversed-phase high-performance liquid chromatography on a C₄ protein column as described previously.26 Citrated blood samples (5 mL) were collected at baseline and 1, 2, 3, and 4 hours after the start of the TP9201 infusion. Samples were frozen immediately after collection and stored at −70°C before processing.

**Statistical Analysis**

Data are expressed as literal values or as mean±SD. Data were analyzed by using either paired or unpaired Student t test (2-tailed), ANOVA (with repeated measures), and other nonparametric tests, where appropriate. Statistical significance was generally set at 2P<0.05, except where noted.

**Results**

**Hemostatic Parameters and Neurological Status**

Alterations in hematologic parameters were selective (Table), most especially in total leukocyte count, which increased significantly within 1 hour of MCA:O in all 3 groups, as previously reported.22,24 There was no change in hematocrit or platelet count after MCA:O. After MCA:O, the neurological score changed from baseline (100) and varied within each group, consistent with the individual neurological status. A single animal in group A displayed a slight deficit after MCA device placement that persisted to MCA:O (postsurgical score 85 compared with 100 in the remainder). That animal suffered a symptomatic hemorrhage (Figure 5).

**Pharmacodynamic Studies and Dose Derivation**

Before device implantation, each naive animal participated in individual pharmacodynamic studies (Figure 1). On the basis of in vitro and ex vivo studies with a separate primate cohort,20 each animal received a constant infusion of TP9201 to achieve full inhibition of platelet aggregation to ADP in citrate and either 70% to 80% inhibition (group A) or 20% to 30% inhibition (group B) of platelet aggregation in normocalcemic conditions (Figure 1). A significant early increase in bleeding time was observed within 2 hours of infusion in group A but not group B (Figure 1). A significantly greater inhibition of platelet aggregation in vitro with TP9201 and ex vivo during each TP9201 infusion in response to ADP was observed under low Ca²⁺ conditions (citrate) than under normocalcemic conditions (heparin) (Figure 2). Logistic regression fits for platelet aggregation inhibition and TP9201 concentrations indicated an IC₅₀ (citrate) of 0.11 μmol/L and an IC₉₀ (heparin) of 2.28 μmol/L (Figure 2). The dose rates chosen for each individual animal within the MCA:O cohorts...
A and B were based on these IC₅₀ values and the plasma TP9201 levels.

**Microvascular Patency**

After placement of the MCA devices, each awake animal received 675 mg/kg +12 mg/kg per minute TP9201 (group A), 170 mg/kg +3 mg/kg per minute TP9201 (group B), or vehicle (group C) before MCA:O and continuously for 3 hours of MCA:O through 1 hour of reperfusion (Figure 3). Steady-state TP9201 blood levels of 2.5 mg/mL or 0.62 mg/mL were reached within 10 to 60 minutes of initiating the infusions in group A and group B, respectively (Figure 3). The patterns of ex vivo platelet aggregation inhibition, bleeding times, and plasma levels were in good agreement with the in vitro aggregation data from the same groups of animals. The anticipated bleeding time increase was observed in group A; however, no change in bleeding time was seen in any group B animal.

Compared with vehicle, both TP9201 regimens produced significant increases in microvascular patency in both the capillary (4.0 to 7.5 μm) and precapillary arteriole/postcapillary venule (7.5 to 30.0 μm) diameter categories by 1 hour of reperfusion (Figure 4). Variation in the number and fractional patency (relative reflow) of larger microvessels (eg, 50.0 to 100.0 μm and >100.0 μm) was due in part to the relatively small numbers of vessels in those categories. However, in each diameter category, the vascular patency in the low-dose group (group B) was as great as that in the high-dose group (group A).

**Hemorrhagic Transformation**

A dose-dependent increase in hemorrhage was observed among the 3 groups (P=0.036) (Figure 5). In group A, 3 animals suffered large parenchymal hemorrhages, which contributed significantly to the early neurological deficit, whereas only 1 animal in group B displayed evidence of a hemorrhagic infarction. The contribution of this hemorrhage to outcome is uncertain. The remainder of the animals in all 3 groups had no evidence of carbon tracer extravasation.

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**Hematologic Data**

<table>
<thead>
<tr>
<th></th>
<th>MCA:O</th>
<th>Reperfusion</th>
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<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>1 h</td>
</tr>
<tr>
<td>Leukocytes (total), ×10⁹/μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>11.3±2.4</td>
<td>24.5±2.7</td>
</tr>
<tr>
<td>Group B</td>
<td>11.0±3.2</td>
<td>17.8±4.6</td>
</tr>
<tr>
<td>Group C</td>
<td>11.0±2.5</td>
<td>19.3±3.4</td>
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<tr>
<td>Hematocrit, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>37.8±1.4</td>
<td>34.8±1.7</td>
</tr>
<tr>
<td>Group B</td>
<td>38.5±0.7</td>
<td>37.2±2.9</td>
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<tr>
<td>Group C</td>
<td>39.1±5.1</td>
<td>38.4±5.3</td>
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<tr>
<td>Platelets, ×10⁹/μL</td>
<td></td>
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<tr>
<td>Group A</td>
<td>426.3±68.0</td>
<td>519.0±61.6</td>
</tr>
<tr>
<td>Group B</td>
<td>362.5±56.2</td>
<td>412.7±48.0</td>
</tr>
<tr>
<td>Group C</td>
<td>493.3±14.5</td>
<td>523.3±37.7</td>
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Values are mean±SD.
Neurological Outcome

Measurable neurological deficits were observed within 5 to 10 minutes after MCA:O in each animal. Subsequently, no difference in the course of the animals was observed in group B compared with group C. Group A showed worse neurological scores compared with group B and group C, owing entirely to the contribution of large parenchymal hematomas in 3 animals (Figure 6).

Discussion

Circulating platelets are activated and accumulate in cerebral microvessels within the ischemic territory after MCA:O.7–10 Fibrinogen and polymorphonuclear leukocytes can participate with activated platelets in microvascular deposits that lead to microvascular obstruction and contribute to the focal no-reflow phenomenon.22,23 It is postulated that microvascular obstruction, due in part to platelet activation and leukocyte adhesion, (1) is a consequence of MCA:O and (2) may lead to local ischemia and augmentation of brain cellular injury in primates. To address the first step of this postulate, we have demonstrated that microvascular patency can be restored by inhibiting the platelet integrin $\alpha_{IIb}\beta_3$-fibrinogen interaction before and during MCA:O. Two dose/concentrations of the well-characterized integrin $\alpha_{IIb}\beta_3$ inhibitor TP9201 encompassing the IC$_{30}$ and IC$_{80}$ of platelet aggregation (heparin) produced microvascular patency; however, symptomatic hemorrhage occurred only at the higher dose. A low risk of detectable hemorrhagic transformation accompanied the low dose/concentration. The present study confirms a role for platelet activation in functional microvascular obstruction generated during ischemic brain injury. A central implication of these experiments is that careful characterization of the precise target and hemorrhagic risk of the specific integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) inhibitor before its application to clinical ischemic stroke is categorically required for its use in acute brain ischemia to avoid excessive clinically significant parenchymal hemorrhage.

![Figure 3](image-url). TP9201 plasma levels obtained during 3 hours of MCA:O and 1 hour of reperfusion (R) in group A (circles) and B (squares) animals. Broken lines indicate the plasma levels predicted from the experiments in Figures 1 and 2 and separate data.

![Figure 4](image-url). Relative reflow (patency) within separate microvessel diameter categories in animals receiving TP9201 at IC$_{30}$ (group A, solid bars), TP9201 at IC$_{80}$ (group B, stippled bars), and no TP9201 (group C, open bars) before and during MCA:O. Cohorts A and B displayed normal patency, which was significantly increased compared with cohort C in the 4.0- to 7.5-$\mu$m-diameter ($P=0.01$ and 0.02, respectively) and 7.5- to 30.0-$\mu$m-diameter ($P=0.02$ and 0.002, respectively) categories. Other comparisons were not significantly different.

![Figure 5](image-url). Intracerebral hemorrhage by 4 hours after MCA:O. In group A, 3 of 4 animals displayed large symptomatic parenchymal hematomas (PH); in group B, 1 of 4 animals displayed a hemorrhagic infarction (HI); and in group C (control), no hemorrhage (nil) was visible ($P=0.036$).

![Figure 6](image-url). Neurological outcome. Group A demonstrated worse neurological scores than did groups B and C by 4 hours after MCA:O. This course was consistent with the significant contribution of symptomatic hemorrhage to the outcome of group A (see text).
Activation of platelets within cerebral microvessels is a consequence of ischemia. These experiments corroborate our initial observation of platelet deposition within the ischemic basal ganglia in the nonhuman primate model.\textsuperscript{9} Inhibition of fibrin generation by heparin and blockade of the platelet ADP receptor by ticlopidine, in combination, reduced microvascular platelet deposition and occlusion formation. At least 3 further findings support the participation of platelets in microvascular occlusions after MCA:O in the nonhuman primate: (1) the time-dependent and monotonic increase in the number of microvessels displaying fibrin accumulation,\textsuperscript{27} (2) the time-dependent accumulation of activated platelets within the ischemic microvasculature,\textsuperscript{7} and (3) the appearance of platelet-fibrin-leukocyte aggregates within ischemic cerebral microvessels, indicating activation of both cell types.\textsuperscript{9} Implied by those observations is the interaction of the platelet integrin $\alpha_{\text{IIb}}\beta_{3}$ receptor with fibrinogen and thrombin generation stimulating both fibrin formation and platelet activation. More recently, Garcia et al\textsuperscript{10} described the accumulation of platelets within microvessels in peripheral ischemic zones after MCA:O in the Wistar rat. However, it has remained unclear whether platelet accumulation per se could cause microvascular obstruction and hence brain tissue injury or whether the obstruction results from the tissue injury itself or both.

Given the redundancy of platelet activation pathways, inhibition of steps upstream from adhesion or aggregation produces a relative blockade of platelet aggregation.\textsuperscript{11,13,14} Hence, the inhibition by aspirin, a noncompetitive inhibitor of cyclooxygenase, and by ticlopidine, a thienopyridine non-competitive ADP receptor antagonist, is relative and may be defeated by agonists other than thromboxane A$_2$ or ADP. The final step of platelet aggregation involves the interaction of the glycoprotein integrin $\alpha_{\text{IIb}}\beta_{3}$ with an RGD sequence on the 2 fibrinogen Aa chains.\textsuperscript{12} This interaction is irreversible, and because the receptor is expressed only on platelets, it is specific. Inhibition of this integrin receptor–ligand interaction by potentially irreversible antiplatelet agents (eg, monoclonal antibodies) may be disadvantageous because of the risk of significant hemorrhage with pathologies in which platelet activation plays a role.\textsuperscript{28,29} This is of primary importance for central nervous system vascular processes in which antiplatelet agents have been shown to contribute to the risk of intracerebral hemorrhage.\textsuperscript{30}

A range of inhibitor subclasses that demonstrates relative species dependence with regard to fibrinogen binding blockade has been developed.\textsuperscript{31,32} Generally, significantly greater potency attends their inhibition of human and nonhuman primate platelet aggregation than that of dogs, rabbits, and rodents.\textsuperscript{31} Although the molecular natures of these species-related differences are not understood, they underscore the relevance of a nonhuman primate model to human clinical studies. The humanized Fab fragment of the monoclonal antibody (c7E3) has been shown to inhibit integrin $\alpha_{\text{IIb}}\beta_{3}$-dependent platelet aggregation, platelet-rich thrombus formation, and reocclusion after coronary artery angioplasty.\textsuperscript{15} Immunogenicity, thrombocytopenia, dose titration, and limitations in the management of hemorrhage associated with the platelet defect are concerns for use of this agent in cerebral nervous system injury.\textsuperscript{33} There is no report testing this agent in experimental cerebral ischemia. Selected snake venom peptides (disintegrins), which are potent integrin $\alpha_{\text{IIb}}\beta_{3}$-blocking agents by virtue of competition for the RGD site on fibrinogen, have therapeutic potential.\textsuperscript{34} Many such peptides are nonspecific, with high affinities for other receptors, including the integrins $\alpha_{\beta_{1}}, \alpha_{\beta_{2}},$ and $\alpha_{\beta_{3}}$.\textsuperscript{35,36} Major side effects include their immunogenicity, significant alterations in hemostasis marked by increased bleeding times, and the potential for intracerebral and gastric hemorrhage.\textsuperscript{37}

A number of small peptide and nonpeptide RGD mimetics, which selectively and reversibly block integrin $\alpha_{\text{IIb}}\beta_{3}$, some of which have been investigated clinically, have been synthesized.\textsuperscript{38–40} TP9201 was selected as a potential therapeutic candidate from a panel of RGD-containing peptide compounds because it does not prolong the bleeding time at doses that inhibit ex vivo platelet aggregation in humans, nonhuman primates, and dogs.\textsuperscript{18–20} It is less active by more than an order of magnitude in guinea pigs, rabbits, rats, and mice. TP9201 also competes with Ca$^{2+}$ for binding to the integrin receptor. This property is conferred by the presence of a positive charge that is 2 residues C-terminal to the RGD sequence\textsuperscript{41} and implies increased inhibition of the fibrinogen–integrin $\alpha_{\text{IIb}}\beta_{3}$ interaction below physiological Ca$^{2+}$ concentrations. The IC$_{30}$ (heparin) is achieved at a TP9201 concentration that produces complete inhibition of platelet aggregation to ADP in hypocalcemic conditions (IC$_{30}$ [citrate]) (Figure 2). The present studies are consistent with earlier results indicating that TP9201 plasma levels at IC$_{30}$ (heparin) are sufficient to achieve appropriate in vivo efficacy\textsuperscript{19,20} while avoiding the deleterious consequences of hemorrhagic transformation and increased bleeding times shown in the present study. Furthermore, in several models, complete large-artery patency could be achieved at IC$_{50}$ (heparin) measured ex vivo in heparinized plasma without bleeding time elevation.\textsuperscript{18–20} Finally, thrombocytopenia did not confound any experiments.

There are few data involving well-characterized focal cerebral ischemia models with highly specific integrin $\alpha_{\text{IIb}}\beta_{3}$ inhibitors. Strict species requirements have dictated that preclinical studies against the platelet receptor have been mostly limited to nonhuman primates.\textsuperscript{31,32} However, intravenous weight-adjusted doses of the organic integrin $\alpha_{\text{IIb}}\beta_{3}$ inhibitor SDZ GPI 562 produced a reduction in ischemic injury volume at 24 hours in one study with a murine MCA:O model.\textsuperscript{42} GPI 562 displayed an IC$_{50}$ of 11 $\mu$mol/L to ADP-induced aggregation of isolated murine platelets in heparin, 5-fold higher than that for isolated baboon platelets to TP9201 ex vivo (Figure 2). A salutary effect on injury volume correlated with the inhibition of $^{11}$In-labeled platelet accumulation at 24 hours and a reduction in fibrin deposition. The latter findings are consistent with the effects of anti–tissue factor strategies during MCA:O in the nonhuman primate.\textsuperscript{33} However, it could not be excluded that GPI 562 at the high doses used also affected the course of tissue injury by independent effects. Hemorrhage confounded the surgical procedures and increased dose-dependently at 24 hours with GPI 562.

The increased microvascular patency at the IC$_{50}$ and IC$_{30}$ of TP9201 confirms the participation of activated platelets in

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cerebral microvascular responses to MCAO.9 Platelet activation and accumulation are in accord with the progressive accumulation of fibrin within target microvessels after MCAO.23,27 The distribution of microvascular occlusions was heterogeneous within the ischemic territory, where patent vessels appeared adjacent to occluded ones. Whereas 10% of microvessels appear activated by 2 hours of MCA:O (eg, vascular endothelial growth factor and integrin αvβ3 expression),43 loss of patency occurs in up to 70% of microvessels, mostly capillaries and postcapillary venules, by 4 hours of MCA:O. This suggests the rapid recruitment of vessels by acute and progressive microvascular occlusion in the ischemic bed.

Hemorrhagic transformation is a natural accompaniment of ischemic stroke and occurs in up to 65% of patients with carotid territory ischemia.43,44 Parenchymal hematomas, most often symptomatic, are associated with anticoagulation45,46 and less so with antiplatelet agents, including aspirin.40 However, integrin αvβ3 inhibitors may produce substantial blockade of platelet aggregation and clinically significant peripheral hemorrhage.15,47 Significant parenchymal hemorrhage with early neurological deterioration at TP9201 plasma concentrations equivalent to IC50 indicates the necessity of competent platelet activation to limit or prevent cerebral hemorrhage. Although the mechanisms of early hemorrhage are not discernible in the present study, this observation underscores the potent effects on hemostasis of GPIIb/IIIa inhibitors at unregulated doses to increase the risk of brain hemorrhage. Although uncertain, it cannot be excluded that the large vessels in group A were the sources of the parenchymal hematomas. The benign character of the single visible hemorrhage in group B suggests that the group A hemorrhages were most probably extensions of microvascular hemorrhages seen in the absence of antithrombotic treatment in this model.48

The finding of complete microvascular patency with 30% inhibition of platelet aggregation and no substantial hemorrhage under conditions that produce microvascular occlusion indicates that only partial blockade of the platelet-fibrinogen interaction is required to produce patency. With near complete inhibition of the integrin αvβ3–fibrinogen interactions, clinically devastating intracerebral hemorrhage results, indicating that the characteristics of this inhibitor and its dose adjustment are critical to the generation of significant intracerebral hemorrhage. However, the present experiments also indicate that dose adjustments for efficacy and cerebral hemorrhagic risk must take into account the degree of platelet aggregation response to inhibitor levels, species considerations, Ca2+ concentration, and specific characteristics of the inhibitor. It is incorrect to assume that an IC50 dose of another inhibitor of the integrin αvβ3–fibrinogen interaction is equivalent to the effect of TP9201 at its IC50. Moreover, these experiments set the stage to test the second step of the hypothesis, that early inhibition of platelet aggregation that produces microvascular occlusion formation leads to a substantial decrease in neuron injury in a clinically relevant stroke model.

Acknowledgments

This study was supported in part by grant R01 NS-26945 (Dr del Zoppo) of the National Institute of Neurological Disorders and Stroke and a grant from Integra LifeSciences Corp. We are very grateful for the expect technical assistance of Pearl Akamine, Jacinta Lucero, and James O. Tolley.

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12. Pytelia R, Perschbacher MD, Ginsberg MH, Plow EF, Ruossli E. Platelet membrane glycoprotein IIb/IIIa inhibitors of unregulated doses to increase the risk of brain hemorrhage. Although uncertain, it cannot be excluded that the large vessels in group A were the sources of the parenchymal hematomas. The benign character of the single visible hemorrhage in group B suggests that the group A hemorrhages were most probably extensions of microvascular hemorrhages seen in the absence of antithrombotic treatment in this model.48

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Platelets have been implicated, but not proven, as injurious participants in ischemic stroke. These blood-borne elements are likely activated on transit through an ischemic microcirculatory bed, and abnormal platelet accumulation has been demonstrated within brain after both focal and global cerebral ischemic insults.1–3 The molecular means by which platelet aggregation and degranulation are achieved have been extensively studied, although little is known about these mechanisms under complex ischemic conditions. The platelet glycoprotein (GP) GPIIb/IIIa is a member of the integrin family (integrin \( \alpha_{\text{IIb}}\beta_3 \)) and the single most abundant species of GP on the platelet surface. This molecule is well recognized as a receptor for fibrinogen and mediates many cohesive platelet interactions. GPIIb/IIIa also actively participates in various types of intraplatelet signaling, for example with other GPs (GPIb/IX/V), leading to aggregation during shear stress.4 The prevailing hypothesis relative to stroke is that microvascular platelet and platelet product deposition GPIIb/IIIa receptor antagonist, enhances and sustains coronary arterial thrombolysis with recombinant tissue-type plasminogen activator in a canine preparation. Circulation. 1991;83:1038–1047.


conclusions drawn about efficacy as a therapeutic agent. It remains to be shown that improved perfusion during the first hour of reperfusion will alter histological and functional outcome in the intact animal. We anticipate with interest further studies to better understand the relative necessity of preserved versus inhibited platelet activation/aggregation in reducing neuronal damage without hemorrhagic sequelae.

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Stroke. 2000;31:1402-1410
doi: 10.1161/01.STR.31.6.1402
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
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