The Nonpeptide Glycoprotein IIb/IIIa Platelet Receptor Antagonist SM-20302 Reduces Tissue Plasminogen Activator–Induced Intracerebral Hemorrhage After Thromboembolic Stroke

Paul A. Lapchak, PhD; Dalia M. Araujo, PhD; Donghuan Song, MD; Justin A. Zivin, MD, PhD

Background and Purpose—Platelet activation and deposition in brain microvessels appear to be key events in the pathogenesis of ischemia-induced neuronal degeneration and behavioral deficits. It has been hypothesized that activated platelets in combination with polymorphonuclear leukocytes and fibrin may play a role in vessel reocclusion leading to the “no-reflow” phenomenon after administration of the thrombolytic tissue plasminogen activator (tPA). We studied the effects of the novel glycoprotein IIb/IIIa platelet receptor antagonist SM-20302 when administered in combination with tPA on infarct and hemorrhage rate and volume to determine whether activated platelets are involved in either infarct or hemorrhage generation after a thromboembolic stroke.

Methods—One hundred thirty-two male New Zealand White rabbits were included in the present study. Rabbits were embolized by injecting a blood clot into the middle cerebral artery via a catheter. Five or 65 minutes after embolization, SM-20302 (5 mg/kg) was infused intravenously. In drug combination studies, tPA was infused intravenously for 30 minutes starting 60 minutes after embolization, and SM-20302 was administered 5 or 65 minutes after embolization. Postmortem analysis included assessment of hemorrhage, infarct size and location, and clot lysis.

Results—In the vehicle control group, the hemorrhage rate after a thromboembolic stroke was 33%. There was a significant increase (109%) in the hemorrhage rate in the group of rabbits treated with the thrombolytic tPA. SM-20302 by itself did not significantly alter the embolism-induced hemorrhage rate when administered either 5 or 65 minutes after embolism. The SM-20302 groups had a 42% and 33% incidence of hemorrhage in the 5- and 65-minute groups, respectively. In groups treated with a combination of drugs, the SM-20302/tPA group had a 31% and 71% incidence of hemorrhage when SM-20302 was administered 5 and 65 minutes after embolization, respectively. SM-20302 in combination with tPA also significantly increased infarct rate, but not hemorrhage or infarct volume.

Conclusions—This study suggests that treatment of thromboembolic stroke with the combination of a platelet inhibitor and tPA may have a beneficial outcome on the basis of the following: First, acute administration of SM-20302 did not significantly increase hemorrhage rate. Second, SM-20302 in combination with tPA significantly reduced tPA-induced intracerebral hemorrhage. Third, there appears to be a specific window of opportunity when a platelet inhibitor must be administered to produce a beneficial effect. Overall, on the basis of our results, we hypothesize that the increased rate of intracerebral hemorrhage observed after tPA administration may be partly due to increased reclosure of cerebral vessels following lysis of the emboli and that reocclusion can be controlled by administration of a platelet inhibitor.

Key Words: ischemia • neuroprotection • platelet aggregation • reperfusion injury • tissue plasminogen activator • vascular diseases • rabbits

Recent evidence obtained from various preclinical animal models suggests that even though thrombolytics such as tissue plasminogen activator (tPA) are beneficial, tPA may not always resume reperfusion rapidly enough to prevent the onset of neuronal degeneration. Additionally, there is substantial evidence indicating that thrombolytics increase the incidence of intracerebral hemorrhage (ICH) in preclinical animal models of stroke and, more importantly, in stroke patients. With these observations in mind, there is a need to develop new drugs that may be used in combination with tPA not only to manage secondary tissue damage but also to reduce the risk of tPA-induced ICH. Because of this, we focused on specific mechanisms involved in tissue damage after an embolic...
stroke.4 We developed an animal model to investigate specific biochemical mechanisms involved in ischemia and hemorrhage after a thromboembolic stroke.3,4 In the model, we found that tPA significantly increases ICH.3,4 The model has proven to be useful in testing the direct effects of novel pharmacological agents since we found that we can significantly reduce tPA-induced hemorrhage by administration of matrix metalloproteinase antagonists and spin trap agents.3,4 The results suggest that tPA-induced hemorrhage may be related to activation of numerous mechanisms after reperfusion-induced injury.3 tPA infusion after an embolic stroke allows for rapid dissolution of the embolus, resulting in reperfusion of a portion of the ischemic brain tissue.1,9 As a result of tPA-induced reperfusion, there is accumulation of platelets and polymorphonuclear leukocytes in the microvasculature in the previously ischemic tissue. Calif10 recently emphasized that even after initial tPA-induced reperfusion is achieved, reocclusion (transient and permanent) occurs often and is associated with high mortality rates in patients.

In the present study we tested the hypothesis that platelet activation and subsequent accumulation in microvessels may be involved in the generation of infarcts and ICH. To our knowledge, a study describing the pharmacological effects of a platelet inhibitor in combination with a thrombolytic has not been conducted in an animal model that has been used successfully to develop a stroke treatment approved by the Food and Drug Administration.11 Therefore, we determined the pharmacological effects of administration of the novel nonpeptide glycoprotein IIb/IIIa platelet inhibitor SM-20302 on hemorrhage and infarct rate in the presence or absence of tPA treatment in a thromboembolic stroke model.

Materials and Methods
This study was conducted in accordance with institutional guidelines. Male New Zealand White rabbits weighing 2 to 3 kg were used. The common carotid artery was catheterized as described previously.3,4 Fresh emboli were prepared, and the rabbits wereembolized according to the procedure of Lapchak et al.3,4 Briefly, whole blood was collected from a donor rabbit, and tracer quantities of labeled 15-μm microspheres were mixed into the blood. It was then allowed to clot for 2 hours at 37°C and cut into pieces of approximately 25 mm (representing 3.2- to 3.8-mg clots). After a 3-hour recovery period, rabbits were embolized. For embolization, a clot was suspended in 0.5 mL of normal saline and transferred to the injection catheter hub with forceps. After the cap was replaced, 5.0 mL of saline was injected to push the clot into the cerebral arterial system. If the animal did not react behaviorally (nystagmus, hemiparesis, seizure) to the embolization, a second blood clot was injected in the same way 3 minutes after the first embolization. If there was no behavioral reaction to either embolization, no further blood clots were administered. Animals that had no behavioral reaction after administration of 2 clots were treated in the same manner as animals responding to emboli. Inclusion or exclusion of animals was based on the criteria described below. After the embolization process was completed, the catheter was ligated close to the neck, and the rest of the catheter and injection port was cut off.

Animals that received all pharmacological treatments but died before euthanasia were included in the study, and the brains were fixed and sectioned as below. The surviving animals were killed 48 hours after embolization. The brains were removed and immersion fixed in 4% paraformaldehyde for at least a week and then examined by a treatment-blinded observer. The right middle cerebral artery (MCA) of each brain was examined for the presence of emboli. The surface blood vessels were then stripped from the right hemisphere of each brain. The cerebellum was also removed from the brain stem. Hemispheres and brain stem were cut into five 5-mm-thick coronal slices, each having 2 faces, for a total of 10 faces. We noted the presence, location, size, and type of each hemorrhage and infarct. We recorded the size of hemorrhage as the number of section faces showing hemorrhage.12,13 Infarction was grossly visible as pale, softer tissue surrounded by pink, normal brain tissue on the brain sections. Three major types of hemorrhage were identified with the use of the grading system we used in previous studies.3,4,14–16 The 3 types of hemorrhage are as follows: (1) necrotic hemorrhagic infarction was indicated by red speckling of an area, usually surrounded by soft infarcted tissue; (2) punctate hemorrhage consisted of isolated small red marks within normal tissue that did not extend through the tissue as a blood vessel would; and (3) parenchymatous ICH was a large homogeneous mass of blood within the tissue. After evaluation for hemorrhage and infarcts, the total radioactivity in the brains was measured by placing the slices into a gamma counter. The surface vessels from the right hemisphere were placed in a separate container and counted. Then the cerebellum, each hemisphere, and brain stem were counted in separate tubes. The amount of radiolabel present in the brain (including the right hemisphere vessels) was compared with that contained in the labeled blood clot at embolization. If <10% of the counts were found in the brain and vessels, it was assumed that the labeled blood clot had not reached the brain.17 The data from these animals were excluded from further analysis. Thrombolysis was defined in 2 ways: by recovery of radioactive label and by visual inspection. Any brains containing <20% of the total recovered radioactivity in the surface vessels of the right hemisphere were said to have undergone thrombolysis of the embolus. Postmortem, we recorded whether a clot was visible in the MCA. This observation correlated with the recovery of radioactivity in our prior studies.3,4,14,16,18–20

Drug Administration
We randomly allocated the rabbits into 6 different treatment groups before the embolization procedure. The randomization sequence was generated with the use of randomization tables, and concealment of the randomization was achieved by use of an independent third party. The randomization sequence was not revealed until all postmortem analyses were complete. Sample size was based on power analysis, with α=0.05 and β=0.90, a coefficient of variation of 15%, and a difference between means of 20%. It was determined that a sample size of approximately 12 to 14 animals per group was required. Our previous experience with this stroke model indicates that we actually need an average of 20 animals per group because of premature losses caused by various preparation difficulties or deaths after embolization before treatments can be fully administered. The treatment groups were as follows: vehicle/vehicle (n=18), vehicle/tPA (n=19), SM-20302 (5 minutes after embolism)/vehicle (n=24), SM-20302 (65 minutes after embolism)/vehicle (n=21), SM-20302 (5 minutes after embolism)/tPA (n=26), and SM-20302 (65 minutes after embolism)/tPA (n=24). The study medications were administered in an open-label fashion, but persons performing assessment of study outcomes remained blinded to the treatment allocation.

The platelet inhibitor SM-20302 was dissolved in normal saline and administered at a dose of 5 mg/kg by intravenous infusion over 30 minutes starting 5 or 65 minutes after embolization. This dose of SM-20302 was chosen on the basis of previous in vivo pharmacological studies that showed that doses in the range of 0.1 to 10 mg/kg inhibited platelet aggregation and maintained vessel patency in vivo.21,22 In the remaining groups of rabbits, we then administered tPA or vehicle 1 hour after embolization according to the aforementioned schedule. The tPA regimen used in this study was as follows: 3.3 mg/kg tPA. 20% as a bolus injection given over 1 minute, followed by the remainder infused over 30 minutes.3,4,23 Genentech, Inc supplied tPA and its vehicle. tPA was supplied as a lyophilized cake in 50-mg configurations, containing 50 mg tPA (29 million IU), 1.7 mg L-arginine, 0.5 g phosphoric acid, and <4 mg polysorbate 80. The tPA was reconstituted with sterile water at a concentration of 1 mg/mL. We analyzed the data with the χ² test corrected for multiple
comparisons using the Bonferroni technique and ANOVA where relevant.

**Results**

**Stroke Success Rate**

Of 175 embolized rabbits included in the study, we found that 132 rabbits (76%) had >10% recovered radioactivity in the brain postmortem and were included in the statistical analyses of the present study (Table 1). The majority of successfully embolized rabbits responded by behavioral manifestations, including nystagmus, pupillary dilation, hemiparesis, or brief uncoordinated jerking movements. Rabbits that did not have behavioral manifestations of embolization, but did have >10% recovered in the brain postmortem, were included in the analyses. The remaining 24% of the rabbits had ≤10% of the label present in the brain postmortem, indicating that the injected blood clot did not reach the brain. The rabbits that did not reach criteria were excluded from the study, and the data were not used in the final analysis. This success rate corresponds well with other studies involving this model.3,4,14,17

**Hemorrhage Rate, Types of Hemorrhage, and Hemorrhage Volume**

Tables 2 and 3 present the hemorrhage rate for the 6 different treatment groups included in this study. There was a statistically significant difference in hemorrhage rate between the SM-20302 groups (P<0.05) and the vehicle control group. The hemorrhage rate in the group treated with the combination of SM-20302/tPA was significantly decreased (P<0.01) by 55% when SM-20302 was administered 5 minutes after embolism; however, SM-20302 did not significantly alter tPA-induced hemorrhage when administered 5 minutes after the start of tPA infusion (ie, 65 minutes after embolism).

Table 2 shows the types of hemorrhage present in each of the experimental groups. Most of the hemorrhages seen were characterized as necrotic hemorrhagic infarction and punctate hemorrhage. In only 1 instance was a parenchymatous ICH observed. Some of the animals had >1 type of hemorrhage present in the brain. For quantitative purposes, we treated each individual hemorrhage observed as a separate entity. Hemorrhages occurred at various locations in individual animals, including the following structures: caudate putamen; thalamus; hippocampus; frontal, parietal, and occipital cortex; hypothalamus; suprachiasmatic area; cerebellum; pons; and midbrain.

Table 4 shows the number of faces with observed hemorrhage, which is a measure of hemorrhage volume. For each animal in the study, the maximum number of faces observed is 10. There were no statistically significant differences among the 6 treatment groups.

**Infarct Rate and Volume**

Table 3 presents the infarct rate data from the 6 experimental groups used in this study. We determined whether SM-20302

<table>
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<tr>
<th>TABLE 1. Effect of Pharmacological Treatments on Percentage of Successful Cerebral Embolization in Rabbits After Embolus Injection</th>
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<tbody>
<tr>
<td><strong>Treatment Group</strong></td>
</tr>
<tr>
<td>Vehicle + vehicle</td>
</tr>
<tr>
<td>Vehicle + tPA</td>
</tr>
<tr>
<td>SM-20302 (5 min) + vehicle</td>
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<tr>
<td>SM-20302 (65 min) + vehicle</td>
</tr>
<tr>
<td>SM-20302 (5 min) + tPA</td>
</tr>
<tr>
<td>SM-20302 (65 min) + tPA</td>
</tr>
</tbody>
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There were no significant differences (P<0.05) among the 6 groups.

<table>
<thead>
<tr>
<th>TABLE 2. Effect of Pharmacological Treatments on Hemorrhage Types and Incidence After Thromboembolic Stroke</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type of Hemorrhage</strong></td>
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<tr>
<td>Vehicle + vehicle</td>
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<tr>
<td>Vehicle + tPA</td>
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*Hln indicates necrotic hemorrhagic infarction; Hn, punctate hemorrhage; and PICH, parenchymatous ICH.

*P<0.01.
TABLE 4. Effect of Pharmacological Treatments on Hemorrhage and Infarct Volume After Thromboembolic Stroke

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Hemorrhage Volume, No. of Faces</th>
<th>Infarct Volume, No. of Faces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle + vehicle</td>
<td>4.00±0.83</td>
<td>6.42±1.26</td>
</tr>
<tr>
<td>Vehicle + tPA</td>
<td>2.65±1.16</td>
<td>5.00±0.57</td>
</tr>
<tr>
<td>SM-20302 (5 min) + vehicle</td>
<td>3.00±0.39</td>
<td>4.74±0.51</td>
</tr>
<tr>
<td>SM-20302 (65 min) + vehicle</td>
<td>3.80±0.96</td>
<td>5.87±2.07</td>
</tr>
<tr>
<td>SM-20302 (5 min) + tPA</td>
<td>4.67±1.38</td>
<td>4.88±0.82</td>
</tr>
<tr>
<td>SM-20302 (65 min) + tPA</td>
<td>2.61±0.29</td>
<td>4.67±0.71</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Neither infarct nor hemorrhage volumes were significantly altered by administration of any drug.

Discussion

The novel nonpeptide glycoprotein IIb/IIIa receptor antagonist SM-20302 was shown to significantly reduce tPA-induced ICH when administered before the thrombolytic but was ineffective at reducing the hemorrhage rate when administered after the start of tPA infusion. SM-20302 by itself did not significantly alter embolism-induced hemorrhage with the use of either treatment regimen.

The finding that the platelet inhibitor affects hemorrhage rate after tPA administration strongly suggests that in our hemorrhage model, platelet activation and aggregation are important steps in reperfusion-induced injury and hemorrhage. In various models, SM-20302 has previously been shown to block the final common pathway in platelet aggregation, thus maintaining vascular integrity and patency.

Our study is in agreement with a previous acute focal cerebral ischemia study of Abumiya et al.26 The researchers showed that the antiplatelet compound TP9201 effectively increased microvascular patency after MCA occlusion. However, they also noted that TP9201 caused a dose-dependent increase in hemorrhagic transformations. Platelet inhibitors have also been shown to be effective in a murine stroke model. Coudhri et al27 showed that SDZ GPI 562 reduced infarct volume after MCA occlusion by reducing microvascular thrombosis. When these findings are taken together, it appears that platelet inhibitors may have multiple benefits in the treatment of cerebral ischemia. First, on the basis of the literature, platelet inhibitors may reduce ischemia-induced neuronal degeneration and possibly behavioral deficits. It is not surprising that in our study SM-20302 did not alter infarct volume because the model is based on injection of a large 3- to 4-mg blood clot directed toward the MCA, and SM-20302 was administered after embolism. Moreover, in our model the initial stroke is independent of the consequences of platelet/polymorphonuclear leukocyte activation and aggregation. Once the blood clot blocks the MCA, a large area of the brain has reduced blood flow and quickly becomes ischemic, and we observe an immediate behavioral response to embolus injection. Second, on the basis of our results with tPA, it appears that platelet inhibitors may reduce the incidence of hemorrhage after administration of thrombolytics. The seemingly paradoxical results concerning hemorrhage rate obtained with the use of different animal models requires further investigation. Nevertheless, in our model SM-20302 was quite effective in reducing tPA-induced hemorrhage. In the present study the majority of hemorrhage was of the necrotic hemorrhagic infarction and punctate hemorrhage types, with only 1 incidence of parenchymatous ICH, and SM-20302 was most effective at reducing necrotic hemorrhagic infarction. However, from a clinical perspective, the most significant type of hemorrhage is parenchymatous ICH.28 Additional studies with a variation of the present model,13 which has a higher incidence of parenchymatous ICH, are required to determine whether SM-20302 can also reduce parenchymatous ICH.

Numerous clinical trials, such as the Strategies for Patency Enhancement in the Emergency Department (SPEED), Evaluation of IIb/IIIa Platelet Receptor Antagonist 7E3 in Preventing Ischemic Complications (EPIC), Global Utilization of Streptokinase and tPA for Occluded Arteries (GUSTO), and Blockade of the Glycoprotein IIb/IIIa Receptor to Avoid Vascular Occlusion (BRAVO) trials, have focused on the effectiveness of antiplatelet compounds in reducing vascular occlusion after coronary
heart disease.10,29–31 The results of the trials have been mixed, and it is becoming increasingly apparent that long-term administration of certain platelet inhibitors can have significant toxic side effects. The toxicity includes peripheral and cerebral hemorrhage30–35 and thrombocytopenia.34 A review of the pertinent literature indicates that the majority of the side effects noted may be related to chronic rather than acute administration of platelet inhibitors. However, there are some exceptions to that statement. For instance, randomized clinical trials have shown that there is an increased risk of hemorrhagic complications in patients treated acutely with antiplatelet agents such as aspirin before thrombolysis.36 This, together with the observation that there was an increase in infarct rate in the 2 groups of rabbits that received SM-20302 and tPA, suggests that this form of combination therapy may not be optimal to treat embolic stroke. However, with that observation in mind, we propose, on the basis of our data, that acute administration of a platelet inhibitor such as SM-20302, which has a relatively long half-life (t1/2 approximately 3 hours24) reduces tPA-induced hemorrhage, possibly as the result of maintaining vascular patency.

In conclusion, we show that effective combination drug treatments can be developed as novel treatments for stroke. Preadministration of the platelet inhibitor SM-20302 significantly reduced tPA-induced hemorrhage. However, the compound significantly increased infarct rate when administered in combination with tPA. Our study suggests that platelet inhibitors should be further investigated as an adjunct therapy to tPA. However, caution should be exercise because platelet inhibitors may paradoxically decrease tPA-induced hemorrhage while increasing infarct size. It is likely that combined therapies of various classes of drugs will be required to optimize stroke management, including additional treatment strategies to provide neuroprotection to neuronal populations that become ischemic after primary vessel occlusion.

Acknowledgments

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References


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