Neuroprotective Effect of SolCD39, a Novel Platelet Aggregation Inhibitor, on Transient Middle Cerebral Artery Occlusion in Rats

Ludmila Belayev, MD; Larissa Khoutorova, BS; Theresa A. Deisher, PhD; Andrey Belayev, BS; Raul Busto, BS; Yongbo Zhang, MD, PhD; Weizhao Zhao, PhD; Myron D. Ginsberg, MD

Background and Purpose—SolCD39 is a soluble form of recombinant human ecto-ATP/ADPase (NTPDase1) and represents a new class of antithrombotic agents. SolCD39 blocks and reverses platelet activation, preventing recruitment of additional platelets into a growing thrombus. The purpose of this study was to examine the effect of solCD39 on neurological deficit, infarct size, and extent of edema after transient middle cerebral artery occlusion (MCAO) in rats.

Methods—Physiologically controlled Sprague-Dawley rats underwent 2-hour MCAO by retrograde insertion of an intraluminal suture coated with poly-L-lysine. The agent (solCD39) was administered intravenously before MCAO or at 1-hour or 3-hour recirculation. Other groups received vehicle (Tris-buffered saline) or human albumin (as a “positive” neuroprotective control; 25%, 0.5% of body weight) at 1-hour recirculation. Neurological status was evaluated during occlusion (at 60 minutes) and daily for 3 days after MCAO. Brains were perfusion-fixed at 72 hours, and infarct volumes and brain swelling were determined.

Results—Pretreatment with solCD39 significantly improved the neurological score at 72 hours compared with the vehicle group (4.4±0.6 versus 7.6±0.6, respectively; \( P=0.008 \)). Cortical infarct areas were significantly reduced at multiple levels by pretreatment with solCD39. Total striatal infarct area was also significantly reduced compared with vehicle by both solCD39 pretreatment (48% mean reduction) and solCD39 treatment at 3-hour recirculation (51% mean reduction). Treatment with SolCD39 significantly reduced total infarct volume (corrected for brain swelling) by an average of 71% to 72% when administered either before ischemia or at 3 hours of recirculation compared with vehicle. Treatment with albumin significantly reduced neurological score and total, cortical, and subcortical infarction at multiple levels, as expected.

Conclusions—Treatment with SolCD39, administered either before or at 3 hours after MCAO, improves neurological score and reduces infarct size compared with vehicle. A pharmacological agent of this type appears to have potential for the treatment of focal ischemic stroke. (Stroke. 2003;34:758-763.)

Key Words: brain edema • cerebral ischemia, focal • middle cerebral artery occlusion • neuroprotection • rats

Stroke is one of the leading causes of death in the world. There is currently no effective treatment for ischemic stroke with the exception of recombinant tissue plasminogen activator. The rationale for thrombolytic therapy is based on the recognition that most ischemic strokes are caused by thrombotic or thromboembolic arterial occlusions.1,2 Therapeutic strategies designed to restore cerebral perfusion in a timely fashion have the potential to limit the cellular, biochemical, and metabolic consequences of cerebral ischemia that ultimately lead to irreversible brain injury. Although intravenous administration of recombinant tissue plasminogen activator within 3 hours of symptom onset improves outcome at 3 months without increasing the risk of death,3 it nonetheless increases the incidence of intracerebral hemorrhage. Platelets become activated and accumulate in brain microvessels of the ischemic microvascular bed after experimental focal cerebral ischemia.4 Microvascular thrombi continue to accumulate even after recanalization of the middle cerebral artery (MCA), contributing to posts ischemic hypoperfusion and ongoing neuronal damage.5 The initial adherence of platelets to the injured vascular wall leads to platelet activation and the release of additional agonists, including ADP, thromboxane A₂, and serotonin. Removal of ADP eliminates platelet recruitment and results in a return of platelets to the resting state.6 Endothelial cell CD39, an ecto-enzyme with ADPase and ATPase activities, rapidly metabolizes ATP and ADP released from activated platelets, thereby abolishing recruitment.7
Physiological Variables During MCA Occlusion

<table>
<thead>
<tr>
<th>Variable</th>
<th>SolCD39 (prior) (n=9)</th>
<th>SolCD39 (1 hour) (n=8)</th>
<th>SolCD39 (3 hours) (n=6)</th>
<th>Vehicle (n=8)</th>
<th>Albumin (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial temp, °C</td>
<td>36.2±0.04</td>
<td>36.2±0.09</td>
<td>36.3±0.13</td>
<td>36.3±0.13</td>
<td>36.1±0.13</td>
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<td>Rectal temp, °C</td>
<td>36.4±0.07</td>
<td>36.3±0.08</td>
<td>36.4±0.11</td>
<td>36.5±0.13</td>
<td>36.3±0.07</td>
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<td>pH</td>
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<td>7.42±0.01</td>
<td>7.41±0.02</td>
<td>7.41±0.00</td>
<td>7.40±0.02</td>
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<td>PO2, mm Hg</td>
<td>109±5</td>
<td>123±8</td>
<td>114±6</td>
<td>113±5</td>
<td>121±6</td>
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<tr>
<td>PCO2, mm Hg</td>
<td>41.8±0.7</td>
<td>39.1±0.5</td>
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<tr>
<td>MABP, mm Hg</td>
<td>108±7</td>
<td>125±4</td>
<td>109±6</td>
<td>119±8</td>
<td>125±2</td>
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<tr>
<td>Plasma glucose, mg/dL</td>
<td>155</td>
<td>49±0.7</td>
<td>45±0.6</td>
<td>48±0.7</td>
<td>48±0.4</td>
</tr>
<tr>
<td>Hematocrit before</td>
<td>48±0.6</td>
<td>49±0.7</td>
<td>45±0.6*</td>
<td>47±0.8</td>
<td>38±0.0*</td>
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<tr>
<td>treatment, %</td>
<td></td>
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<tr>
<td>Hematocrit after</td>
<td>47±0.8</td>
<td>38±0.0*</td>
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<td>treatment, %</td>
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MABP indicates mean arterial blood pressure. Values are mean±SEM. *Different from vehicle group (P<0.05, Student t test).

Recently, a soluble form of the extracellular region of CD39, termed soluble CD39 (solCD39), has been developed, which retains ADPase enzymatic activity.7 SolCD39 effectively depletes ADP from the platelet releasate, thereby inhibiting platelet recruitment. Previous studies have shown that solCD39 blocks ADP-induced human platelet aggregation in vitro7 and inhibits platelet and fibrin accumulation and improves postischemic cerebral perfusion in vivo.8

In this study we examined the effect of solCD39 on neurological deficit, infarct size, and extent of brain swelling after transient MCA occlusion (MCAO) in rats.

Materials and Methods

Animal Preparation

Thirty-five adult male Sprague-Dawley rats (weight, 270 to 330 g; Charles River Laboratories, Wilmington, Mass) were fasted overnight but allowed free access to water. After administration of atropine sulfate (0.5 mg/kg IP), anesthesia was induced with 3.5% halothane in a mixture of 70% nitrous oxide and 30% oxygen. Rats were orally intubated, immobilized with pancuronium bromide (0.6 mg/kg IV), and mechanically ventilated. Temperature probes were inserted into the rectum and the left temporalis muscle, and separate heating lamps were used to maintain rectal and cranial temperatures at normothermic levels. In all rats, polyethylene catheters were introduced into the femoral artery and vein for blood pressure recording, blood sampling, and drug infusion.

MCAO Occlusion

MCAO was induced as described by Zea Longa et al9 and modified by us.10 Under an operating microscope, the right common carotid artery was exposed through a midline neck incision and was carefully dissected free from surrounding nerves and fascia from its bifurcation to the base of the skull. The occipital artery branches of the external carotid artery were then isolated, and these branches were dissected and coagulated. The internal carotid artery was isolated and carefully separated from the adjacent vagus nerve, and the pterygopalatine artery was ligated. Next, a 4-cm length of 3-0 monofilament nylon suture was inserted via the proximal external carotid artery into the internal carotid artery and thence into the circle of Willis, effectively occluding the MCA.

In producing MCAO, we used a previously described poly-L-lysine–coated suture.10 The suture was inserted 20 to 23 mm from the bifurcation of the common carotid artery, according to the animal’s body weight. After the intraluminal suture was placed, the neck incision was closed with a silk suture.

The animals were then allowed to awaken from anesthesia and were returned to their cages. Rats that did not demonstrate a right body weight. After administration of atropine sulfate (0.5 mg/kg IP), anesthesia was induced with 3.5% halothane in a mixture of 70% nitrous oxide and 30% oxygen. Rats were orally intubated, immobilized with pancuronium bromide (0.6 mg/kg IV), and mechanically ventilated. Temperature probes were inserted into the rectum and the left temporalis muscle, and separate heating lamps were used to maintain rectal and cranial temperatures at normothermic levels. In all rats, polyethylene catheters were introduced into the femoral artery and vein for blood pressure recording, blood sampling, and drug infusion.

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The animals were then allowed to awaken from anesthesia and were returned to their cages. Rats that did not demonstrate a right
upper extremity deficit during this recovery period were excluded from further study (see Behavioral Testing). After 2 hours of MCAO, rats were reanesthetized with the same anesthetic combination. Temperature probes were reinserted, and the intraluminal suture was carefully removed. The neck incision was closed with silk sutures, and the animals were allowed to survive for 3 days with free access to food and water.

**Drug Administration**

The drug (solCD39, 8 mg/kg, 0.56 mL) or vehicle (Tris-buffered saline, 0.56 mL) was administered intravenously at different time points. Human serum albumin (25%, 0.5% of body weight) was given at 1-hour recirculation.

**Treatment Groups**

SolCD39 was given before MCAO (n=9) or at 1-hour recirculation (n=8) or 3-hour recirculation (n=6). In other groups, vehicle (n=8) or human serum albumin (n=4) was given at 1-hour recirculation. The human-albumin group served as a “positive control” because our previous studies have shown this agent to be highly neuroprotective.11–13

**Behavioral Testing**

Behavioral tests were performed in all 35 rats before MCAO, during occlusion (at 60 minutes), and 1, 24, 48, and 72 hours after treatment. The battery consisted of 2 tests that have been used previously to evaluate various aspects of neurological function: (1) the postural reflex test, to examine upper body posture while the animal is suspended by the tail; and (2) the forelimb placing test, to examine sensory-motor integration in forelimb placing responses to visual, tactile, and proprioceptive stimuli.13 Neurological function was assessed on a scale of 0 to 12 (normal score = 12), as previously described.15 Rats with convulsions or sustained disturbances of consciousness were excluded from the study; most of these cases prove to have subarachnoid hemorrhage secondary to suture-induced arterial rupture. In the present study 3 animals were excluded for the aforementioned reasons.

**Infarct Assessment**

Animals were allowed to survive for 3 days. Brains were then perfusion-fixed as previously described19 with a mixture of 40% formaldehyde, glacial acetic acid, and methanol (1:1:8 by volume), and brain blocks were embedded in paraffin. Ten-micrometer-thick sections were cut in the coronal plane and stained with hematoxylin and eosin. To quantify infarct volume, histological sections were digitized at 9 standardized coronal levels (MCID image analysis system, Imaging Research Inc), from which data were exported to a UNIX-based workstation for further processing. An investigator blinded to the experimental groups outlined the zones of infarction (which were clearly demarcated) as well as the left- and right-hemisphere contours at each level. Software developed by us was then used to quantify infarct size and brain swelling. To correct for changes in tissue water content (swelling), the following equation was applied to each coronal section:

$$A_{\text{corrected}} = A_{\text{infarct}} \times \left(1 - \frac{A_I - A_C}{A_C}\right)$$

where $A_I$ is the ipsilateral hemisphere area and $A_C$ is the contralateral hemisphere area. Infarct volumes were then determined by integrating infarct areas with the use of the trapezoidal rule.

**Statistical Analysis**

Neurological score, cortical and subcortical infarct areas, total infarct volumes, and brain swelling were compared by 1-way ANOVA, and, in the case of cortical and subcortical infarct areas, repeated-measures ANOVA, with appropriate post hoc tests to correct for multiple comparisons. Physiological variables were compared by Student’s t test. *P<0.05 was regarded as significant. Values are presented as mean±SEM values.

**Results**

**General Physiological Variables**

All animals of this study showed similar values for rectal and cranial temperatures, blood pressure, arterial blood gases, and plasma glucose before, during (Table), and after MCAO. Hematocrit in the albumin-treated rats was 47±1.3% at baseline and was reduced to 38.0±0.0% by albumin treatment (Table).

**Neurological Assessment**

Before MCAO, neurological score was normal (score=0) in all animals. High-grade behavioral deficits (score=10 to 11) were present in all animals when tested at 60 minutes of MCAO (Figure 1); thus, no animals required exclusion on the basis of an inadequate degree of cerebral ischemia. Pretreatment with solCD39 significantly improved the neurological score at 72 hours compared with the vehicle group (Figure 1). There was no significant neurological improvement when solCD39 was administered at 1 hour or 3 hours of recirculation. Treatment with albumin significantly improved neurological score at 1, 24, and 72 hours compared with vehicle (Figure 1).

**Infarct Volume and Brain Swelling**

Cortical infarct areas were significantly reduced by treatment with solCD39 pretreatment compared with vehicle at 3 coronal levels (Figure 2A). Total cortical infarct area was also significantly reduced by solCD39 pretreatment compared with vehicle (4.7±4.5 versus 42.7±12.1 mm³, respectively; *P=0.02).

Subcortical infarct areas were not significantly different in the solCD39 treatment groups than in the vehicle group at various coronal levels. However, total striatal infarct area was significantly reduced in the solCD39 pretreatment, 3-hour solCD39, and albumin-treated groups compared with vehicle (Figure 2B).
Treatment with solCD39 significantly reduced total (cortical+subcortical) infarct areas at various coronal levels when administered before ischemia or at 3 hours of recirculation compared with vehicle (Figure 3A). Total infarct volume corrected for brain swelling was also significantly reduced by solCD39 in the same groups (Figure 3B). Albumin treatment also showed reduction of total infarct areas at multiple levels (Figure 3A) and total infarct volume (Figure 3B). Figure 4 displays pixel-based infarct-frequency maps for the 4 groups, and Figure 5 shows the results of Fisher’s exact test applied on a pixel-by-pixel basis to compare vehicle-treated and solCD39-treated groups. This confirmed a significant neuroprotective effect of solCD39 when administered either before ischemia or at 3 hours of recirculation.

Brain swelling was not significantly different between any solCD39 treatment group compared with the vehicle group. Four animals died during the experiment: 3 in solCD39-treated groups (1 from each group, all at 24 hours) and 1 in the vehicle-treated group (at 48 hours). None died in the albumin-treated group. These animals were not included in the histological analysis. Autopsy revealed a large ipsilateral hemispheric infarct and extensive brain edema in all instances.

**Discussion**

We have demonstrated that the administration of solCD39, a novel platelet aggregation inhibitor, reduced infarct volume and improved neurological deficits resulting from reversible MCAO. The protective effect of solCD39 in this study could not be explained by differences in body or brain temperatures, arterial pressure, or arterial blood gases because these variables were carefully controlled and did not differ among groups. The human albumin–treated group in this study also exhibited high-grade neuroprotection, confirming our previous reports.11–13

Considerable experimental evidence with the use of thrombolytic agents in animal stroke models has shown that autologous clots can be effectively lysed by thrombolytics and that functional neurological recovery results.16,17

In the present study we used a novel soluble recombinant form of human CD39 with potent ADPase and ATPase activities and strong inhibitory effects on platelet aggregation.7 In in vitro studies, solCD39 blocks ADP-induced human platelet aggregation and inhibits collagen and thrombin receptor agonist peptide-induced platelet reactivity.18 In mice, solCD39 has a circulating half-life of approximately 2 days.18

The present study shows that solCD39 confers histological neuroprotection when administered either before MCAO or at 3 hours after the onset of recirculation. In previous studies with CD39 wild-type (CD39+/+) mice, treatment with solCD39 (either before or up to 3 hours after stroke) reduced infarct size, and solCD39 inhibited platelet accumulation in the ipsilateral cerebral hemisphere after induction of stroke.8 In this study a dose-response analysis confirmed the efficacy of the 8-mg/kg dose in inhibiting fibrin accumulation in the ipsilateral cerebral hemisphere after induction of stroke. Thus, we used this dose in the present study.

A curious aspect of the present results, however, is the lack of apparent benefit of solCD39 when administered at 1-hour recirculation, a finding that cannot be ascribed to the presence of data outliers. One possible explanation of this result is that...
oxidative/peroxidative reaction products generated during the early reperfusion period may have inactivated soICD39. Native endothelial cell ATPDase activity is irreversibly lost on endothelial cell activation or exposure to oxidative stress. Similarly, CD39 expressed in Cos-7 cells exhibits ATPDase activity that is lost on exposure of the Cos-7 cells to oxidative stress. The soICD39 used in this study has been shown to be maximally active under the same physiological conditions as endogenous endothelial cell ATPDase. The benefit we observed with soICD39 pretreatment may be ascribed to both prevention of platelet-embolus formation and reduction of ATP-mediated astrocytic or neural damage. Washout or inactivation of deleterious oxidation products generated during early reperfusion must have occurred before administration of soICD39 at the 5-hour (3-hour recirculation) time point, allowing for the beneficial effects of soICD39 to be recovered at this later time.

Platelet activation occurs in patients with acute ischemic stroke. Antiplatelet therapy might reduce the volume of brain damaged by ischemia and reduce the risk of early recurrent ischemic stroke. However, antiplatelet therapy might also increase the risk of fatal or disabling intracranial hemorrhage. Aspirin is the most widely studied antiplatelet agent in ischemic stroke. Two large recent clinical trials, the International Stroke Trial (IST) and the Chinese Acute Stroke Trial (CAST), demonstrated that treatment with aspirin 160 to 300 mg/d, started within 48 hours of stroke onset, reduced both stroke recurrence risk and mortality.

The effects of soICD39 and aspirin have been examined with regard to development of intracerebral hemorrhage after focal cerebral ischemia in mice. In this previously published study, aspirin did not increase postischemic blood flow or reduce infarction volume but did increase intracerebral hemorrhage. In

Figure 5. Maps of Fisher's exact test performed on a pixel-by-pixel basis to compare the vehicle-treated group with each of the soICD39-treated groups shown in Figure 4. Color scales denote \((1-P)\), where \(P\) is the significance level of intergroup difference. The lower threshold of the color bars has been set to \((1-P)=0.95\), so that colored pixels are those with \(P<0.05\). The groups in which soICD39 was administered before ischemia (soICD39-prior; A) or at 3 hours of recirculation (soICD39-3h; C) show widespread regions of significant histological protection, while the group in which soICD39 was administered at 1 hour of circulation (soICD39-1h; B) does not.
contrast, solCD39, at doses that did not exacerbate intracerebral hemorrhage, significantly improved cerebral blood flow at 24 hours and reduced infarction volume.8

Stroke in humans is commonly associated with impaired sensorimotor ability and reduced cognitive function. Approximately 70% to 85% of all patients experience hemiparesis immediately after stroke.23 After focal cerebral ischemia, rodents also exhibit a neurological deficit characterized by sensorimotor dysfunction, which has been noted as well by previous investigators.24–26 In the present study pretreatment with solCD39 significantly improved the neurological score at 72 hours compared with vehicle. There were no adverse behavioral side effects observed with solCD39 administration. These data agree with the findings of other investigators who noted significant neurological improvement after solCD39 treatment given either before or up to 3 hours after focal cerebral ischemia in mice.9

We did not find a strong correlation between behavior and histology in the present study. In fact, there are several published studies in which behavioral outcomes are improved in the absence of a corresponding change in histology27 or, conversely, histological protection was not accompanied by neurological improvement.28 Among the factors that could account for the lack of a close behavioral-histological association are functional compensation or recovery.26 For some tests, even when a behavioral deficit has been identified soon after stroke, there is partial to full functional recovery over time despite histopathological findings.28 In addition, the timing of the behavioral tests and histological tissue collection may influence the degree of correlation between these 2 end points.

In summary, our results demonstrate the neuroprotective efficacy of solCD39 in an in vivo model of temporary focal cerebral ischemia as judged by neurological score and infarct size. A pharmacological agent such as solCD39 thus may have potential utility in treating focal ischemic stroke in the clinical setting.

Acknowledgments
These studies were supported by a grant from Immunex Corporation, Seattle, Wash, and by program project grant NS 05820 of the National Institutes of Health (Dr Ginsberg). The authors thank Guillermo Fernandez for technical assistance.

References
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*Stroke.* 2003;34:758-763; originally published online February 6, 2003;
doi: 10.1161/01.STR.0000056169.45365.15
*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

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