Siagoside Selectively Attenuates Morphological and Functional Striatal Impairments Induced by Transient Forebrain Ischemia in Rats

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Background and Purpose: Transient forebrain ischemia induced in rats by the four-vessel occlusion method is known to produce severe neural damage in the hippocampus and striatum and a behavioral syndrome the major symptom of which is a working memory deficit. Recent evidence suggests that monosialogangliosides can ameliorate postischemic symptoms. Our purpose was to study the effect of siagoside, the inner ester of GM1 ganglioside, on some behavioral and morphological impairments induced by four-vessel occlusion in rats.

Methods: Rats were injected daily with 5 mg/kg i.p. siagoside starting 4 hours after the cerebral ischemia. After 14 days the rats were tested for working memory in a water T maze or scored for apomorphine-induced stereotypy. The rats were killed 21 days after the cerebral ischemia. Histological and computer-assisted morphometric analyses were performed on cresyl violet-stained brain sections, which were graded according to a neuropathologic score, and on sections stained with a monoclonal antiserum against dopamine and cyclic adenosine-3',5'-monophosphate-regulated phosphoprotein, a marker for striatal dopaminergic neurons.

Results: Siagoside treatment reduced the stereotypy score induced by low doses of apomorphine and the extent of striatal lesions but did not affect the working memory deficit or the extent of hippocampal lesions.

Conclusion: Daily siagoside treatment after acute cerebral ischemia attenuates some morphological and functional deficits related to striatal damage. These effects can be interpreted as a selective protective action on striatal neural populations or as a modulatory action on neural systems involved in striatal control. These data are consistent with preliminary clinical reports showing that monosialogangliosides enhance motor recovery after acute ischemic stroke. (Stroke 1992;23:234-241)

The sialylated glycosphingolipid GM1 has been found in a variety of tissues, with particularly high levels observed in membranes of neural cells. Although detailed information on its biological function is not available, growing evidence suggests that GM1 is involved in neural differentiation and repair after peripheral denervation or brain injury. When administered intraperitoneally, monosialogangliosides cross the blood–brain barrier, are incorporated into the neural cell membrane, and enhance behavioral as well as morphological recovery following damage to various brain regions. Some of these behavioral effects were found shortly (24–48 hours) after injury, suggesting that monosialogangliosides may have protective effects. On the other hand, several reports indicate that chronic (2–8 weeks) treatment with monosialogangliosides produces long-term behavioral recovery from injury, a phenomenon often associated with neural regeneration, axonal sprouting, and reduction of the extent of damage in the lesioned areas.

These properties of monosialogangliosides have recently been tested in a series of studies on experimental brain ischemia in mammals. In rodents, administration of GM1 or siagoside, a GM1 internal
ester, before or immediately after the induction of brain ischemia ameliorated posts ischemic mortality in gerbils\textsuperscript{19} and passive avoidance impairment in rats.\textsuperscript{24,25} These changes were associated with reduced cerebral edema\textsuperscript{19,24} and reduced areas of lesion in the striatum\textsuperscript{23,25} and hippocampus.\textsuperscript{22}

We report the effects of repeated administrations of siagoside to rats on intermediate- and long-term behavioral recovery from transient forebrain ischemia induced by the four-vessel occlusion procedure according to Pulsinelli and Brierley.\textsuperscript{26} The effects of siagoside treatment were assessed by studying working memory performance in a T maze, which has been demonstrated to be affected by transient forebrain ischemia.\textsuperscript{27-29} In addition, since transient forebrain ischemia has been reported to damage dopaminceptive striatal neurons,\textsuperscript{30,31} we examined apomorphine-induced stereotypy, an indicator of dopamine receptor activation in the striatum.\textsuperscript{32,33}

Ischemic brain damage was assessed histologically by grading cresyl violet–stained sections according to a neuropathologic score\textsuperscript{26,28} and by evaluating the levels of immunoreactivity of dopamine and cyclic adenosine-3’,5’-monophosphate-regulated phosphoprotein (DARPP-32)\textsuperscript{34} in dopaminceptive neurons of the striatum using semiquantitative image analysis.\textsuperscript{35}

\textbf{Materials and Methods}

Male specific pathogen-free Sprague-Dawley rats weighing 250–300 g were kept, one per cage, under standardized conditions of humidity, temperature, and lighting (lights on at 8 AM and off at 8 PM) and given food pellets and water ad libitum. The rats were subjected to transient forebrain ischemia consisting of 30 minutes’ occlusion of the common carotid arteries following permanent occlusion of the vertebral arteries. A two-stage preparation procedure was employed. In the first stage the rats were anesthetized with 100 mg/kg i.p. ketamine and the vertebral arteries were electrocoagulated. On the following day cerebral ischemia was produced in the animals while awake. Changes in the electrocorticogram and cardiovascular parameters were similar to those reported by other authors.\textsuperscript{26} All rats included in the study were scored as having “complete ischemic coma,” defined by an isoelectric electrocorticogram, absence of the righting reflex, and absence of locomotor activity, during carotid occlusion. A detailed description of the surgical procedure and characterization of the ischemia model are provided elsewhere.\textsuperscript{26} Sham-operated rats were obtained by occluding the vertebral arteries without transient clamping of the carotid arteries.

Rats were administered either 5 mg/kg i.p. siagoside or an equivalent volume (1 ml/kg) of saline vehicle 4 hours after carotid occlusion and daily for 21 days. Siagoside (Fidia, Abano Terme, Italy) was dissolved in saline immediately prior to the injection. For functional analysis the four treatment groups were four-vessel–occluded rats treated with saline (n=14), four-vessel–occluded rats treated with siagoside (n=15), sham-operated rats treated with saline (n=14), and sham-operated rats treated with siagoside (n=13). For histological analysis another set of rats (n=17 per group) was killed 7 days after cerebral ischemia together with a control group of intact naive rats (n=5) matched for age.

No effects of treatment on daily body weight or food and water intake were observed, and therefore these values are not reported.

The T maze procedure was modified from that described by Hepler and colleagues\textsuperscript{37} to study the effect of four-vessel occlusion–induced cerebral ischemia on working memory in rats. The rats were tested between 14 and 21 days after the cerebral ischemia in a white waterproof Plexiglas T maze filled to a depth of 30 cm with water at 24–26°C made opaque by adding approximately 2 g/l powdered milk. On day 1 the rats were allowed to swim for 3 minutes in the maze. A submerged platform was introduced on day 2, and each rat was trained individually to swim to the end of an arm, where the platform was encountered. From day 3 to day 8 trial-dependent memory discrimination (working memory) was examined using a discrete-trial, rewarded alternation design. Each trial consisted of two runs: a forced run, in which the rat was directed into one arm by blocking the entrance to the other with a white Plexiglas panel, and a choice run, in which the rat was allowed to choose either arm. A correct response was recorded when the animal chose the arm blocked during the forced run. The choice run followed 20 seconds after the forced run, and the intertrial interval was 2 minutes. The time for each rat to reach the platform in the choice run was also recorded. Each rat received at least 6 days of testing, 12 trials/day, the criterion being 10 correct responses in 12 trials. Results were analyzed using a two-way analysis of variance (ANOVA) for repeated measures followed by post hoc Newman-Keuls tests where appropriate. Working memory was tested between 8 AM and 3 PM.

Apomorphine-induced stereotypy was measured according to Costall and colleagues\textsuperscript{32} with modification.\textsuperscript{33} Testing began on day 14 after cerebral ischemia and was carried out daily for 4 days. Each morning at 10 AM the rats were placed in individual cages in a quiet room, and 2 hours later they were injected subcutaneously with a dose of apomorphine (0.05, 0.10, 0.25, or 0.50 mg/kg) determined using a balanced randomized design. At the end of the experiment each rat had received all the apomorphine doses. Beginning 10 minutes after the injection, a rater unaware of the treatment observed each rat for 1 hour at 5-minute intervals using a stereotypy scale. Apomorphine-induced stereotypies were scored as 0: asleep or not moving, occasional sniff, rare episodes of locomotion; 1: discontinuous sniffing, exploratory activity; 2: continuous sniffing and small head movements, periodic exploratory activity; 3: continuous sniffing and small head movements with the nose below the horizontal plane (head
down), discontinuous licking, biting, and gnawing; or 4: continuous licking, biting, and gnawing, head down, no exploratory activity. Data were analyzed using a three-way ANOVA with treatment groups as the main factor and apomorphine dose and time after injection as the repeated factors, followed by Newman-Keuls tests where appropriate.

All rats used in the functional experiments were killed 21 days after cerebral ischemia. In addition, a set of four-vessel-occluded rats was killed 7 days after cerebral ischemia to gauge the extent of the ischemic lesion and the effects of siagoside treatment. The rats were anesthetized with 100 mg/kg i.p. ketamine and killed by means of intracardiac perfusion with 100 ml warm saline followed by 100 ml ice-cold 4% paraformaldehyde plus 3% picric acid in 0.1 M phosphate buffer (pH 7.2). The brains were rapidly dissected out and postfixed overnight in the same fixative. The following day the brains were cut on a cryotome (50-μm-thick sections). Sections (12 per rat) were taken at various rostrocaudal levels of the precommissural striatum and dorsal hippocampus. Then the sections were either stained with cresyl violet or processed according to the indirect immunoperoxidase procedure using the avidin-biotin complex (Vectastain, Vector Laboratories, Inc., Burlingame, Calif.) as a detection system, the chromophore being diaminobenzidine. The immunocytochemical procedure has been described elsewhere.26 A neuropathologic score for each region was calculated as the mean of the results obtained in both hemispheres of each rat within a group. The areas of striatal DARPP-32 immunoreactivity hypostaining were analyzed as previously described.23 Briefly, a television camera connected to a microscope allowed the acquisition and digitization of the image of the entire striatum at low magnification (coronal section, size of the field 23.0 mm²). The area of the entire striatum was interactively selected by means of a light pen and measured. Subsequently, an interactive discrimination procedure was performed to separate on a densitometric basis DARPP-32–positive from –negative areas. Then the percent DARPP-32–negative area per striatum was calculated. Atrophy in the hippocampus and striatum was analyzed by measuring on a digitized image the total regional areas in four-vessel–occluded and sham-operated rat brains at the same rostrocaudal levels and calculating the area for four-vessel–occluded rats as a percentage of the area for sham-operated rats at that level. In this way, we obtained a shrinkage index for a given area at a given time after ischemic injury that can be considered as an integrated measure of postlesion tissue rearrangement.25 Results were analyzed using nonparametric Mann-Whitney U and Dunn tests.

Results

The percent correct responses (choice accuracy) in the T maze arm discrimination test are shown in Figure 1, left panel. The results of testing on day 1 were not included in the analysis. Two-way ANOVA for repeated measures showed that the choice accuracy for all groups improved during testing ($F_{3,50} =26.97$, $p<0.01$). However, the choice accuracy for four-vessel–occluded rats was impaired relative to sham-operated rats ($F_{2,50} =8.35$, $p<0.01$). Analysis with a post hoc Newman-Keuls test indicated that

![Graph of effects of 5 mg/kg/day i.p. siagoside treatment on arm choice performance of four-vessel–occluded rats tested in water T maze 14–21 days after cerebral ischemia. Data are presented as mean±SEM percent correct responses (left panel) and swim time (right panel) summarized over 6-day block of 12 trials/day. Sham-operated rats treated with saline or siagoside did not differ at any point so they were combined to form group designated sham-operated rats. ○, sham-operated rats; ▲, four-vessel–occluded rats treated with saline; ●, four-vessel–occluded rats treated with siagoside. Post hoc Newman-Keuls test: *$p<0.05$ different from sham-operated rats.](http://stroke.ahajournals.org/lookup/doi/10.1161/01.STR.23.2.236)
The time course of stereotypy scores measured after the acute administration of apomorphine at various doses is shown in Figure 2. Three-way ANOVA revealed that all main factors and interactions were highly significant \( (p<0.01) \). The interaction between treatment groups and apomorphine dose \( (F_{9,72} = 5.10, p<0.01) \) was further studied by means of the post hoc Newman-Keuls test. Both ischemic groups showed scores significantly \( (p<0.01) \) higher than the two sham-operated groups at all apomorphine doses tested. At 0.05 and 0.10 mg/kg apomorphine the scores obtained in four-vessel-occluded rats treated with siagoside were significantly \( (p<0.01) \) lower than the scores in four-vessel-occluded rats treated with saline, while no difference was found at the 0.25 and 0.50 mg/kg doses. No significant difference was found between sham-operated rats treated with saline and sham-operated rats treated with siagoside. These results indicate that brain ischemia enhances apomorphine-induced stereotypy and that siagoside treatment partially counteracts these effects when apomorphine is administered at low doses.

Histological analysis of the hippocampal subregions of four-vessel-occluded rats revealed that the neuropathologic score increased from day 7 to day 21. In particular, the CA1 field showed the most severe neural damage on day 21. In no significant difference was observed between the scores of four-vessel-occluded rats treated with siagoside and four-vessel-occluded rats treated with saline in any hippocampal subregion. In the hippocampus of sham-operated rats no lesions were present. Regional atrophy was severe in the CA1 field, which showed a shrinkage index of about 80% on day 7 and about 74% on day 21 (Table 1). Less marked changes were found in the CA3 and CA4 and dentate gyrus fields (Table 1). No significant effect of siagoside on the shrinkage index was observed in any hippocampal subregion. Morphometric analysis of the striatum revealed disappearance of DARPP-32 immunoreactivity in patches that were mainly located in the dorsolateral region of the striatum (Figure 3); areas of immunoreactivity disappearance in saline-treated
TABLE 1. Effects of Siagoside Treatment on Severity and Distribution of Ischemic Brain Damage Assessed As Neuropathologic Score and Shrinkage Index in Various Areas of Dorsal Hippocampus 7 and 21 Days After Transient Forebrain Ischemia in Rats

<table>
<thead>
<tr>
<th>Hippocampal subregion</th>
<th>Neuropathologic score</th>
<th>Shrinkage index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 days</td>
<td>21 days</td>
</tr>
<tr>
<td>Field CA1-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>2.4±0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Siagoside</td>
<td>2.5±0.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Field CA3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.2±0.1</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>Siagoside</td>
<td>0.3±0.1</td>
<td>0.6±0.3</td>
</tr>
<tr>
<td>Field CA4 and dentate gyrus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.1±0.1</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>Siagoside</td>
<td>0.2±0.2</td>
<td>0.5±0.2</td>
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Neuropathologic score: 0, no lesion; 1, few neurons damaged; 2, many neurons damaged; 3, majority of neurons damaged. Shrinkage index, hippocampal subregion area per section as percent of values measured in sham-operated rats. Hippocampal subregion areas in sham-operated rats: CA1-2 field, 3.18±0.15 mm²; CA3 field, 1.60±0.07 mm²; CA4 field and dentate gyrus, 2.00±0.09 mm². Values are presented as mean±SEM. Number of animals per group was 7-9. Rostrocaudal level was -3.3 mm from bregma.

Discussion

We examined the effects of repeated administrations of siagoside, a monosialoganglioside derivative, on some behavioral changes and histological damage caused by transient forebrain ischemia induced in rats by the method of Pulsinelli and Brierley. Treatment with siagoside significantly reduced the area of DARPP-32 immunoreactivity disappearance both 7 and 21 days after carotid occlusion (Figure 3 and Table 2). The shrinkage index in saline-treated rats was modest (97%) on day 7 and more severe (87%) on day 21 (Table 2). Siagoside treatment significantly reduced the shrinkage index of the striatum 21 days after carotid occlusion, but not 7 days (Table 2).

To our knowledge, the first study on long-term behavioral consequences of transient forebrain ischemia was carried out by Volpe and colleagues testing four-vessel-occluded rats in an eight-arm radial maze 1–3 months after carotid occlusion. That study demonstrated an impairment of working memory and further suggested that the bilateral loss of CA1 neurons observed in these animals could account for this deficit. These findings have been observed in four-vessel-occluded rats tested in a water T maze, whereas siagoside appeared to decrease the stereotypies induced in ischemic animals by low doses of apomorphine. Histological examination revealed no effect of treatment on the severity of postischemic morphological neural damage in various hippocampal subregions. However, the disappearance of DARPP-32 immunoreactivity was reduced in the striatum of four-vessel-occluded rats treated with siagoside.

FIGURE 3. Microphotographs of dopamine and cyclic adenosine monophosphate-regulated phosphoprotein immunoreactivity in striatum of sham-operated rat treated with saline (a), four-vessel-occluded rat treated with saline (b), and four-vessel-occluded rat treated with siagoside (c) 21 days after cerebral ischemia. Avidin-biotin complex and 3,3'-diaminobenzidine chromophore were used as detection system. Antibody dilution 1:2,000. Calibration bar=500 μm. Distance from bregma: 1.2 mm.
Replicated in a 12-arm radial maze28 and a T maze29 and agree with the results obtained in our experiments. However, in all these studies the accuracy of performance of four-vessel-occluded rats improved to some extent during training, suggesting a residual learning capacity.28 Recent evidence indicates that monosialoganglioside treatment can improve performance in other tests involving learning, such as passive avoidance.24,25 In our experiments daily treatment with siagoside did not significantly affect arm discrimination learning in four-vessel-occluded rats. Failure to show clear improvements in the working memory performance of siagoside-treated four-vessel-occluded rats is consistent with the lack of beneficial effects on neural damage in the CA1 field. This hippocampal subregion is relevant for trial-dependent discrimination in rats.27–29 Striatal lesions do not appear to be involved in the posts ischemic working memory deficit. Recent results showed that radiofrequency lesions of the dorsal striatum did not affect trial-dependent discrimination28,40; instead such lesions impair trial-independent discrimination.41

In contrast, ischemic striatal lesions seem to be involved in the increased sensitivity to apomorphine observed in four-vessel-occluded rats. Decreased levels of DARPP-32 immunoreactivity associated with a reduction in dopamine D1 but not D2 binding site densities have been described 7 days after carotid occlusion.30,31 Our findings confirm and extend these observations, showing areas of DARPP-32 immunoreactivity disappearance in the dorsal striatum 21 days after cerebral ischemia. A protein selectively expressed in neurons that contain D1 receptors, DARPP-32 can be used as a marker for this subtype of dopaminoceptive neuron.31,34 Because simultaneous pharmacological activation of the D1 and D2 receptors is required to produce the typical stereotypy pattern in rats,42 this unbalanced concentration of D1 versus D2 receptors observed in four-vessel-occluded rats may be one cause of the changes in apomorphine-dependent stereotypy. Treatment with siagoside significantly reduced the intensity of stereotypy induced by low doses of apomorphine. This effect may be related to the reduction by siagoside of striatal neural damage assessed by the areas of DARPP-32 immunoreactivity disappearance or the extent of striatal shrinkage. Because cell death in the striatum is reported to occur within 3–6 hours after an ischemic insult, it is suggested that siagoside, administered 4 hours after carotid occlusion, can act by reducing the severity of metabolic derangement of damaged cells in the ischemic “penumbra” and eventually altering their morbidity. Hypotheses regarding the mechanisms of action involved in such an effect focused on the maintenance of Na,K-ATPase activity in the membrane of damaged neurons18,19 and the reduction of acute edema.19,24 Ca+ influx,43 and glutamate neurotoxicity.44 Alternatively, siagoside can directly affect dopamine receptor regulation since recent studies have demonstrated that GM1 treatment prevents the development of striatal dopamine receptor supersensitivity45,46 and is effective in reducing the apomorphine-dependent rotational behavior induced by nigrostriatal pathway interruption.4,5,10,16 However, increased apomorphine effects have been described after lesions in the neocortex17 and dentate gyrus,15 areas that are also partially damaged during transient cerebral ischemia. Thus, it is possible that the increased sensitivity to apomorphine is due to a lesion-induced reduction of the extrastriatal control over striatal functions.

The beneficial effects described so far are of particular relevance because transient forebrain ischemia in rats can be considered a model for the arrest syndrome and acute ischemic stroke in humans.26,27 Encouraging preliminary results from a multicenter clinical trial showed that recovery of the neurological disability score is enhanced on day 14 in patients with acute ischemic stroke treated with GM1 within 12 hours after onset47 (however, see Hoffbrand et al48).

In conclusion, treatment with monosialoganglioside derivative siagoside produced beneficial effects on the development of intermediate- and long-term behavioral impairments induced by transient forebrain ischemia. As previously demonstrated in other lesion paradigms, the monosialogangliosides enhanced functional recovery in a symptom-specific manner. The present results confirm the view4,5,10–17 that 1) monosialogangliosides tend to attenuate morphological neural damage in only some brain regions or neural systems, 2) monosialoganglioside treatment is most effective in improving functional impairment when behavior-related neural systems are only par-
tially damaged, and 3) the effects of these treatments develop over time following injury.

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