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A synthetic ceramide analog ameliorates spatial cognition deficit and stimulates biosynthesis of brain gangliosides in rats with cerebral ischemia

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Abstract

A synthetic ceramide analog, L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (L-PDMP) upregulates ganglioside biosynthesis in several cell lines. In cultured cortical neurons, neurotrophic effects of L-PDMP on neurite outgrowth and synaptic activity were demonstrated. In addition, it was found that L-PDMP could ameliorate the spatial cognition deficit in rats with ischemia. To elucidate this effect, we evaluated the effect of L-PDMP on brain ganglioside biosynthesis and its therapeutic efficacy against spatial cognition deficit in rats made ischemic. Rats were trained for 2 weeks, using an 8-arm radial maze task, and then forebrain ischemia was induced. L-PDMP was injected i.p. at 40 mg/kg twice a day starting from day 1 or 3 after ischemia induction for 6 or 4 days, respectively. The first study showed significantly reduced spatial cognition deficit at 12 h after the final drug administration, and L-PDMP tended to attenuate apoptosis in hippocampal CA1. To examine the effect of L-PDMP on brain ganglioside biosynthesis, *N*-[^{3}H]acetyl-D-mannosamine was infused into the lateral ventricle via an injection cannula at 12 h after the final drug administration. After 4 h, the brain gangliosides were purified and analyzed. Upregulation of ganglioside biosynthesis by L-PDMP was observed on days 3 and 5 after ischemia. These results are an indication that L-PDMP may ameliorate spatial cognition deficit by upregulating ganglioside biosynthesis in ischemic brain.

Keywords: L-PDMP; Ganglioside biosynthesis; Spatial cognition; 8-Arm radial maze; Apoptosis

1. Introduction

Gangliosides, a family of sialic acid-containing glycosphingolipids, are abundant in cerebral nervous tissue. It has often been reported that exogenous gangliosides can elicit neurite outgrowth and neural repair in vitro and in vivo (Pepeu et al., 1994; Tsuji et al., 1992; Wu et al., 1991). In particular, GM1 and GQ1b have been found not only to enhance nerve growth factor (NGF)-induced neurite outgrowth, but also to display NGF-like activities themselves (Ferrari et al., 1993; Maysinger et al., 1993; Mutoh et al., 1998; Tsuji et al., 1983). The recent development of GM2/GD2 synthase knockout mice revealed that a lack of complex gangliosides induces abnormal conduction velocity in somatosensory nerves (Takamiya et al., 1996). The mice also exhibited decreased central myelination, and axonal degeneration (Sheikh et al., 1999). Moreover, GM1 reduced glutamate, aspartate, γ-amino-*n*-butyric acid (GABA) and glycine efflux from the cerebral cortex after transient cerebral ischemia in rats (Phillis and O'Regan, 1995), and has been developed clinically for the treatment of neuronal dysfunction.

Various studies have been conducted on excitatory amino acids and their receptors to explain the neuronal cell death (necrosis) after cerebral ischemia. The mechanism of ische-

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mia-induced neuronal damage and the efficacies of antagonists were reviewed by Hara et al. (1994). Recently, using caspase-1 knockout or transgenic mice (Friedlander et al., 1997) and Bcl-2 transgenic mice (Martinou et al., 1994), it was shown that the neuronal cell death induced by cerebral ischemia includes apoptosis. GM1 prevented apoptotic cell death by enhancing TrkA dimerization and consequent autophosphorylation in PC12 cells (Ferrari and Greene, 1998) and decreased the severity of ischemic brain lesions in experimental models (Frontczak-Baniewicz et al., 2000; Hicks et al., 1998). Also in neuron-rich cortical cultures, GM1 and other gangliosides attenuated serum deprivation-induced neuronal apoptosis (Ryu et al., 1999).

As shown in Fig. 1, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is a synthetic analog of ceramide that possesses two chiral centers at the C1 and C2 positions and thus forms four isomers (Inokuchi and Radin, 1987). We previously demonstrated that the glucosylceramide synthase inhibitor, D-*threo*-PDMP (D-PDMP; Inokuchi and Radin, 1987; Inokuchi et al., 1989, 1990), inhibited functional synapse formation (Mizutani et al., 1996), neurite outgrowth, autophosphorylation of Trk, and Trk-initiated intracellular protein kinase cascades (Mutoh et al., 1998). These suppressive effects were specifically reversed by the addition of GQ1b or GM1. Conversely, it was found that the enantiomeric form of D-PDMP, L-*threo*-PDMP (L-PDMP), increased the cellular content and biosynthesis of gangliosides in B-16 melanoma cells (Inokuchi et al., 1989, 1995) and stimulated neurite outgrowth (Usuki et al., 1996) and functional synapse formation with a concomitant increase of ganglioside biosynthesis in primary cultured rat embryonic cortical neurons (Inokuchi et al., 1997).

We previously reported that a 6-day regimen of L-PDMP (40 mg/kg, i.p., twice a day) ameliorated the spatial cognition deficit in the 8-arm radial maze task, using our transient ischemia model in which apoptosis is induced in the hippocampal CA1 by repeated cerebral ischemia (10-min occlusion twice with a 1-h interval, Inokuchi et al., 1997, 1998; Iwasaki et al., 1998).

To further examine the ameliorating effects of L-PDMP on the spatial cognition deficit induced by repeated cerebral ischemia, the present study was designed to investigate whether L-PDMP could suppress repeated cerebral ischemia-induced apoptosis in the hippocampal CA1, which plays an important role in spatial cognition. Further, we studied how L-PDMP affects the rates of biosynthesis of brain gangliosides and their chemical contents after repeated cerebral ischemia.

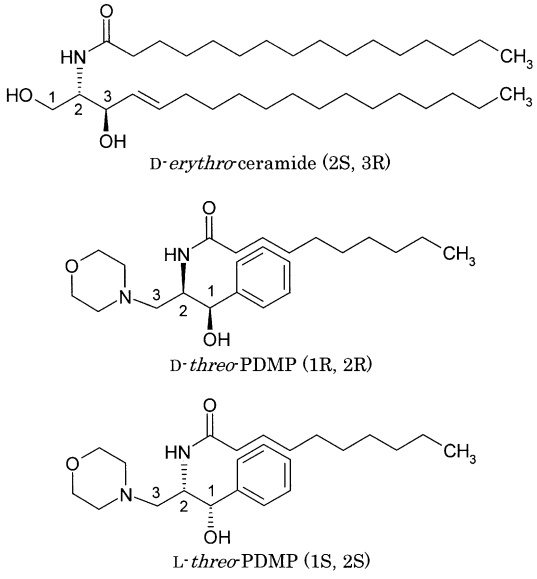

Fig. 1. Structural comparison of ceramide and PDMP isomers.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 200–250 g, supplied by Kyu-Do (Saga, Japan), were used for this experiment. The animals were kept under a constant light–dark cycle (light 07:00–19:00 h) with a restricted diet (CE-2, Clea Japan, Tokyo) in an air-conditioned room (23 ± 2 °C, 60% humidity). All procedures for animal care and use were carried out based on the regulations dictated by the Experimental Animal Care and Use Committee at Facilities for Experimental Animals of Fukuoka University.

2.2. Eight-arm radial maze task

The 8-arm radial maze task was performed as described previously (Inokuchi et al., 1997). Each rat was placed on a platform (25 cm diameter) in the middle of an 8-arm radial maze, in which each arm had been baited with a food pellet. Rats visited each arm and ate the food pellet. They learned not to re-enter an arm that had been visited during the same test. An image motion analyzer, AXIS-30 (Neuroscience, Japan), was used to quantify the task performance (Iwasaki et al., 1996). Behavioral observation was discontinued after 10 min even if the animal did not finish the task. The number of correct choices and of errors was used to assess the performance of the animal in each session. An error was defined as a re-entry into an already visited arm. Rats that had made seven or more correct choices and either one or no errors during the first eight choices in each of three consecutive sessions were used in the subsequent behavioral experiment. Training was performed at 24-h intervals and rats that had not reached the above criteria within 14 days were excluded.

2.3. Repeated cerebral ischemia

Repeated cerebral ischemia was induced using a slightly modified version of Pulsinelli and Brierley's (1979) meth-

ods. Briefly, the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and both vertebral arteries were electrocauterized and the common carotid arteries were exposed. The next day, after confirming that the animals showed no abnormal behavior, both common carotid arteries were clamped for 10 min twice at a 1-h interval using aneurysm clips. Body temperature was maintained at 37 °C using a heating pad and heating lamp during the operation and occlusion until the righting reflex reappeared. Any rats that failed to demonstrate a loss of righting reflex during occlusion were excluded from the subsequent experiments.

2.4. Drug administration

L-PDMP was synthesized as described previously (Inokuchi and Radin, 1987) and purified by crystallization. The purity of L-PDMP was above 99.5% by chiral column chromatography. The drug administration protocol is shown in Fig. 2. L-PDMP was dissolved in 5% Tween80 in saline at a concentration of 20 mg/ml and injected at 40 mg/kg i.p. twice a day. For the experiments on behavior, L-PDMP or vehicle was administered for 6 days, starting 24 h after repeated ischemia (Group 1), or for 4 days, starting on day 3 after repeated ischemia (Group 2). For the metabolic radiolabeling of brain gangliosides, L-PDMP or vehicle was administered for 2, 4 or 6 days, starting 24 h after repeated ischemia (Groups 3, 4, and 5, respectively).

2.5. Histochemistry

Following observation of the 8-arm radial maze task at 7 days after induction of ischemia, each five rats from the administration groups except for Group 2-vehicle were anesthetized with pentobarbital and perfusion-fixed transcardially with 4% formaldehyde. The animals were then decapitated and the brains were dissected. The dissected brains were immersed in the same fixative and embedded in paraffin. Then 5-μm brain slices were cut and stained using the apoptosis detection system, Fluorescein (Promega, USA), utilizing the principle of the terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay (Ben-Sasson et al., 1995; Gavrieli et al., 1992). Briefly, the tissue sections were treated with TdT incubation buffer (0.5 U/μl TdT, 5 μM fluorescein 12-dUTP) for 60 min at 37 °C. The fragmented DNA of

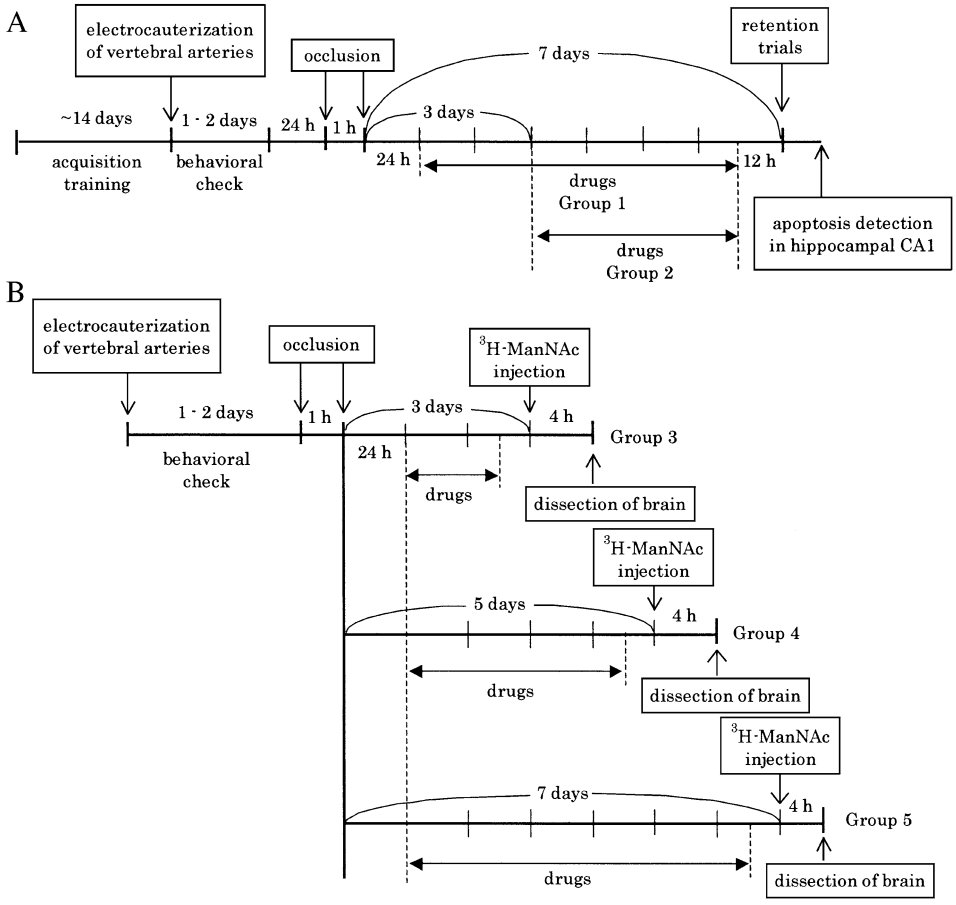

Fig. 2. Experimental protocols. (A) Protocol for behavioral experiments to evaluate the efficacy of L-PDMP against spatial cognition deficit in rats with cerebral ischemia. (B) Protocol for metabolic labeling of brain gangliosides in rats with cerebral ischemia.

apoptotic cells was visualized by the incorporation of fluorescein-12-dUTP (green) at the 3′-OH DNA ends by TdT. Propidium iodide (1 μg/ml, red, Sigma) was used for counterstaining. TUNEL-positive cells in the hippocampal CA1 were observed under a fluorescence microscope (DMRBE, Leica) to detect apoptosis.

2.6. Metabolic radiolabeling of cortical gangliosides

Cortical gangliosides were radiolabeled according to the method described by Doyle et al. (1993). In brief, the rat was anesthetized with 50 mg/kg sodium pentobarbital i.p. and immobilized in a stereotaxic apparatus. A guide cannula was implanted 1 mm dorsal to the ventricle according to the brain coordinates of Paxinos and Watson (1998) and, when in place, the infusion cannula was introduced into the left lateral ventricle. The [6-^{3}H] *N*-acetyl-D-mannosamine (ManNAc) precursor (18 μCi/ 1.2 nmol; specific activity, 15 Ci/mmol; American Radiolabeled Chemicals USA) was infused in an unrestrained animal via the indwelling cannula at 12 h after final administration of L-PDMP. Four hours later, the animal was killed, and its cortex was dissected.

2.7. Total lipid extraction and ganglioside purification

Cortical gangliosides were extracted and purified according to the methods described by Svennerholm and Fredman (1980), and Ladisch and Gillard (1985), respectively. In brief, the dissected cortex was homogenized in 20 volumes of chloroform (C)/methanol (M)/water (4:8:3 v/v/v), and total lipids extracted and taken to dryness under N_2. The total lipid extract was dispersed in 4 ml isopropyl ether/1-butanol (6:4 v/v), 2 ml of 50 mM NaCl was added, and the sample was mixed by vortex and ultrasonication for several minutes. The sample was centrifuged to separate the two phases, and the upper organic phase was removed using a Pasteur pipette. The ganglioside-containing lower aqueous phase was re-extracted with the original volume of fresh organic solvent mixture. Following removal of the upper phase, the lower aqueous phase was lyophilized to concentrate the samples. Then, the lyophilized aqueous phase was re-dissolved in a small volume of C/M (1:1 v/v) and passed through a Sephadex LH-20 column (D: 10 mm, H: 120 mm) eluted with C/M (1:1 v/v). The ganglioside-containing peak was collected and taken to dryness. The ganglioside mixture was re-dissolved with ultrasonication in a small volume of C/M (1:1 v/v) and centrifuged. The supernatant was applied to a high-performance thin-layer chromatography (HPTLC) plate with C/M/0.2% $CaCl_2$–$2H_2O$ (40:40:11 v/v/v) (Ando et al., 1987). The radiolabeled ganglioside on the HPTLC plate was analyzed from the radioluminogram (BAS2000, Fuji Film, Japan), and the amount of ganglioside biosynthesis was converted from the specific radioactivity of a [^{3}H]ManNAc precursor. To measure cortical ganglioside contents, each ganglioside on the HPTLC plate was visualized with 0.2% orcinol in 4 N H_2SO_4, and quantified with a densitometer (CS-9000, Shimadzu, Japan) at 530-nm wavelength.

2.8. Statistical analysis

Data for the 8-arm radial maze task were evaluated by Wilcoxon's rank sum test. Data for ganglioside biosynthesis and content in cerebral cortex were analyzed by Tukey's test.

3. Results

3.1. Effect of L-PDMP on spatial cognition deficit induced by repeated cerebral ischemia in rats

As shown in Fig. 3, vehicle-treated repeated cerebral ischemia rats showed a significant deficit in spatial cognition in the 8-arm radial maze task on the 7th day after reperfusion. On the other hand, a 6-day regimen with L-PDMP (40 mg/kg L-PDMP, twice a day) treatment starting at 24 h after ischemia (Group 1) resulted in a significant increase in correct choices and a decrease in errors in the radial maze task (Fig. 3). A 4-day regimen with L-PDMP (40 mg/kg L-PDMP, twice a day) treatment starting at 72 h after ischemia (Group 2) did not show significant improvement (Fig. 3).

3.2. Effect of L-PDMP on neuronal apoptosis in hippocampal CA1 pyramidal cells after repeated cerebral ischemia

After behavioral evaluation in the 8-arm radial maze task, the rat's brain was fixed and apoptosis was identified in the hippocampal CA1 by TUNEL assay. Apoptotic cells were found in vehicle-treated rats on the 7th day after ischemia (mean ± S.E.M.: 78.4 ± 5.7 TUNEL-positive cells/mm^2). As shown in Fig. 4, continuous treatment with L-PDMP tended to attenuate apoptotic cell death in the hippocampal CA1. In Group 1, apoptotic cells were fewer than 25/mm^2 in three of five animals (Group 1: 48.6 ± 24.4 TUNEL-positive cells/mm^2). In Group 2, apoptotic cells were fewer than 25/mm^2 in one of five animals (Group 2: 61.2 ± 13.2 TUNEL-positive cells/mm^2).

3.3. Effect of L-PDMP on biosynthesis of cortical gangliosides after repeated cerebral ischemia

The effects of L-PDMP on the biosynthesis of cortical gangliosides in rats with cerebral ischemia during these critical treatment periods were examined, as shown in Fig. 2B. Table 1 shows the biosynthetic rates of cortical gangliosides in normal rats, compared with those after GM3 taken as 100%. The most prominent increases of ganglioside

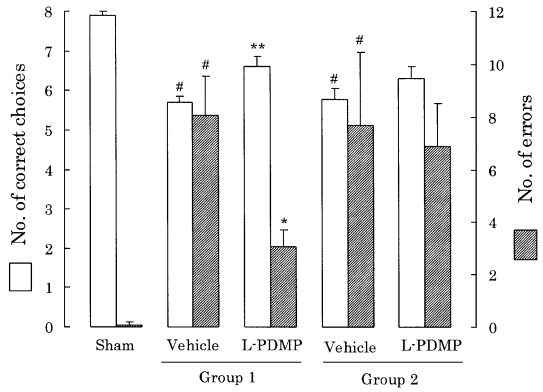

Fig. 3. Effects of L-PDMP on spatial cognition deficit induced by repeated cerebral ischemia. Sham (sham-operated rats, $n=11$), Group 1-vehicle, L-PDMP (i.p. injections of vehicle or 40 mg/kg L-PDMP twice a day for 6 days from 24 h after ischemia, $n=13$), Group 2-vehicle, L-PDMP (i.p. injection of vehicle or 40 mg/kg L-PDMP twice a day for 4 days from 3 days after ischemia, $n=9$ and $n=10$, respectively), Values are means (± S.E.M.) of the number of correct choices or the number of errors. #$P<0.001$ vs. sham-operated rats, *$P<0.05$ and **$P<0.01$ vs. vehicle-treated rats (Wilcoxon's rank sum test).

biosynthesis by i.p. treatment with L-PDMP in the ischemic rat cortex were observed on days 3 and 5. The increases caused by L-PDMP tended to return to the normal level by day 7. L-PDMP treatment could elevate all of the a- and b-series ganglioside biosynthesis examined here in the range of 150–250%. Especially the biosynthesis of b-series gangliosides (GD1b, GT1b and GQ1b) in the cerebral cortex of L-PDMP-treated rats was significantly increased compared to that in vehicle-treated rats (Fig. 5).

Table 1
Relative biosynthesis rates of cortical gangliosides in normal rats

Ganglioside	% of GM3 biosynthesis
GM3	100.0 (21.3)
GM1	319.8 (75.1)
GD1a	186.1 (21.0)
GD1b	346.9 (147.1)
GT1b	93.0 (9.5)
GQ1b	54.5 (16.3)

The data are mean (S.E.M.) values.
Each value is a relative percent of GM3.

3.4. Effect of L-PDMP on ganglioside contents in cerebral cortex after repeated cerebral ischemia

The contents of major gangliosides in the cerebral cortex were measured on days 3, 5, and 7 after transient ischemia. The chemical contents of GM3, GM1, GD1a, GD1b, GT1b and GQ1b ganglioside were 11.3 ± 0.8, 56.9 ± 8.8, 125.3 ± 4.7, 68.1 ± 4.9, 120.9 ± 11.5 and 39.7 ± 3.5 μg/100 mg wet tissue, respectively, in normal rats. The ganglioside contents

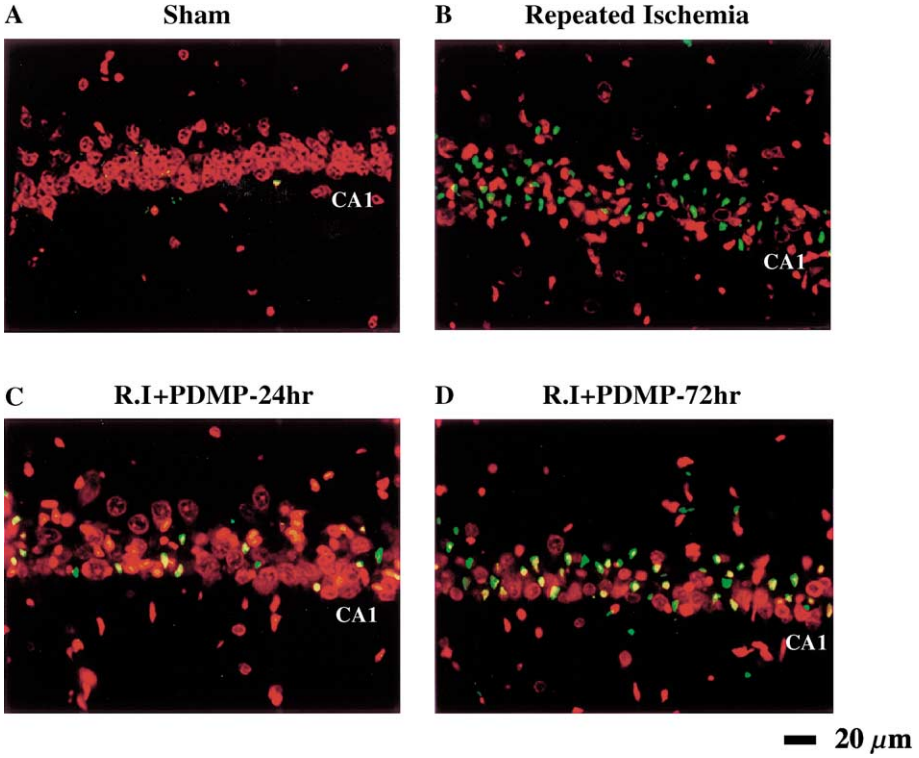

Fig. 4. Hippocampal CA1 in rats with repeated cerebral ischemia 7 days after reperfusion. Green fluorescence shows fragmented DNA in an apoptotic cell. Typical photomicrographs of sham-operated rats (A), repeated ischemia (R.I.) + vehicle (Group 1) (B), R.I. + L-PDMP (Group 1) (C), R.I. + L-PDMP (Group 2) (D). Group 1: i.p. injections of vehicle or 40 mg/kg L-PDMP twice a day for 6 days from 24 h after ischemia, Group 2: i.p. injections of 40 mg/kg L-PDMP twice a day for 4 days from 3 days after ischemia.

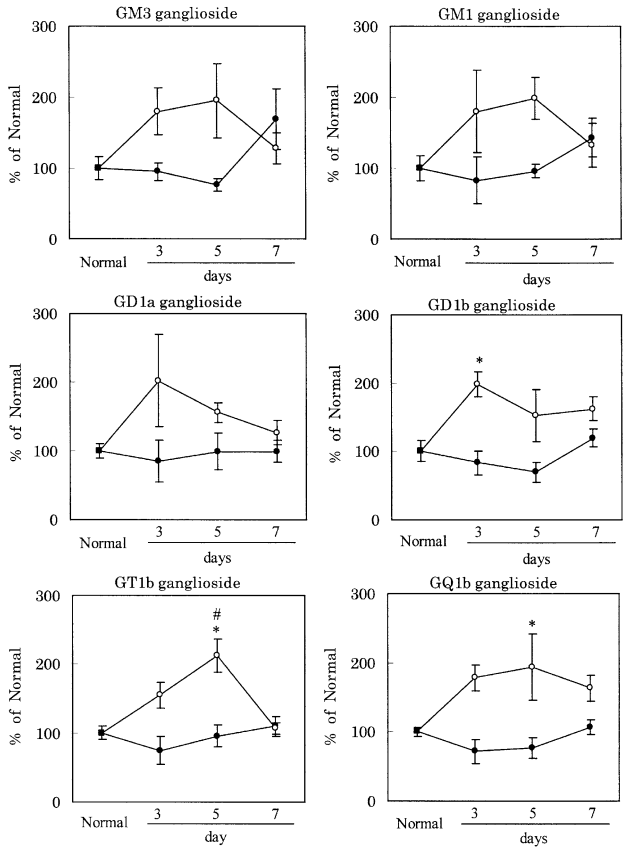

Fig. 5. Effects of L-PDMP on biosynthesis of cortical gangliosides in rats with cerebral ischemia. Normal rats: (■, $n=6$), Vehicle group (●, i.p. injections of vehicle twice a day from 24 h after ischemia): day 3 ($n=5$), day 5 ($n=6$), day 7 ($n=4$), L-PDMP group (○, i.p. injections of 40 mg/kg L-PDMP twice a day from 24 h after ischemia): day 3 ($n=4$), day 5 ($n=6$), day 7 ($n=4$). Data are means ± S.E.M. of % of normal control. *$P<0.05$ vs. vehicle group, #$P<0.05$ vs. normal rats (Tukey's test).

did not change from day to day, and those in L-PDMP-treated rats were not significantly different from those in vehicle-treated rats or normal rats (data not shown).

4. Discussion

In the present study, amelioration by L-PDMP of the ischemia-induced deficit of spatial cognition was confirmed even when treatment was started 24 h after repeated cerebral ischemia (Fig. 3). On the other hand, since no effect on behavior was observed with L-PDMP treatment that started on day 3 after ischemia (Fig. 3), it is considered that it is important to start drug administration between 24 h and 3 days after ischemia in this model. In order to examine the cerebral translocation of L-PDMP, the cerebral concentrations were examined by HPLC analysis after i.p. injection of 40 mg/kg L-PDMP to rats. The cerebral concentration of intact L-PDMP reached that used in various in vitro experiments, Cmax 50 μM (unpublished observations).

The successful results of L-PDMP treatment in ischemic rats encouraged us to investigate whether L-PDMP is able to stimulate ganglioside biosynthesis in vivo under a similar drug administration schedule. As a result of metabolic radiolabeling of gangliosides in the ischemic brain, we found that L-PDMP stimulated the biosynthesis of major gangliosides involving GM3 at the top of the biosynthetic pathway (Fig. 5). In particular, the biosynthesis of b-series gangliosides was significantly increased compared with that in vehicle-treated rats on days 3 and 5 after ischemia. These results correlate with the acceleration of GM3, GD3 and GQ1b synthases and functional synapse formation by L-PDMP in primary cultured cortical neurons (Inokuchi et al., 1997). Since the upregulation of L-PDMP returned to the normal level by day 7, L-PDMP might have a positive effect in the acute ischemic period. The chemical contents of cortical gangliosides were not changed by L-PDMP treatment (data not shown). In primary cultured cortical neurons, it was clear that L-PDMP upregulated ganglioside biosynthesis from the results of metabolic radiolabeling, but no similar change in ganglioside content was observed (Inokuchi et al., 1997). These results suggest that the daily biosynthesis of gangliosides in cerebral neuronal tissue is small, and that the upregulation of biosynthesis for several days has no effect on the ganglioside content. In this experiment, the effects of L-PDMP could not be evaluated in the hippocampus and cerebellum since the amounts of metabolic radiolabeled gangliosides varied widely between normal rats. It may be necessary to modify the radiolabeling method for gangliosides to analyze the hippocampus and the cerebellum.

GM1 and GQ1b enhanced depolarization-induced acetylcholine release from synaptosomes (Ando et al., 1998) and facilitated the tetanus-induced long-term potentiation in rat hippocampal CA1 neurons as positive modulators (Furuse et al., 1998). With transient cerebral ischemia, decreases in acetylcholine release from the dorsal hippocampus were observed after ischemia (Iwasaki et al., 1996, 1998). Though we could not evaluate the ganglioside biosynthetic rates in the whole hippocampus, the supposed upregulation of ganglioside biosynthesis by L-PDMP in the local hippocampal area might normalize the decreased acetylcholine release and synaptic function.

Recently, we found that the repeated cerebral ischemia-induced deficit of spatial cognition is associated with apoptosis in hippocampal CA1 pyramidal cells, and that the key mechanism of promoting apoptosis starts within 3 days of reperfusion (Iwasaki et al., 1998). In fact, it is reported that the expression of apoptosis-regulating molecules such as Bax and caspase-3 in CA1 neurons is seen in the period from 24 to 72 h following cerebral ischemia (Hara et al., 1996; Ni et al., 1998). In this study, L-PDMP tended to prevent apoptosis in hippocampal CA1 on day 7 after ischemia, when treatment was started 24 h after repeated cerebral ischemia (Fig. 4). Interestingly, L-PDMP stimulated cortical ganglioside biosynthesis on day 3 after

ischemia when the drug treatment was started 24 h after ischemia (Fig. 5). Stimulation of ganglioside biosynthesis possibly participates in a cascade of neuronal cell death during the period in which cell death after ischemia shifts from necrosis to apoptosis.

The pharmacological properties of many new drugs such as cerebrovascular enhancers and nootropics have been evaluated, using this dementia model induced by repeated cerebral ischemia. Those drugs have almost shown ameliorative efficacy on the spatial cognition deficit with pre-ischemic administrations, but not with post-ischemic treatments. It is known that AMPA receptor antagonists are neuroprotective in cerebral ischemia, even when administered 6 to 24 h following ischemia (Kawasaki-Yatsugi et al., 1997; Li and Bucham, 1993; Sheardown et al., 1993). In relation to therapy for traumatic ischemic brain injuries in humans, there is a strong incentive to discover a drug possessing such a wider "therapeutic window" (Hara et al., 1994). Thus, L-PDMP or its analogs might be valuable for clinical use in the post-ischemic treatment of cerebrovascular diseases.

In conclusion, it is suggested that stimulation of in vivo ganglioside biosynthesis in the ischemic rat brain by L-PDMP might protect neural activity and result in amelioration of the ischemia-induced deficit of spatial cognition. Although L-PDMP shows a possibility to attenuate apoptosis in the hippocampus, the relationships between the upregulation of ganglioside biosynthesis and apoptotic cell death are still open to investigation. Therefore, further studies to elucidate the action of L-PDMP will lead to understanding of the dynamic regulatory mechanisms of ganglioside biosynthesis and their neuronal function. We are currently searching for the primary molecule in neurons that directly interacts with the ceramide analogs.

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The cerebral penetration of administered L-PDMP was analyzed by T. Kubo.

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