Spermidine Alleviates Severity of Murine Experimental Autoimmune Encephalomyelitis

Xiaoli Guo,1 Chikako Harada,1 Kazubiko Namekata,1 Atsuko Kimura,1 Yoshinori Mitamura,2 Hiroshi Yoshida,3 Yoh Matsumoto,4 and Takayuki Harada1,3

PURPOSE: To assess the effects of spermidine on the severity of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), with a focus on optic neuritis often associated with MS and EAE.

METHODS: Myelin oligodendrocyte glycoprotein-induced EAE mice were administered with or without spermidine at 30 mM in drinking water for 25 days. Clinical signs of EAE were scored daily, and visual functions were measured by multifocal electroretinograms. Histopathology analysis of the spinal cord and optic nerve was performed after mice were killed on day 25. Hydrogen peroxide (H2O2) was detected using the probe 2′,7′-dichlorofluorescin diacetate (DCFDA) in the optic nerve. The effect of spermidine on H2O2-induced retinal ganglion cell apoptosis was investigated by lactate dehydrogenase assay.

RESULTS: Daily clinical scoring revealed that the severity of EAE was significantly attenuated in the spermidine-treated group, which was confirmed by milder demyelination and improved axon survival in the spinal cord of spermidine-treated mice. Visual functions were significantly improved in spermidine-treated mice compared with vehicle-treated mice. Spermidine treatment ameliorated the extent of demyelination in the optic nerve and prevented cell loss in the retinal ganglion cell layer. Furthermore, fewer DCFDA-labeled cells were found in the optic nerve in the spermidine-treated EAE mice, and in vitro analysis revealed that spermidine reduced H2O2-induced retinal ganglion cell apoptosis, suggesting that spermidine alleviated the severities of EAE, particularly of optic neuritis, by acting as an antioxidant.

CONCLUSIONS: The results from this study suggest that oral spermidine administration could be a useful treatment for MS. (Invest Ophthalmol Vis Sci. 2011;52:2696–2703) DOI: 10.1167/iovs.10-6015

From the Departments of 1Molecular Neurobiology and 4Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo, Japan; 2Department of Ophthalmology, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima, Japan; and 3Department of Neuro-ophthalmology, Tokyo Metropolitan Neurological Hospital, Fuchu, Tokyo, Japan.

Supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science for Young Scientists, the Welfare and Health Funds from Tokyo Metropolitan Government, and the Ministry of Health, Labor, and Welfare of Japan.

Submitted for publication June 8, 2010; revised November 1, 2010; accepted November 19, 2010.

Disclosure: X. Guo, None; C. Harada, None; K. Namekata, None; A. Kimura, None; Y. Mitamura, None; H. Yoshida, None; Y. Matsumoto, None; T. Harada, None.

Corresponding author: Takayuki Harada, Department of Molecular Neurobiology, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183-8526, Japan; harada-tk@igakukken.or.jp.

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) characterized by progressive immune-mediated destruction of the myelin sheath and accumulated neurologic disability. The pathophysiological concept of MS includes genetic and environmental factors as well as dysfunction in immune regulation. Recently reactive oxygen species (ROSs) have also been demonstrated to be involved in the pathogenesis of MS.1,2 Increased oxidative stress can lead to axonal degeneration3,4 and is associated with relapses and disease progression in MS.5 It has been suggested that antioxidants are agents worthy of investigation for treating neurodegenerative diseases such as MS.1,6

Optic neuritis, which is an acute inflammatory demyelinating syndrome of the CNS, is one of the major complications in MS. Indeed, for some patients, an episode of optic neuritis may be an early sign of MS. Since it can cause severe visual loss, especially in the optic-spatial form of MS or neuromyelitis optica,7,8 and this loss is irreversible currently, it draws much attention to finding a treatment that will restore the visual function.

Polyamines have a plethora of effects on many aspects of neuronal behavior. They were previously reported to have an effect on neurogenesis,9 brain development,10 neuronal survival,11 and peripheral nerve regeneration.12 Among them, spermidine is a naturally occurring polyamine essential for life.13 Recent studies have shed new light on its functions. For example, increased synthesis of spermidine promoted optic nerve regeneration in vivo.14 Moreover, spermidine, along with spermine, which can be converted from spermine, was reported to play key roles in mediating protection against oxidative damage caused by hydrogen peroxide (H2O2) in cultured mouse fibroblasts.15 Eisenberg et al.16 demonstrated that administration of spermidine extended the lifespan of yeast, flies, worms, and human immune cells by upregulating the lysosomal/vacuolar degradation pathway, referred to as autophagy, which leads to enhanced resistance to oxidative stress and decreased cell death. In view of the pleiotropic protection functions of spermidine, especially its antioxidant property, reported in the literature and the involvement of ROSs in the pathogenesis of MS, we hypothesized that spermidine might be beneficial for suppressing experimental autoimmune encephalomyelitis (EAE) disease signs. In the present study, we investigated the effect of spermidine treatment on EAE with a particular interest in optic neuritis. Our results demonstrated that spermidine alleviated the severities of optic neuritis and other EAE symptoms partly by acting as an antioxidant, which supports the notion that oxidative stress plays a key role in MS.

MATERIALS AND METHODS

Mice

Female C57BL/6J mice were maintained at the animal facilities of the Tokyo Metropolitan Institute for Neuroscience and were 6 to 8 weeks
of age at the time of immunization. Animal treatments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**EAE Induction, Spermidine Administration, and Clinical Scoring**

EAE was induced in mice with myelin oligodendrocyte glycoprotein (MOG)35–55, mixed with 500 μg heat-killed *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI) emulsified in complete Freund’s adjuvant. Each mouse also received intraperitoneal injections of 500 ng pertussis toxin (Seikagaku, Tokyo, Japan) immediately and 48 hours after the immunization. Spermine (Sigma, St. Louis, MO) was added to drinking water at 30 mM for the treatment group throughout the whole experimental period. Control mice were given pure drinking water. Drinking water was replaced every 2–3 days, and spermidine was freshly added from 1 M aqueous stock (spermidine/HCl, pH 7.4), which was kept at -20°C for no longer than 1 month. Clinical signs were scored daily as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, flaccid tail; 3, impairment of righting reflex; 4, partial hind limb paralysis; 5, complete hind limb paralysis; 6, partial body paralysis; 7, partial forelimb paralysis; 8, complete forelimb paralysis or moribund; and 9, death.

**Multifocal Electroretinograms**

The second-order kernel of multifocal electroretinograms (mERGs), which is a sensitive indicator of inner retinal dysfunction,20 was measured as previously reported.19 Briefly, on day 25 after immunization, mice were anesthetized by an intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (25 mg/kg). Pupils were dilated with 0.5% phenylephrine hydrochloride and 0.5% tropicamide. mERGs were recorded using a commercially available system (VERIS 6.0; Electro-Diagnostic Imaging, Redwood City, CA).

**Histopathology and Quantification**

On day 25 after immunization, mice were anesthetized with diethyl ether and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.5% picric acid. Eyes, optic nerves, and lumber spinal cords were removed, postfixed, and processed as previously reported.17–19 Briefly, eyes were embedded in paraffin wax, and sagittal sections through the optic nerve at the thickness of 7 μm were collected and stained with hematoxylin and eosin (HE). To quantify the number of neurons in the ganglion cell layer (GCL) of the retina, cells were counted from one ora serrata through the optic nerve to the other ora serrata. Optic nerves were embedded in paraffin wax, sectioned at a thickness of 7 μm, and stained with luxol fast blue (LFB) followed by HE. Spinal cords were embedded in paraffin wax, sectioned at a thickness of 7 μm, and stained with LFB followed by HE. Immunohistochemistry was performed using the following primary antibodies: rabbit anti-iba1 (1.0 μg/mL),22 mouse anti-GFAP (50 μg/mL; Progen, Toowong, Australia), goat anti-myelin basic protein (MBP; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-NF200 (1:400; Sigma, St. Louis, MO). Quantitative analysis of the immunopositive cell number or stained region was carried out (NIH ImageJ software 1.43u, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

**Detection of ROS in the Optic Nerve**

Detection of H2O2 using the probe 2'-7' dichlorofluorescin diacetate (DCFDA; Molecular Probes, Eugene, OR) was performed as previously reported.23 Briefly, mice were anesthetized 6 days after MOG immunization, and the optic nerves were immediately dissected and processed for fluorescent staining. After a brief rinse in PBS, tissues were incubated in 10 μM DCFDA for 30 minutes at 37°C. Tissues were washed with PBS, fixed with cold 4% paraformaldehyde for 2 hours, and processed for cryomicroscopy. Sections were then observed under a microscope (BX51; Olympus, Tokyo, Japan) equipped with objectives (Plan Fluor) connected to a camera (DP70 Olympus).

**Effect of Spermidine on H2O2-Induced Retinal Ganglion Cell Death**

Retinal ganglion cell (RGCs) were isolated by using a previously described two-step panning method.24 Isolated RGCs were seeded at density of 5 × 10^4 cells/well and cultured with 0.1 mL of medium on a 96-well culture plate. After 2 days, they were unstimulated (control) or stimulated with 2.5 mM H2O2, in the presence or absence of 4 mM spermidine for 16 hours. After incubation, 50 μL of culture medium was collected and analyzed using a lactate dehydrogenase (LDH) cytotoxic test kit (Wako, Osaka, Japan), to estimate cell damage. LDH activity after H2O2 stimulation was normalized by subtracting control values from unstimulated cells.

**Cell Culture**

Primary astrocytes were obtained as previously reported.25 Astrocytes were treated with or without LPS (1 μg/mL) in the presence or absence of 4 mM spermidine for 16 hours. Concentrations of chemokines (MCP-1, RANTES, MIP-1α) in the cell culture media were determined by enzyme-linked immunosorbent assays (ELISA).25

**Statistics**

Data are presented as mean ± SE. Student’s t-test was used for statistical analyses, and results were considered to be significant at *P < 0.05*.

**Results**

**Effect of Spermidine on the Severity of EAE**

We first examined the effect of spermidine on EAE clinical signs during the course of spermidine treatment from 0 to 25 days after the disease induction. All vehicle-treated (*n* = 16) and spermidine-treated (*n* = 16) mice developed EAE with an incidence of 100% and showed a similar mean disease onset (9.3 ± 0.5 vs. 9.3 ± 0.3 days; *P = 0.97*). However, the severity of EAE presented by the clinical scores in spermidine-treated mice was significantly reduced compared with vehicle-treated mice after day 14 (Fig. 1). The mean clinical scores of vehicle- and spermidine-treated mice on day 25 were 4.3 ± 0.1 and 2.9 ± 0.3, respectively (*P = 0.0008*). We also investigated the histopathology of spinal cord on day 25. In vehicle-treated mice, LFB&HE staining revealed numerous inflammatory cell infiltrates and severe demyelination in the white matter of the lumbar spinal cord (Fig. 2B), which was confirmed by MBP staining (Fig. 2E). In addition, the level of NF200 staining for intact axons was largely reduced (Fig. 2H), indicating severe nerve fiber damage in vehicle-treated mice. In contrast, in spermidine-treated mice, the number of infiltrating cells was

**FIGURE 1.** Effect of spermidine on clinical scores of EAE mice. Mice were treated with vehicle or spermidine for 25 days after immunization with myelin oligodendrocyte glycoprotein. Results of 16 independent animals are presented as mean ± SE.
reduced, and the extent of demyelination was less severe (Figs. 2A–F, 3A). Moreover, the NF200 staining in spermidine-treated mice was more intense than vehicle-treated mice (Figs. 2G–I, 3B), indicating a higher rate of surviving axons in spermidine-treated mice. Furthermore, the numbers of GFAP-positive astrocytes and iba1-positive microglia cells were increased in vehicle-treated mice, whereas they were drastically suppressed in spermidine-treated mice (Figs. 2J–O, 3C–D). These results suggest that spermidine ameliorated the major EAE signs in both behavioral and histologic aspects.

Effect of Spermidine on the Severity of Optic Neuritis

To assess the effect of spermidine on the severity of optic neuritis in MOG-induced EAE mice, we first examined the visual function using mfERGs, an established noninvasive

**Figure 2.** Representative histology of the spinal cords in EAE mice. Ventral region of lumbar spinal cords was stained with luxol fast blue (LFB) and hematoxylin and eosin (HE) (A–C), anti-myelin basic protein (MBP) (D–F), anti-NF200 (G–I), anti-GFAP (J–L), and anti-iba1 (M–O) antibody. Bar: (G–I) 40 μm; (A–F, J–O) 220 μm.

**Figure 3.** Quantitative analysis of the histopathology of the spinal cords in EAE mice. (A, B) Quantitative analysis of demyelination (A) and axon degeneration (B) in the white matter of the spinal cord. (C, D) Quantitative analysis of GFAP-positive (C) and iba1-positive (D) cells in the spinal cord. GFAP and iba1-positive cells were counted per unit area (0.143 mm²) in the middle region of the ventral horn. ***P < 0.001, **P < 0.01, *P < 0.05.
method. Figure 4A shows the averaged responses of the second-order kernel in each group. The visual function in vehicle-treated EAE mice was impaired in all visual fields, which was clearly improved by spermidine treatment (Fig. 4A).

The quantitative analysis confirmed that the visual function was significantly improved in spermidine-treated mice (2.7 ± 0.4 nV/deg²; n = 8) compared with vehicle-treated EAE mice (1.3 ± 0.2 nV/deg²; n = 8; P = 0.015; Fig. 4B). We next analyzed the histopathology of the optic nerve. In vehicle-treated EAE mice, inflammatory cells and demyelination, as revealed by LFB&HE and MBP staining, appeared distinctively in the optic nerve lesion, while they were almost absent in spermidine-treated mice (Figs. 5A–I, 6A). Moreover, a higher rate of surviving axons was demonstrated by NF200 staining in spermidine-treated mice compared with vehicle-treated mice. *P < 0.01.

**Figure 5.** Representative histopathology of the optic nerves in EAE mice. Optic nerves were stained with luxol fast blue (LFB) and hematoxylin and eosin (HE) (A–F), anti-myelin basic protein (MBP) (G–I), anti-NF200 (J–L), anti-GFAP (M–O), and anti-iba1 (P–R) antibody. (D–F) Higher magnifications of (A–C), respectively. Bar: (D–F) 45 µm; (A–C, G–R) 110 µm.

**Figure 6.** Quantitative analysis of the histopathology of the optic nerve in EAE mice. Quantitative analysis of demyelination (A), axon degeneration (B), GFAP-positive (C), and iba1-positive (D) areas in the optic nerve. *P < 0.01, **P < 0.05.
Spermidine-treated mice (Figs. 5J–L, 6B). In addition, fewer activated GFAP-positive astrocytes or iba1-positive microglials appeared in spermidine-treated mice (Figs. 5M–R, 6C–D). Taken together, these data demonstrate that spermidine treatment attenuates EAE-induced optic neuritis in both histologic and functional aspects.

**Effect of Spermidine on the Protection of Retinal Neurons**

Since spermidine protected optic nerves from EAE-induced optic neuritis, we further examined whether it also protected retinal neurons in EAE mice. We administered vehicle and spermidine to EAE mice and performed histologic evaluation. EAE induced a mild cell loss in the GCL in vehicle-treated mice, but such degeneration was not obvious in spermidine-treated mice (Fig. 7A). Quantitative analysis revealed that the number of surviving cells in the GCL was significantly increased after the spermidine treatment (from $351 \pm 11$ in vehicle-treated mice to $423 \pm 20$ in spermidine-treated mice; $P = 0.007$) (Fig. 7B). These results suggest that spermidine protects RGCs during EAE.

We next investigated the possible mechanism contributing to the protective effect of spermidine in EAE. To this aim, we focused on the role of ROSs since ROSs are known to be involved in the pathogenesis of MS. A drastic rise in the $\text{H}_2\text{O}_2$ level, which was detected by DCFDA staining, was found in the optic nerve of vehicle-treated EAE mice on day 6 after MOG immunization (upper panels in Fig. 8A). However, this vast change was not detected in the optic nerve of spermidine-treated EAE mice (upper panels in Figs. 8A and 8B), suggesting that spermidine ameliorates EAE disease signs by exerting its antioxidant effects. We further examined this possibility by using an in vitro model. RGCs were subjected to oxidative stress by $\text{H}_2\text{O}_2$, and we investigated whether spermidine treatment has any protective effects on $\text{H}_2\text{O}_2$-induced RGC death. By examining extracellular LDH activities, $\text{H}_2\text{O}_2$-induced apoptosis was clearly detected in nontreated RGCs ($0.55 \pm 0.03; n = 8$); however, spermidine treatment significantly reduced $\text{H}_2\text{O}_2$-induced RGC apoptosis ($0.14 \pm 0.06; n = 8; P = 0.009$ (Fig. 8D). Taken together, these results suggest that the protective effect of spermidine in EAE is attributed to its antioxidant function.

Spermidine has also been reported to have anti-inflammatory effects. Since inflammation is implicated in the pathogenesis of MS, we also examined the possibility that spermidine may attenuate the severity of EAE partly by exerting its anti-inflammatory function. At day 6, when spermidine shows its antioxidant effect on EAE mice, infiltrated...
cells are still sparse in the optic nerve, and no difference was found between the vehicle- and spermidine-treated groups (lower panels in Figs. 8A and 8C), suggesting that antioxidant effects may be more dominant than anti-inflammatory effects. We also used an in vitro model and measured the release of three key chemokines from primary cultured astrocytes that were treated with or without spermidine, using an ELISA. Secretion of MCP-1, RANTES, and MIP-1α was dramatically induced in astrocytes after LPS stimulation (Fig. 9). Interestingly, such LPS-induced secretion was significantly reduced with spermidine treatment, suggesting a possible anti-inflammatory function of spermidine in ameliorating the severity of EAE.

**DISCUSSION**

It has been known that spermidine can serve as a free-radical scavenger not only in vitro but also in vivo. In the present study, we demonstrated that spermidine treatment attenuated clinical scores of EAE mice and reduced the extents of demyelination in the spinal cords. In addition, spermidine prevented neural cell death in the GCL and reduced the extents of demyelination in the optic nerve of EAE mice. The results from the second-order kernel of mfERGs, which is a sensitive indicator of inner retinal function including RGCs, demonstrated that visual function was significantly improved in spermidine-treated EAE mice. Furthermore, the level of H2O2, as detected by the DCFDA probe, was significantly reduced in the optic nerves of EAE mice by the treatment of spermidine. Also, in vitro analysis revealed that spermidine protected RGCs from H2O2-induced cell death, suggesting that spermidine ameliorated the severity of EAE by acting as an antioxidant.

The role of ROSs, such as superoxide and H2O2, in the pathogenesis of MS has been emerging. Several antioxidants such as tempamine and thymoquinone have been shown to reduce the severity of EAE. In addition, suppression of mitochondrial oxidative stress by SOD2 gene delivery provided long-term neuroprotection in experimental optic neuritis. Moreover, we have previously shown that loss of apoptosis signal-regulating kinase 1 (ASK1), a mitogen-activated protein kinase kinase (MAPKKK) that is activated in response to various stimuli including ROSs, protects RGCs from ischemic injury. Thus, antioxidants in combination with inhibitors of ROSs’ downstream signals, such as ASK1 inhibitor, might prove effective for the treatment of MS.

In addition to oxidative stress, imbalanced glutamate metabolism can also lead to axonal degeneration in MS. Agents that antagonize glutamate receptor activity might be effective for the treatment of MS. One of the potential targets in this regard is glutamate transporters, which are responsible for the removal of glutamate from the extracellular fluid. We recently showed that loss of glutamate/aspartate transporter (GLAST), a glia-type glutamate transporter, leads to RGC loss and optic nerve degeneration, the histopathology similar to normal tension glaucoma. Interestingly, memantine, an N-methyl-D-aspartate (NMDA) receptor antagonist, partially protected RGCs in GLAST-deficient mice. Thus, it will be intriguing to study EAE in mice overexpressing glutamate transporters and determine whether this is an effective strategy for treating MS. Naturally, inhibiting both oxidative stress and glutamate neurotoxicity may lead to more effective treatment for MS. Indeed, a novel water-soluble fullerene derivative (ABS-75) attached to an NMDA receptor antagonist, which combines antioxidant and anti-excitotoxic properties, was shown to block axonal damage and reduce disease progression in a chronic progressive EAE model.

One point that needs to be addressed is that spermidine has anti-inflammatory effects. Spermidine has been reported to play an important role in the suppression of inflammatory mediators. In addition, it was shown that spermine, which is converted from spermidine, protected mice against lethal sepsis partly by attenuating surrogate inflammatory markers such as IL-6, MCP-1, and soluble tumor necrosis factor-alpha receptor I and II in peritoneum and serum. We have previously shown that inflammatory markers such as MCP-1 and TNFα are elevated during the course of EAE. Moreover, we demonstrated that the re-

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**FIGURE 9.** Effect of spermidine on chemokine release from astrocytes. Astrocytes isolated from wild-type mice were treated with or without spermidine (4 mM) under LPS stimulation (1 μg/mL) for 16 hours. Concentrations of MCP-1 (A), RANTES (B), and MIP-1α (C) in culture medium were measured by ELISA. **P < 0.001, *P < 0.01.**
lease of several key chemokines from astrocytes was significantly reduced with spermidine treatment (Fig. 9). Thus, it is possible that spermidine may attenuate the severity of EAE partly by exerting its anti-inflammatory function in addition to its antioxidant effects. However, it has been reported that oxidative stress occurs before the infiltration of inflammatory cells in the optic nerve. Together with the findings that no difference in the number of infiltrated cells was found between the vehicle- and spermidine-treated groups at the time when spermidine clearly suppressed the rise in the H_{2}O_{2} level (Fig. 8), we suggest that spermidine might first act as an antioxidant reagent before exerting its anti-inflammatory function. The extent of reduced oxidative stress in the optic nerve on day 6 with the treatment of spermidine (Fig. 8) indicates that antioxidant property of spermidine played a significant role in reducing the severity of EAE, particularly of optic neuritis.

Spermidine is a natural component of our diet, and several foods are known to be rich in spermidine, including soy beans, tea leaf, and mushrooms. Evidence suggests that eating a diet rich in spermidine results in increased blood spermidine levels. Thus, the benefits of spermidine could be easily attainable for most people through dietary modifications or by supplementation, although oral administration of spermidine was given before the disease onset of EAE in our study. Taken together, our present study suggests that spermidine might be suitable for the treatment of MS and optic neuritis.

References


