Introduction

The concept that the adult mammalian central nervous system is incapable of self-repair/regeneration had to be reevaluated after the detection of neuronal stem cells, defined by their ability to rapidly self-renew and differentiate into the 3 major cell types, that is, neurons, astrocytes, and oligodendrocytes, in the adult brain (1). The subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus are the most important regenerative centers in the adult brain (2). Cells derived from the subgranular zone move into the granule cell layer of the dentate gyrus and sprout axons to the CA3 region of the hippocampus (3). In the subgranular zone, neural stem/progenitor cells (NPCs) are generally classified into 2 different cell populations (4 – 6). One is the radial glia-like cell (type 1 cell), that expresses fibrillary acidic protein (GFAP), nestin, brain lipid-binding protein, and the SRY-related high mobility group box transcription factor SOX2. These cells cross into the granule cell layer and rarely enter into the cell cycle. The other is

In Vivo and In Vitro Treatment With Edaravone Promotes Proliferation of Neural Progenitor Cells Generated Following Neuronal Loss in the Mouse Dentate Gyrus

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Abstract. Edaravone is clinically used in Japan for treatment of patients with acute cerebral infarction. To clarify the effect of edaravone on neurogenesis in the hippocampus following neuronal injury in the hippocampal dentate gyrus, we investigated the effect of in vitro and in vivo treatment with edaravone on the proliferation of neural stem/progenitor cells prepared from the mouse dentate gyrus damaged by trimethyltin (TMT). Histological assessment revealed the presence of large number of nestin(+) cells in the dentate gyrus on days 3 – 5 post-TMT treatment. We prepared cells from the dentate gyrus of naïve, TMT-treated mice or TMT/edaravone-treated mice. The cells obtained from the dentate gyrus of TMT-treated animals were capable of BrdU incorporation and neurosphere formation when cultured in the presence of growth factors. The TMT-treated group had a larger number of nestin(+) cells and nestin(+)GFAP(+) cells than the naïve one. Under the culture condition used, sustained exposure of the cells from the damaged dentate gyrus to edaravone at 10^{-11} and 10^{-8} M promoted the proliferation of nestin(+) cells. The systemic in vivo treatment with edaravone for 2 days produced a significant increase in the number of nestin(+) cells among the cells prepared from the dentate gyrus on day 4 post-TMT treatment, and as well as one in the number of neurospheres formed from these cells in the culture. Taken together, our data indicated that edaravone had the ability to promote the proliferation of neural stem/progenitor cells generated following neuronal damage in the dentate gyrus.

Keywords: dentate gyrus, edaravone, neural stem/progenitor cell, neurogenesis, neuronal damage
the amplifying progenitor (type 2a cell), which expresses nestin and SOX2 but not GFAP; and it enters into the cell cycle more often. The type 2a cells are proposed to be derived from the type 1 ones. Adult hippocampal neurogenesis in mammals is regulated by the proliferation of the NPCs under physiological and pathological conditions including exercise (7), enriched living conditions (8, 9), and aging (9, 10). These findings suggest that the mechanisms controlling life-long neurogenesis in the postnatal central nervous system are adapted to complex extrinsic inputs. In addition to physiological conditions, pathological conditions such as psychosocial stress (11, 12), seizure (13), and hippocampal neurodegeneration (14, 15) are also known to affect neurogenesis in the hippocampal dentate gyrus.

The organotin trimethyltin chloride (TMT) produces neuronal degeneration in both human and rodent central nervous systems (16). The pattern of neurodegeneration by a single exposure to TMT is different between rats and mice: whereas in rats TMT damages granule neurons of the dentate gyrus and pyramidal cells in the CA1 and CA3c/CA4 subfields (17), this damage is restricted to the granule neurons of the dentate gyrus in mice (18–21). Our previous studies using mice also demonstrated that TMT treatment markedly produces enhanced neurogenesis in the dentate gyrus to regenerate the granule cell layer (15). These previous findings lend support to the proposal that the TMT-exposure mouse model is a very sensitive animal model for studies on neuronal regeneration following neurodegeneration in the dentate gyrus.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a low molecular weight free radical scavenger that readily crosses the blood–brain barrier (22, 23). Edaravone interacts biochemically with a wide range of free radicals, mainly the hydroxyl radical (•OH), and protects neurons from ischemic injury in animal models (24, 25). Based on clinical trials in patients with acute cerebral infarction, edaravone was successfully used to treat a patient with acute cerebral ischemia (26). In the middle cerebral artery occlusion/reperfusion–treated rats, edaravone has the ability to decrease cell proliferation and NPCs in the subventricular zone as well as to protect neurons from ischemic injury though reduction in reactive oxygen species (27). In addition to the in vivo experimental data, our previous data that a direct exposure to edaravone at 50 μM suppresses the proliferation of NPC derived from the mouse embryonic hippocampus through scavenging endogenously generated free radicals (28) suggest that edaravone may not be beneficial, for it may suppress neurogenesis enhanced following neuronal injury. However, the in vivo effect of edaravone on neurogenesis enhanced by neuronal injury in the hippocampal dentate gyrus has been little investigated.

In the hippocampus, the subgranular zone of the dentate gyrus is the most important regenerative center. Indeed, the dentate gyrus has the ability to regenerate granule neurons after neuronal loss there. In the CA region of the hippocampus, otherwise, no newly-generated pyramidal neurons have been found following neuronal damage. Therefore, the present study was designed to make an ex vivo assessment as a step toward clarifying the effect of edaravone on neurogenesis following neuronal injury in the hippocampal dentate gyrus. To this end, we herein used the TMT-treated mouse with predominant neuronal loss in the hippocampal dentate granule cell layer and followed the neuronal regeneration there (15). The essential importance of the present findings is that edaravone had the ability to promote the proliferation of NPCs in the subgranular zone following neuronal loss in the hippocampal dentate gyrus, thus indicating a possible beneficial effect for it on neuronal regeneration in the damaged region.

Materials and Methods

Materials

Edaravone was kindly donated by Mitsubishi Pharma Co. (Osaka). Edaravone was dissolved in 1 M NaOH and titrated to pH 7.0 with 1 M HCl. Dulbecco’s modified Eagle’s medium, Nutrient Mixture F12 (1:1, DMEM/F-12), was supplied by Invitrogen Co. (Eugene, OR, USA). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from Peprotech (Rocky Hill, NJ, USA). Fetal cow serum (FCS) was from JRH Biosciences (Lenexa, KS, USA). Poly-l-ornithine and rabbit polyclonal antibody against GFAP came from Sigma-Aldrich Co. (Eugene, OR, USA). Mouse monoclonal antibodies specific for nestin (Millipore Co., Boston, MA, USA), β-tubulin III (Chemicon International, Temecula, CA, USA), and neuronal nuclear antigen (NeuN; Chemicon International) were also used. TMT and Protein Assay Rapid kit were supplied by Wako Pure Chemical Industries, Ltd. (Osaka). All other chemicals used were of the highest purity commercially available.

Animal treatment

The protocol used here met the guidelines of The Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Setsunan University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.
Adult male Std-ddY mice weighing 26–28 g were housed in metallic breeding cages in a room with a light (12 h)–dark (12 h) cycle and a humidity of 55% at 23°C and given free access to food and water for at least 4 days before use. The animals were intraperitoneally injected with TMT (2.8 mg/kg) dissolved in phosphate-buffered saline (PBS) and then returned to their home cages until the time of decapitation. Edaravone (30 mg/kg, i.p.) was intraperitoneally injected into the mice every 12 h, starting from day 2 post-TMT treatment, for the desired number of days.

**Cell culture**

Ten brains were quickly removed and immersed in saline at 2°C, after which 0.5-mm slices of the brains were prepared to dissect the dentate gyrus of the hippocampus under microscopic observation (15). The dissected dentate gyrus was suspended in suspension medium consisting of DMEM/F-12 supplemented with 10% (vol/vol) FCS, 0.12% sodium bicarbonate, 100 U/mL penicillin, 16.5 mM glucose, and 0.25 mM N-acetyl-L-cysteine by pipetting and then centrifuged at 500 × g for 5 min. For dissociation of the cells, the pellet was resuspended in Dulbecco’s PBS containing 100 U/mL penicillin, 0.1 mg/mL streptomycin, 33 mM glucose, 2 μg/mL papain, 0.5 mg/mL DNase, and 0.18 mg/mL neutral protease (Worthington Biochemical Co., Lakewood, NJ, USA) and then incubated at 37°C for 5 min. After gentle trituration and centrifugation at 500 × g for 5 min, the cells obtained were washed twice with the suspension medium by gentle trituration and centrifugation and subsequently washed once again with growth medium (see below) from which the insulin, EGF, and bFGF had been excluded. For use in the floating culture system as neurosphere assay, the cells finally obtained (15,000 cells/0.5 mL) were put into each well (1.9 cm²) of a culture plate [Nalge Nunc dish, low cell bind (24-well Cs7); Nunc, Denmark] and usually cultured for the desired periods in growth medium consisting of DMEM/F-12 supplemented 0.12% sodium bicarbonate, 100 U/mL penicillin, 16.5 mM glucose, 20 nM progesterone, 30 nM sodium selenite, 60 nM putrescine, 100 μg/mL apotransferrin, 25 μg/mL insulin, 20 ng/mL EGF, and 20 ng/mL bFGF, with a half medium change every 4 days. For use in the monolayer culture system, the cells were seeded at a density of 15,000 cells/0.5 mL in 4-well plates (Nunc, Denmark) that had been previously coated with poly-L-ornithine and then cultured for the desired periods in the growth medium. The cultures were always maintained at 37°C in 95% (vol/vol) air – 5% (vol/vol) CO₂. After seeding, the cells were exposed to no FCS at all to avoid possible influences of hitherto unidentified factors present in the FCS.

**Immunostaining of cultured cells**

The cells were washed with TBST and fixed with 4% (wt/vol) paraformaldehyde for 20 min at 4°C. After having been blocked for 1 h at room temperature with 5% (vol/vol) goat serum in Tris-buffered saline (pH 7.5) containing 0.03% (wt/vol) Tween 20 (0.03% TBST), the cells were incubated at 4°C overnight with the primary antibody against GFAP (1:200), nestin (1:200), β-tubulin III (1:100), or BrdU (1:1000). After a wash with 0.03% TBST, they were then reacted with fluorescence-labeled secondary antibodies [Texas Red–conjugated anti-rabbit IgG (1:200) for GFAP and BrdU; FITC-conjugated antimouse IgG (1:200) for nestin and β-tubulin III] for 2 h at room temperature. Finally, they were counterstained with Hoechst 33342 (5 μg/mL) for 20 min at room temperature. Stained cells were observed by confocal laser scanning microscopy (FV1000-D; Olympus, Tokyo). The number of cells positive for each antibody was counted in 5 different visual fields randomly selected on each coverslip. The number of cells was determined as the average of those found in the visual fields. “X-positive” and “X-negative” cells were reported as “X(+)” and “X(−)” cells, where “X” refers to a given antigen.

**BrdU incorporation**

Cell proliferation was assessed by determining BrdU incorporation into the cells during the culture period. Cells were exposed to 0.1 μM BrdU and then centrifuged at 300 × g for 10 min. After removal of the medium, the cells remaining in the dish were evaluated for their BrdU level by using a Cell Proliferation ELISA kit according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany).

**Preparation of brain slices**

Mice were anesthetized with chloral hydrate (500 mg/kg, i.p.) and perfused via the heart with saline, followed by 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brains were quickly removed and further fixed with the same fixative solution at 4°C overnight. Post-fixed brains were embedded in paraffin, cut as sagittal sections of 3-μm thickness with a microtome, and placed on Matsunami-adhesive silane-coated glass slides (Matsunami Glass Ind., Ltd., Kyoto). The paraffin-embedded brain sections were then deparaffinized with xylene, rehydrated by immersion in ethanol of graded decreasing concentrations of 100% – 50% (vol/vol), and finally washed with water. Sections so obtained were subjected to the immunohistochemical procedures described below.

**Immunohistochemical evaluation**

For immunostaining for NeuN and nestin, the sections
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(Lateral 1.4 – 1.6 mm) were first heated for 10 min in 10 mM sodium citrate buffer (pH 7.0) by use of a microwave oven, washed with 0.03% TBST, and subsequently incubated with 0.03% (vol/vol) H2O2 in methanol for 5 min. After having been blocked with 5% goat serum, the sections were incubated with primary antibody against NeuN (1:300) or nestin (1:400) at 4°C overnight. After a wash with 0.03% TBST, they were next reacted with biotinylated secondary antibody against mouse IgG (1:300) for 30 min at room temperature and subsequently incubated with the reagents of the streptavidin-biotin complex peroxidase kit for 1 h at room temperature.

Data analyses

All data were expressed as the mean ± S.E.M., and the statistical significance was determined by using the Welch’s t-test or Dunnett’s test.

Results

Preparation of NPCs from the dentate gyrus

Our previous report indicated that the acute systemic treatment with TMT produces a dramatic neuronal loss in the dentate granule cell layer on day 2 post-treatment and cognitive impairment in mice (15). Following the TMT-induced neuronal loss in the dentate gyrus, a marked increase in the number of BrdU-incorporating cells and of nestin(+) cells, NeuroD(+) cells, and doublecortin(+) cells, which are neurogenesis-related markers, is seen in the dentate gyrus. However, there has still been no direct evidence for an increase in the NPC population in the dentate gyrus of TMT-treated animals. To address this issue, we prepared NPCs derived from the dentate gyrus of naïve and TMT-treated animals.

First, we determined the time course of nestin expression in the dentate gyrus following TMT treatment, in order to confirm the time points for enhanced expression of nestin(+) cells following neuronal loss in the dentate gyrus (Fig. 1). Immunostaining for NeuN revealed dramatic neuronal loss in the granule cell layer of the dentate gyrus on days 3 – 5 post-TMT treatment and a significant recovery of the granule cell layer on day 30 post-TMT treatment. The highest level of nestin was seen in the dentate gyrus on days 3 – 5 post-TMT treatment and the expression returned to the naïve level on day 10 and afterward post-TMT treatment.

Based on the data shown in Fig. 1, cells were subsequently prepared from the dentate gyrus of naïve and TMT-treated animals on day 3 post-treatment. Immunostaining for nestin and GFAP revealed that nestin(−)GFAP(+) cells and nestin(+)GFAP(+) cells were mainly observed in the preparation from naïve animals (Fig. 2a). In the addition to nestin(+)GFAP(+) cells, nestin(+)GFAP(−) cells were evident in the preparation from TMT-treated animals. The total number of surviving cells obtained from the dentate gyrus was significantly greater in the TMT-treated group than in the naïve group (Fig. 2b). Interestingly, the TMT-treated group had a markedly larger number of nestin(+)GFAP(−) cells and nestin(+)GFAP(+) cells compared to the naïve group (Fig. 2c) [nestin(+)GFAP(−) cells (% of total cells): naïve (n = 4), 1.17 ± 0.06; TMT (n = 5), 21.1 ± 6.0 (P < 0.05); nestin(+)GFAP(+) cells (% of total cells): naïve (n = 4), 3.35 ± 1.18; TMT (n = 5), 13.2 ± 3.2 (P < 0.05)]. Although no significant change in total GFAP(+) cells was seen between naïve and TMT-treated ones, the ratio of nestin(−)GFAP(+) cells to total cells was decreased in

![Fig. 1. Dramatic increase in nestin(+) cell number during neuronal degeneration and regeneration in the dentate gyrus following acute treatment with TMT in vivo. Animals were given TMT (2.8 mg/kg, i.p.) and then killed on the indicated days post-treatment for preparation of hippocampal sagittal sections, which were used for immunostaining for NeuN and nestin. These experiments were carried out at least 4 times with similar results obtained under the same experimental conditions. GCL, dentate granule cell layer; Hilus, dentate hilus; ML, dentate molecular layer. Scale bar = 200 μm.](image-url)
the TMT-treated group [nestin(−)GFAP(+) cells (% of total cells): naïve (n = 4), 18.4 ± 3.1; TMT (n = 5), 6.73 ± 2.74 (P < 0.05)].

Culture of NPCs derived from the dentate gyrus

For a trial experiment to prepare NPCs derived from the dentate gyrus of naïve and TMT-treated animals, we cultured the cells obtained from the dentate gyrus of the respective animals in the monolayer culture system in the presence of growth factors including bFGF and EGF for 28 days in vitro (DIV) and then determined the total number of surviving cells and nestin(+) cells (Fig. 2d). At 14 DIV, the number of total surviving cells and nestin(+) cells in the TMT-treated group had increased...
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5- to 6-fold over those in the naïve group. At 14 DIV, these increases were 3-fold.

To determine the proliferative activity of the cultured cells, we examined BrdU incorporation into them. In both naïve and TMT-treated groups, BrdU was predominantly incorporated into nestin(+) cells cultured for 14 DIV (Fig. 3a). The incorporation of BrdU was significantly increased in the cells of the TMT-treated group compared with that of the naïve group throughout the culture period (Fig. 3b).

We next cultured the cells in the floating culture system in order to perform the neurosphere assay. In both naïve and TMT-treated groups, marked round spheres were formed and continued to grow and proliferate to form large neurospheres in proportion to time in culture up to 30 DIV (Fig. 4a). As expected, the number of neurospheres was significantly greater in the TMT-treated group than the naïve group throughout the period of culture from 5 to 30 DIV (Fig. 4b). The neurospheres obtained by culturing either group for 30 DIV were positive for nestin (Fig. 4c).

In vivo treatment with edaravone increased nestin(+) cells and β-tubulin III(+) cells in the dentate gyrus of TMT-treated mice

The above finding that nestin(+) cells, but not GFAP(+) cells, were increased in number under the culture conditions in the presence of edaravone prompted us to test whether in vivo treatment with edaravone would increase the number of nestin(+) cells in the dentate gyrus of

![Fig. 4.](image)

Sustained in vitro exposure to edaravone promoted the proliferation of NPCs derived from the dentate gyrus of TMT-treated mice

To test the effect of edaravone on the proliferation of NPCs in the dentate gyrus after neuronal degeneration, we cultured cells derived from the dentate gyrus of TMT-treated mice by using the monolayer system with medium containing edaravone or not. Edaravone at 10 pM or 10 nM was significantly effective in increasing the total number of cells cultured for 7 or 14 DIV, but not effective at 1 DIV (Fig. 5a). In addition to total cells, nestin(+) cells were significantly increased in number by edaravone at both concentrations during culture for 7 or 14 DIV (Fig. 5b). However, no significant change due to edaravone was seen in the number of GFAP(+) cells throughout the culture periods used (Fig. 5c).

To determine if sustained exposure to edaravone damages cells, we performed Hoechst 33342 staining in the cells after edaravone exposure. No change in damaged cells with nuclear condensation was observed by edaravone, at least at the concentrations used (data not shown).
TMT-treated mice. After mice had been given edaravone or saline from day 2 to 4 post-TMT treatment, cells were prepared from the dentate gyrus for nestin immunostaining and culturing in the floating system. Immunostaining revealed that both nestin(+) and nestin(+GFAP(+) cells were significantly increased in number in the edaravone-treated group compared with their numbers in the saline-treated group (Fig. 6). However, no significant change was seen in the GFAP(+) cell number between saline- and edaravone-treated groups. When cells were cultured for 9 and 18 DIV in the floating system, the number of neurospheres formed was significantly greater in the edaravone-treated group than in the saline-treated one (Fig. 7: a and b). Nestin(+) cells were found in the neurospheres formed by the cells derived from either group (Fig. 7c). In addition to nestin(+) cells, β-tubulin III(+) cells were increased in number among the cells obtained from the dentate gyrus of mice consecutively treated with edaravone from days 2 to 9 post-TMT treatment [β-tubulin III(+) cells (% of total cells): saline (n = 6), 15 ± 5; edaravone, 43 ± 6 (n = 6, P < 0.05)].

Discussion

The important proposition stemming from the present study is that edaravone promotes the proliferation of NPCs generated following neuronal loss in the hippocampus. This proposition is based on the following findings: The in vitro sustained exposure to edaravone promoted the proliferation of nestin(+) cells among the cultured NPCs derived from the dentate gyrus damaged by TMT. The systemic injection of edaravone into mice promoted the generation of NPCs positive for nestin, as well as that of immature neurons positive for β-tubulin III, in the dentate gyrus damaged by TMT treatment. These findings suggest that edaravone had the beneficial effect of enhancing neurogenesis there following the hippocampal injury. The beneficial effect of edaravone is supported by previous data showing that edaravone protects NPCs positive for nestin around the area of traumatic cerebral cortex injury (29). The present data are the first to show that exposure to edaravone of cultured NPCs to edaravone increased the number of nestin(+) cells during culture in the presence of EGF and bFGF, thus allowing us to propose the idea that edaravone has the ability to promote the proliferation of NPCs. It is thus likely that the observed in vivo effect of edaravone was due to both protection and proliferation of NPCs generated following dentate neuronal loss.

Nestin(+) cells are known to increase in number around the damaged cerebral cortex following cryoinjury (30), ablation injury (31), or controlled cortical impact (32). In the present study, we used TMT-treated mouse as a model for neuronal degeneration/regeneration in the hippocampal dentate gyrus. This model shows predominant neuronal loss in the dentate granule cell layer on day 2.
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Fig. 6. Effect of in vivo treatment with edaravone on the number of nestin(+) and GFAP(+) cells prepared from the dentate gyrus of TMT-treated mice. Animals were given TMT (2.8 mg/kg, i.p.) and then injected with either saline or edaravone (30 mg/kg, i.p.) every 12 h for a consecutive 2 days starting from day 2 post-TMT treatment. On day 4 post-TMT treatment, the dentate gyrus was dissected from the 10 animals of saline- or edaravone-treated group for preparation of cells, which were then double-immunostained for nestin and GFAP. The data for antigen-positive cells in the edaravone-treated group are presented as the percentage of those in the saline-treated group as the control. Values are expressed as the mean ± S.E.M. from 5 – 6 separate experiments. *P < 0.05, **P < 0.01, significantly different from the value obtained for saline-treated animals. In the saline-treated group, the percentages of nestin(+) cells, GFAP(+) cells and nestin(+)/GFAP(+) cells to total cells were 22%, 73%, and 20%, respectively.

post-TMT treatment (degeneration stage, day 0 to 2 post-TMT treatment) and promotes neurogenesis in the dentate gyrus to regenerate the granule cell layer after the neuronal loss there (initial time window in regeneration stage, day 0 to 7 post-TMT treatment; ref. 15). In the histological assessment using this model, we demonstrated that BrdU-incorporating cells positive for nestin or doublecortin were dramatically increased in number in the dentate gyrus at the initial time window during the regeneration stage. The current histological findings showing that TMT treatment produced a large increase in the nestin(+) cell number in the dentate gyrus on days 3 to 5 post-treatment strongly support the above-mentioned previous findings. In addition to the histological assessment, the ex vivo findings indicating that a larger number of nestin-positive neurospheres was formed by cells isolated from the dentate gyrus of the TMT-treated group rather than from that of the naive one let us propose the idea that there existed a large number of NPCs there following the dentate neuronal loss induced by TMT. Of further interest, a large increase in number of nestin(+)/GFAP(−) cells and nestin(+)GFAP(+) cells, but not in that of total GFAP(+) cells, was observed for the TMT-treated group (Fig. 2c). These ex vivo findings suggest that both the radial glia-like cells (type 1 cells) with expression of GFAP and nestin and amplif-
ing progenitors (type 2a cells) positive for nestin but not for GFAP increased in number in the dentate gyrus at the initial time window of the regeneration stage of TMT-treated animals. Thus, the TMT-treated mouse is very useful for evaluation of NPCs including type 1 and type 2a cells generated following dentate neuronal loss.

In the present study, sustained in vitro exposure to edaravone promoted the proliferation of nestin(+) cells derived from the dentate gyrus of TMT-treated mice cultured in the monolayer culture system (Fig. 5). A previous report demonstrated that in cultures of NPCs derived from embryonic hippocampus, sustained in vitro exposure to edaravone (50 μM) or tempol (200 μM) produces a large suppression of neurosphere formation and BrdU incorporation into the NPCs as well as reduces the level of endogenous reactive oxygen species in the NPCs, suggesting that endogenously-generated reactive oxygen species promote the proliferative activity of the embryonic NPCs. Since edaravone at higher concentrations (> 10 μM) was capable of suppressing cell proliferation under the same experimental conditions (data not shown), edaravone at different concentrations has an opposite effect on NPC proliferation. In light of the IC_{50} values for the scavenging effect of edaravone (formation of linoleic acid-conjugated dienes caused by hydroxyl radical, 32.0 μM; iron-dependent peroxidation in rat brain homogenates and mitochondrial homogenates, 15.0 and 2.3 μM, respectively; and iron-dependent peroxida-
tive disintegration of mitochondrial membranes, 39.0 μM; ref. 33), it is most likely that the inhibition of the NPC proliferation by edaravone at high concentration is attributable to the scavenging of free radicals. However, the present observations revealed that the NPC proliferation was enhanced by edaravone at quite low concentrations (10 pM and 10 nM), a finding that allows us to propose the idea that the promoting effect of edaravone was due to some mechanism other than the scavenging of free radicals. Indeed, accumulating evidence suggests that edaravone modulates inflammatory processes, matrix metalloproteinase levels, and nitric oxide production in addition to its free radical-removing effect (34). In particular, a recent report provided direct evidence to confirm that edaravone promotes nitric oxide synthesis/release following acute endothelial damage in peripheral microvessels, thus indicating it to have a beneficial effect on some neurodegenerative disorders (35). In addition, previous reports of ours indicated that endogenous or exogenous nitric oxide promotes the proliferation of NPCs derived from the embryonic mouse hippocampus (28, 36) as well as from the dentate gyrus of the adult mouse (data not shown). Hence, the possibility that the promoting effect of edaravone on NPC proliferation was due to enhanced nitric oxide synthesis/release is feasible. However, further studies need to certify the actual mechanism underlying the promoting effect of edaravone on NPC proliferation.

In the present study, ex vivo observations that in vivo treatment with edaravone increased the number of nestin(+)GFAP(+) cells in the dentate gyrus on day 4 following TMT treatment suggest that edaravone promoted type 1 cell proliferation at the initial time window of the regeneration stage after neuronal loss in the dentate gyrus. Interestingly, following the TMT insult, β-tubulin III(+) cells, probably immature neurons, were increased in number in the dentate gyrus by in vivo treatment with edaravone. This result may be evidence for enhanced differentiation into neuronal cells. This neuronal differentiation-facilitating effect of edaravone would be an important role for enhancement of neurogenesis after neuronal loss, and the mechanism underlying it must be elucidated in future studies.

In conclusion, we provided, for the first time, evidence for the promoting effect of edaravone on NPC proliferation in the dentate gyrus following neuronal loss caused by in vivo treatment with TMT. Hence, it is possible that edaravone is capable of beneficially promoting neurogenesis after neuronal damage in the dentate gyrus of adult animals. Details of the mechanisms underlying these effects of edaravone described presently are now a matter of focus for future studies on adult neurogenesis, with the goal being the development of new regenerative medical techniques for the treatment of brain insults.

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