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Systemic administration of a free radical scavenger, edaravone, protects against light-induced photoreceptor degeneration in the mouse retina

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ABSTRACT

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a free radical scavenger, is used for the clinical treatment of acute cerebral infarction. In this study, we investigated the protective effects of edaravone against light-induced retinal damage in the mouse. Retinal damage in the mouse was induced by exposure to white light at 8000 lx for 3 h after dark adaptation. Photoreceptor damage was evaluated by measuring the outer nuclear layer thickness at 5 days after the light exposure and recording the electroretinogram (ERG). Retinal cell damage was also detected by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and the expression of 8-hydroxy-2-deoxyguanosine (8-OHdG) and the phosphorylation of mitogen-activated protein kinases (MAPKs) such as extracellular signal regulated protein kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 were analyzed in the retinal samples by immunohistochemistry and immunoblotting. According to evaluation of outer nuclear layer thickness, 3 mg/kg, i.p. of edaravone and 1 mg/kg. i.v. of edaravone significantly protected against light-induced photoreceptor degeneration at 5 days after exposure to light. In ERG measurement, 3 mg/kg, i.p. of edaravone inhibited retinal dysfunction at 5 days after exposure to light. In addition, 3 mg/kg, i.p. of edaravone decreased the numbers of TUNEL-positive cells, 8-OHdG, phosphorylated JNK, and phosphorylated p38, but not that of phosphorylated ERK, in the whole retina at 6 h after light exposure. These findings suggest that oxidative stress plays a pivotal role in light-induced retinal damage and that systemic administration of edaravone may slow the progression of photoreceptor degeneration.

1. Introduction

Excessive light exposure leads to photoreceptor degeneration in many animals (Noell et al., 1966; Shahinfar et al., 1991), and can be a risk factor for onset and progression of age-related macular degeneration and possibly some forms of retinitis pigmentosa (Cruickshanks et al., 1993).

Photoreceptor cell death is an irreversible injury and can cause night blindness, constriction of the visual field, and finally the loss of central vision. Photoreceptor cell death has been reported to be induced by various factors (Wenzel et al., 2005), such as calcium levels, nitric oxide (NO), reactive oxygen species, mitochondria, and so on. In particular, the retina consumes significant amounts of oxygen in the human body and easily produces reactive oxygen species such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). The oxidation capacity of these radicals is weak, but they react to metals within a living body and UV light and immediately change to hydroxyradicals (•OH). Hydroxyradicals are the most reactive of the radicals and injure DNA and the cell membrane. Thus, oxidative stress disrupts the normal balance of antioxidation and oxidation response in the human body, and is involved in the progression of many diseases, including retinal diseases. Oxidative stress can contribute to neuronal toxicity and has been implicated in neuronal cell death (Pettmann and Henderson, 1998). Many researchers have investigated the efficacy of antioxidants such as ascorbate (Li et al., 1985) (Organisciak et al., 1985), dimethylthiourea (Organisciak et al., 1992), thioredoxin (Tanito et al., 2002), phenyl-*N*-*tert*butylnitrone (Ranchon et al., 2001; Tomita et al., 2005), and 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (Tanito et al., 2007) against light-induced retinal damage. Thus, oxidative stress is likely to be involved in the pathogenesis of light-induced retinal damage.

Edaravone, a potent free radical scavenger, has been shown to have protective effects against cerebral ischemia/reperfusion injuries in a variety of experimental animal models (Nishi et al., 1989). The clinical efficacy of edaravone against ischemic brain attack has been demonstrated by the presence of significant improvements in functional outcome in a randomized, placebo-controlled, double-blind study, and has been prescribed clinically in Japan for the treatment of acute brain infarction since 2001 (Toyoda et al., 2004). In our previous study, we showed the protective effect of edaravone against *N*-methyl-D-aspartate

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(NMDA)-induced retinal damage, and we found that edaravone has more potent antioxidant potential than other antioxidants, such as Trolox and *N*-acetylcysteine (NAC) (Inokuchi et al., 2009). We examined the effects of edaravone on photoreceptor degeneration induced by light exposure because edaravone has intense antioxidant potential and possible application in clinical practice.

In this study, to demonstrate the protective effects of edaravone against photoreceptor cell death by exposure to light, we examined histological and electrophysiological analyses and the underlying mechanism using an *in vivo* mouse model.

2. Materials and method

2.1. Animals

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and they were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. Male albino ddY mice (Japan SLC, Hamamatsu, Japan), aged 9 to 10 weeks, were used in this study. They were kept under controlled lighting conditions (12 h:12 h light/dark).

2.2. Exposure to light

After dark adaptation for 24 h, the pupils were dilated with 1% cyclopentolate hydrochloride eye drops (Santen, Osaka, Japan) 30 min before exposure to light. Nonanesthetized mice were exposed to 8000 lx of white fluorescent light (Toshiba, Tokyo, Japan) for 3 h in cages with reflective interiors. The temperature during the exposure to light was maintained at 25 ± 1.5 °C. After the exposure to light, all mice were returned to darkness for 24 h and then placed in the normal light/dark cycle.

2.3. Treatment with edaravone

Edaravone was dissolved in saline and was intraperitoneally (i.p.) treated at a dose of 3 mg/kg or an identical volume of saline at 30 min before and just after exposure to light, and then twice daily for 5 days after light exposure. Furthermore, edaravone (1 mg/kg) or saline was administered intravenously (i.v.) at 30 min before and just after exposure to light. Edaravone was administered repeatedly because its half-life was 5.4 min (Komatsu et al., 1996), and free radicals are generated during and after light exposure.

2.4. Electroretinogram

Electroretinogram (ERG) was recorded 5 days after light exposure (Mayo, Aichi, Japan). Mice were maintained in a completely dark room for 24 h. They were intraperitoneally anesthetized with a mixture of ketamine (120 mg/kg) (Daiichi-Sankyo, Tokyo, Japan) and xylazine (6 mg/kg) (Bayer Health Care, Tokyo, Japan). The pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen). Flash ERG was recorded in the left eyes of the dark-adapted mice by placing a golden-ring electrode (Mayo) in contact with the cornea and a reference electrode (Nihon Kohden, Tokyo, Japan) through the tongue. A neutral electrode (Nihon Kohden) was inserted subcutaneously near the tail. All procedures were performed in dim red light, and the mice were kept warm during the entire procedure. The amplitude of the a-wave was measured from the baseline to the maximum a-wave peak, and the b-wave was measured from the maximum a-wave peak to the maximum b-wave peak.

2.5. Histological analysis

In mice under anesthesia produced by an intraperitoneal injection of sodium pentobarbital (80 mg/kg) (Nakalai Tesque, Kyoto, Japan), each eye was enucleated and kept immersed for at least 24 h at 4 °C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections per eye (thickness, 5 μm) cut through the optic disc of each eye were prepared in the standard manner, and stained with hematoxylin and eosin. The damage induced by light exposure was then evaluated, with six sections from each eye used for the morphometric analysis described below. Light-microscope images were photographed, and the thickness of the outer nuclear layer from the optic disc was measured at 240-μm intervals on the photographs in a masked fashion by two observers (SI and SN). Data from three sections (selected randomly from the six sections) were averaged for each eye.

2.6. TUNEL staining

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed according to the manufacturer's protocols (In Situ Cell Death Detection Kit; Roche Biochemicals, Mannheim, Germany) to detect the retinal cell death induced by exposure to light. The mice were anesthetized with 80 mg/kg, i.p. of pentobarbital sodium 48 h after exposure to light for 3 h. The eyes were enucleated, fixed overnight in 4% paraformaldehyde, and immersed for 2 days in 25% sucrose with phosphate-buffered saline (PBS). The eyes were then embedded in a supporting medium for frozen-tissue specimens (OCT compound; Tissue-Tek, IL, USA). Retinal sections 10-μm thick were cut on a cryostat at −25 °C and stored at −80 °C until staining. After being washed twice with PBS, sections were incubated with terminal deoxyribonucleotidyl transferase (TdT) enzyme at 37 °C for 1 h. The sections were washed 3 times in PBS for 1 min at room temperature. Light-microscope images were photographed, and labeled cell counts in the outer nuclear layer at a distance between 480 and 720 μm from the optic disc were obtained in the superior area of the retina. The number of TUNEL-positive cells in outer nuclear layer was averaged for two sections per eye.

2.7. Western blot analysis

In vivo, mice were euthanized using 80 mg/kg, i.p. of sodium pentobarbital, and the eyeballs were quickly removed. The retinas were carefully separated from the eyeballs and quickly frozen in dry ice. To extract protein, the tissue was homogenized in cell-lysis buffer using a homogenizer (Physcotron; Microtec Co. Ltd., Chiba, Japan). The lysate was centrifuged at $12{,}000 \times g$ for 20 min and the supernatant used for this study. The protein concentration was measured by comparison with a known concentration of bovine serum albumin using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). A mixture of equal parts of an aliquot of protein and sample buffer with 10% 2-mercaptoethanol was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated protein was then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, MA, USA). For immunoblotting, the following primary antibodies were used: phosphorylated-p38 mouse monoclonal antibody (Promega, Tokyo, Japan), phosphorylated-JNK rabbit polyclonal antibody (Cell Signaling, Tokyo, Japan) (1:1000), phosphorylated-ERK rabbit polyclonal antibody (Cell Signaling) (1:1000), p38 mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:1000), JNK rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) (1:1000), ERK rabbit polyclonal antibody (Cell Signaling) (1:1000) and β-actin mouse monoclonal antibody (Sigma-Aldrich, Tokyo, Japan) (1:4000). The secondary antibody used was either goat anti-rabbit HRP-conjugated (1:2000) or goat anti-mouse HRP-conjugated (1:2000). The immunoreactive bands were visualized using

SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology). The band intensity was measured using a Lumino Imaging Analyzer (Fujifilm, Osaka, Japan).

2.8. Immunohistochemistry

The eyes were enucleated as described in the histological analysis section, fixed in 4% paraformaldehyde overnight at 4 °C, immersed in 25% sucrose for 48 h at 4 °C, and embedded in optimum cutting temperature (OCT) compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan). Transverse 10-μm-thick cryostat sections were cut and placed on slides (MAS COAT, Matsunami Glass Ind. Ltd., Osaka, Japan.). Immunohistochemical staining was performed in accordance with the following protocol. Briefly, tissue sections were washed in 0.01 M of PBS for 10 min, and followed by preincubation with 10% normal goat serum. We used the following antibodies: 8-OHdG monoclonal antibody (Japan Institute for the Control of Aging, Shizuoka, Japan). These were diluted in PBS (1:20) and then incubated with the tissue sections' primary antibodies for 2 h at 37 °C. Then endogenous peroxidase was quenched by treating the sections with 3% hydrogen peroxide in absolute methanol for 10 min. The sections were washed and then incubated with biotinylated anti-rabbit IgG or anti-mouse IgG. They were subsequently incubated with the avidin–biotin–peroxidase complex for 30 min, and then developed using diaminobenzidine (DAB) peroxidase substrate for 10 min. We confirmed the staining by comparison with the negative control. Images were obtained using a digital camera (COOLPIX 4500; Nikon, Tokyo, Japan), and 8-OHdG-positive cells in outer nuclear layer were counted at 480 μm to 720 μm from the optic nerve. The numbers of 8-OHdG-positive cells in outer nuclear layer were averaged for two sections per eye.

2.9. Statistical analysis

Data are presented as the means ± S.E.M.. Statistical comparisons were made using Student's *t* test [using STAT VIEW version 5.0 (SAS Institute, Cary, NC, USA)]. $P<0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effect of edaravone on light-induced photoreceptor degeneration

In the histological evaluation, Fig. 1 shows the representative retinal images between 480 μm and 720 μm from the optic nerve in the superior area at 5 days after light exposure. The outer nuclear layer thickness was remarkably thinned in the saline-treated group

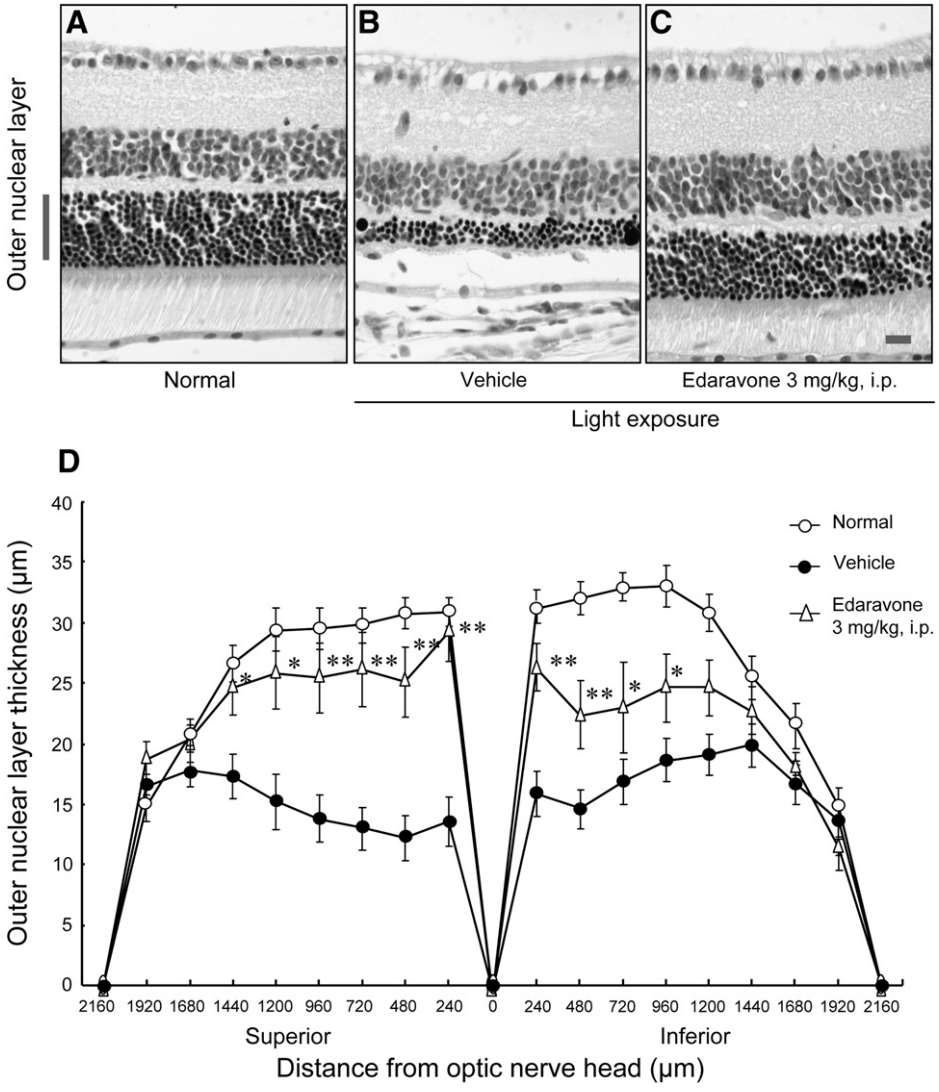

Fig. 1. Effects of edaravone (i.p.) on retinal damage induced by exposure to light in mice. (A) Nontreated, (B) light exposure (8000 lx) plus vehicle-treated, and (C) light exposure plus edaravone-treated (3 mg/kg, i.p.) retinal cross sections at 5 days after light exposure in mice. (D) Measurement of thickness in the outer nuclear layer at 5 days after light exposure. Data are shown as means ± S.E.M., $n=6$ or 8. $^{*}P<0.05$, $^{**}P<0.01$ vs light exposure plus the vehicle-treated group. The scale bar represents 25 μm.

(Fig. 1B) versus the nontreated group (Fig. 1A), but the group treated with 3 mg/kg, i.p. of edaravone significantly suppressed the photic damage (Fig. 1C) versus the saline-treated group (Fig. 1D). Edaravone protected significantly at the retinal superior area from 240 μm to 1440 μm and at the inferior area from 240 μm to 960 μm (Fig. 1D). The outer nuclear layer thickness was reduced by 48% versus the nontreated retina, but edaravone inhibited outer nuclear layer degeneration to 24% versus saline. Similar to Fig. 1, edaravone, when administered intravenously at 1 mg/kg (administered at before and just after light exposure), significantly inhibited the reduction of the outer nuclear layer thickness compared with the saline-treated group (Fig. 2D). Edaravone protected significantly at the retinal superior area from 240 μm to 1440 μm, and at the inferior area from 240 μm to 1440 μm (Fig. 2D). In the present study, we compared two administration routes (i.p. and i.v.), and 3 mg/kg, i.p. of edaravone was more efficacious than 1 mg/kg, i.v. of edaravone. Hence, we used the i.p. route after that.

3.2. Light-induced TUNEL-positive cells

To show light-induced apoptotic cell death and the effect of edaravone on cell death, we investigated the numbers of TUNEL-positive cells at 48 h after light exposure (Fig. 3). TUNEL-positive cells were observed only in the outer nuclear layer, not in any other retinal area (Fig. 3B). At the same time, in the normal retina, TUNEL-positive cells were not observed in any retinal area. Quantitative analysis showed that exposure to light in the mouse retina significantly increased the number of TUNEL-positive cells in the outer nuclear layer at 48 h (compared to the nontreated normal retina) (Fig. 3D). Edaravone (3 mg/kg, i.p.) significantly reduced the number of TUNEL-positive cells (52% reduction versus the saline-treated group) (Fig. 3C and D).

3.3. Light-induced 8-OHdG-positive cells

To confirm the antioxidative effects of edaravone on the mouse retina after light exposure, we evaluated the numbers of 8-OHdG-positive cells in the outer nuclear layer. Based on the results of Figs. 1 and 2, we evaluated from 480 μm to 720 μm in the retinal superior area. The number of 8-OHdG-positive cells increased in the non-treated retina at 24 h after exposure to light in the outer nuclear layer (Fig. 4B and E), but almost none were seen in the nontreated retina (Fig. 4A and D). Edaravone (3 mg/kg, i.p.) significantly reduced the number of 8-OHdG-positive cells (Fig. 4G; 37% reduction versus the vehicle-treated retina).

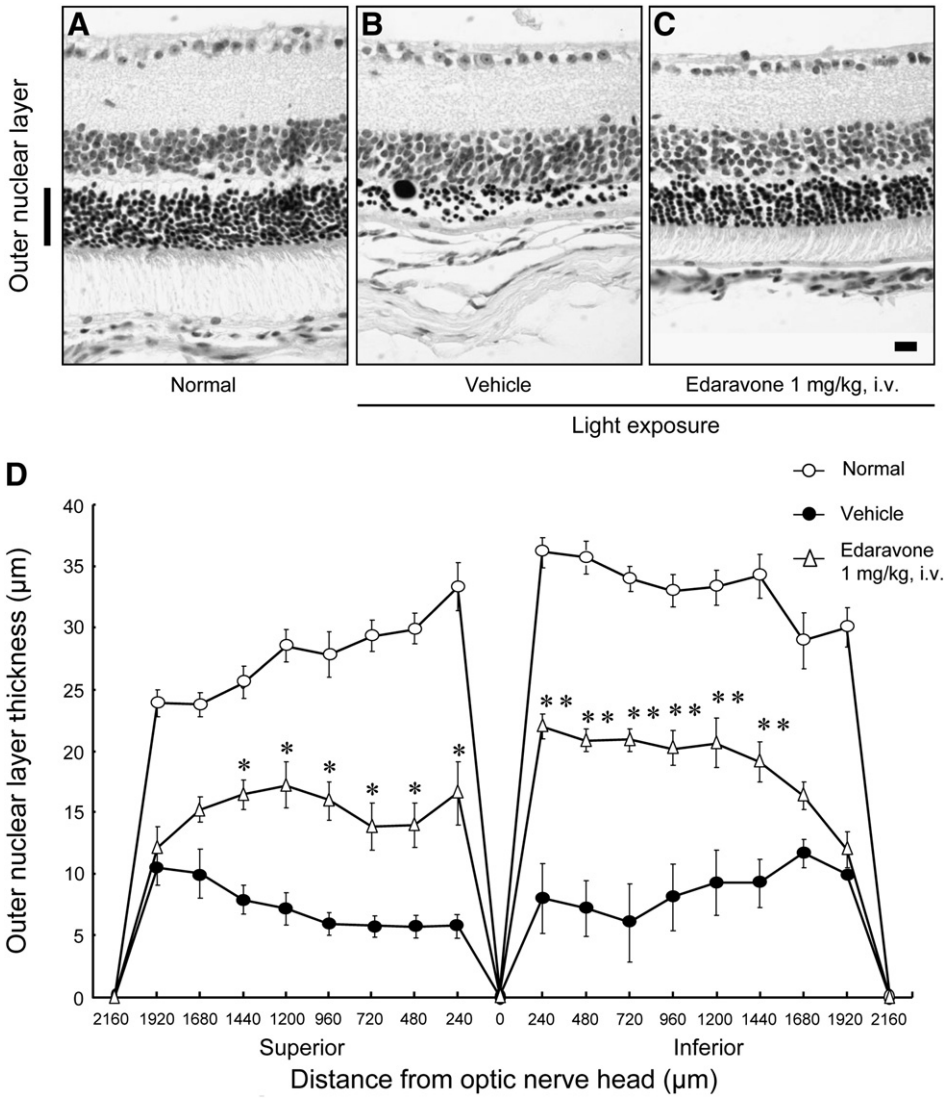

Fig. 2. Effects of edaravone (i.v.) on retinal damage induced by exposure to light in mice. (A) Nontreated, (B) light exposure (8000 lx) plus vehicle-treated, and (C) light exposure plus edaravone-treated (1 mg/kg, i.v.) retinal cross sections at 5 days after light exposure in mice. (D) Measurement of the thickness in the outer nuclear layer at 5 days after light exposure. Data are shown as means ± S.E.M., $n = 6$ or 8. $^{*}P<0.05$, $^{**}P<0.01$ vs light exposure plus the vehicle-treated group. The scale bar represents 25 μm.

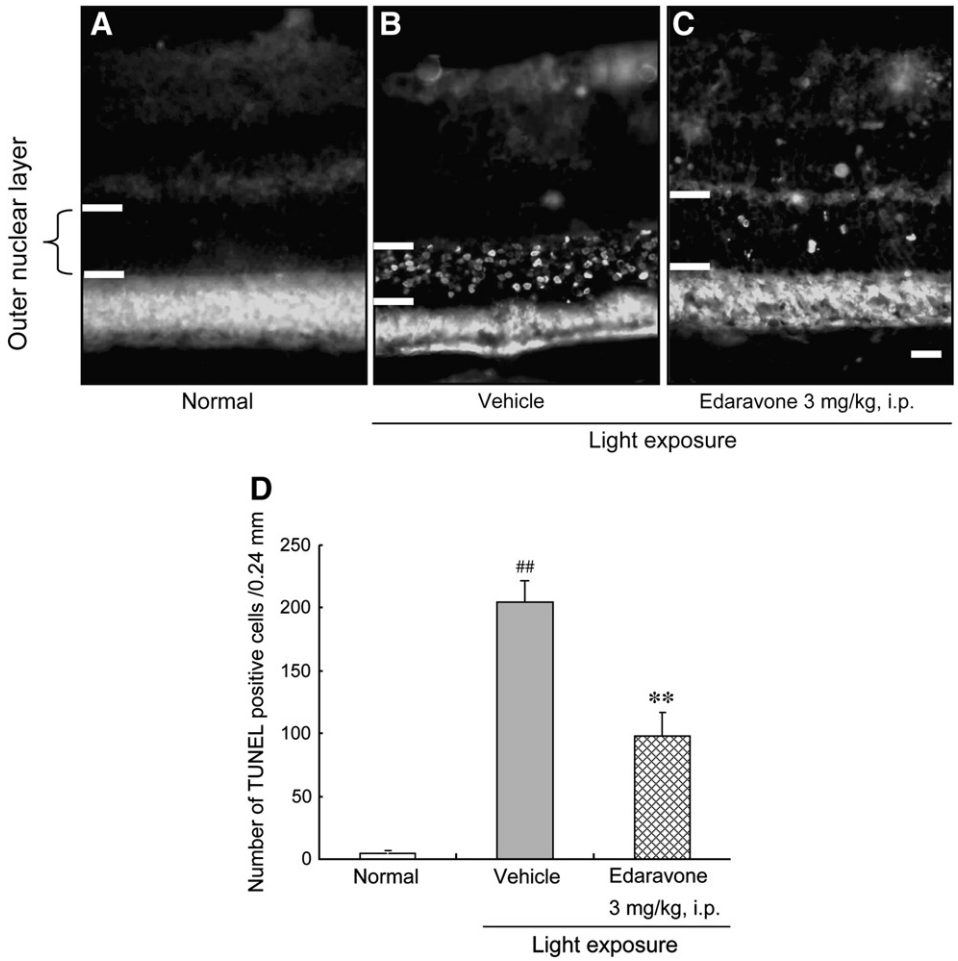

Fig. 3. Effects of edaravone on light-induced expression of TUNEL-positive cells in the mouse retina. (A) Nontreated, (B) light exposure (8000 lx) plus vehicle-treated, and (C) light exposure plus edaravone-treated (3 mg/kg, i.p.) retinal cross sections at 48 h after light exposure. (D) Quantitative analysis of the number of TUNEL-positive cells in the outer nuclear layer at 48 h after light exposure. Data are shown as means ± S.E.M., $n = 7$ or 8. $^{**}P<0.01$ vs light exposure plus the vehicle-treated group. $^{\#\#}P<0.01$ vs the nontreated group (normal). The scale bar represents 25 μm.

3.4. Electroretinogram (ERG)

The effects of edaravone on light-induced photoreceptor degeneration were examined with electrophysiologic analyses. In the nontreated retina, the amplitudes of the a- and b-waves increased as the light intensity increased, but they were attenuated in the retina exposed to light (Fig. 5). Both the a- and b-wave amplitudes were significantly reduced at 5 days after 8000 lx white light exposure for 3 h, and both the a- and b-waves decreased by 76% and 73%, respectively, compared with the nontreated retina at 0.98 log cds/m^2 (Fig. 5B, C). At 0.98 log cds/m^2, the group treated with 3 mg/kg, i.p. of edaravone showed significantly less reduction in the a- and b-wave amplitudes (47% and 41% reduction, respectively) compared with the saline-treated group (Fig. 5B, C). In the a- and b-wave amplitudes, edaravone protected significantly against light-induced retinal dysfunction from −1.02 to 0.98 cds/m^2.

3.5. Light-induced phosphorylations of p38, JNK, and ERK

To clarify the mechanism of action of edaravone, the activities of mitogen-activated protein kinases (MAPKs), which are signals related to oxidative stress, were measured using immunoblotting. Phosphorylated p38, phosphorylated c-Jun N-terminal kinases (JNK), and phosphorylated extracellular signal regulated protein kinases (ERK1/2) were all markedly increased (versus the untreated retina) in the retina at 6 h after exposure to light, against total p38, JNK, and ERK, respectively (Fig. 6A–C). Phosphorylation of p38, JNK, and ERK1/2 in the light-exposed retina increased 2.5-, 3.5-, and 2.7-fold, respectively, versus the nontreated retina. Edaravone (3 mg/kg, i.p.) significantly reduced the light-induced phosphorylation of p38 and JNK (Fig. 6A, B, D, E). However, edaravone did not reduce the phosphorylation of ERK (Fig. 6C, F).

4. Discussion

Light-induced retinal cell death is caused by an increase in intracellular calcium levels, nitric oxide (NO), rhodopsin mutation, and free radicals, including reactive oxygen species (Wenzel et al., 2005).

In the present study, to investigate the role of oxidative stress in retinal damage after exposure to light, we focused on the effects of edaravone, a free radical scavenger, against retinal damage by light exposure. Edaravone has been previously reported to protect several organs, such as the brain (Mizuno et al., 1998; Shichinohe et al., 2004), kidney (Matsuyama et al., 2006), liver (Taniguchi et al., 2007), and retina (Inokuchi et al., 2009; Song et al., 2008) from free radical-induced damage. Therefore, edaravone can be expected to have protective effects against free radical-related diseases. In the present study, we found edaravone demonstrated remarkable neuroprotective effects against excessive light-induced photoreceptor degeneration in mice.

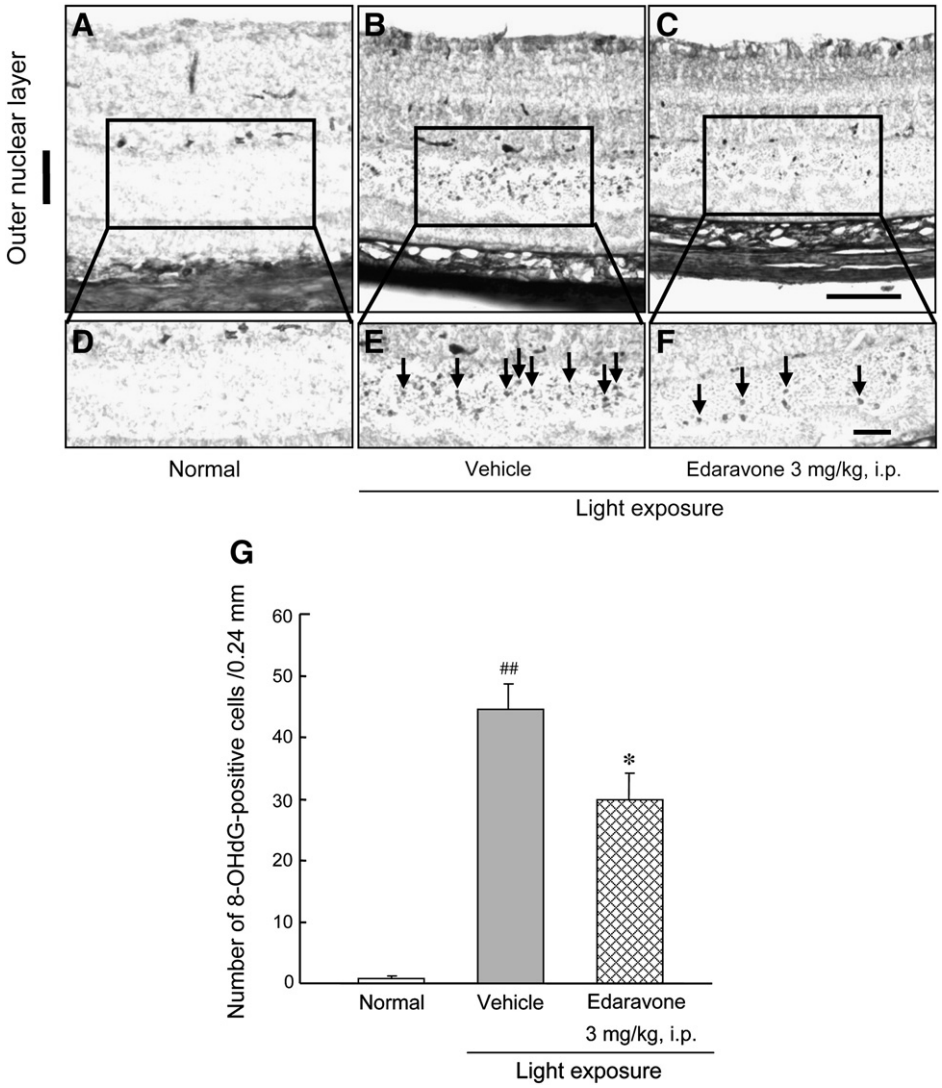

Fig. 4. Effects of edaravone on light-induced expression of 8-OHdG-positive cells in the mouse retina. Expression of 8-OHdG-positive cells in (A and D) nontreated, (B and E) light exposure (8000 lx) plus vehicle-treated, and (C and F) light exposure plus edaravone-treated (3 mg/kg, i.p.) retinal cross section at 24 h after light exposure. Arrows (E and F) show the 8-OHdG-positive cells. (G) Quantitative analysis of the number of 8-OHdG-positive cells in the outer nuclear layer at 24 h after light exposure. Data are shown as means ± S.E.M., $n = 8$ or 10. $^{*}P<0.05$ vs vehicle plus the light-exposed group. $^{\#\#}P<0.01$ vs nontreated retina (normal). The scale bars represent 50 μm (A, B, and C) and 25 μm (D, E, and F).

Excessive light exposure induces many reactive oxygen species, including free radicals, and their production can be overcome by a retinal defensive mechanism, such as superoxide dismutase (SOD) (Dong et al., 2006). It has been reported that SOD is induced in the rat retina after light exposure (Yamamoto et al., 1999), and mutant SOD1 mice, which have a gain of catalytic functions such as increased peroxidase activity, are highly susceptible to environmental light-induced retinal degeneration (Mittag et al., 1999). These reports indicate that many superoxide radicals ($O_2^{\bullet -}$) are induced by light exposure, and they relate to retinal dysfunction or cell death (Hashizume et al., 2008). Additionally, superoxide radicals ($O_2^{\bullet -}$) can change to hydroxyl radicals (•OH), which can critically injure DNA and the cell membrane. For these reasons, it is conceivable that oxidative stress is associated with a light-induced retinal damage mechanism.

Takamatsu et al. (Takamatsu and Watanabe, 1997) investigated the maximum drug concentration (C_{max}) in the blood plasma after treatment with edaravone (3 mg/kg, i.p.). The C_{max} of edaravone was 1728.7 ng/ml (9.9 μM) at just after continuous treatment for 30 min. In a previous study, we examined the *in vitro* effects of edaravone against the production of various radical species ($O_2^{\bullet -}$, •OH, and H_2O_2) in rat retinal ganglion cells (RGC-5) (Inokuchi et al., 2009). Edaravone protected RGC-5 against radical-induced cell death, and showed a protective effect from 1 μM to 10 μM. Moreover, edaravone scavenged the intracellular ·OH radicals more strongly than the other radicals examined ($O_2^{\bullet -}$and H_2O_2). Our *in vitro* data were consistent with their report; therefore, in this experiment we used doses of 3 mg/kg, i.p. and 1 mg/kg, i.v.

At 5 days after light exposure, the saline-treated (control) mice showed significant decrease of the outer nuclear layer thickness, and the edaravone treatment showed a protective effect against light-induced photoreceptor degeneration. According to a previous report, the main course of light-induced cell death was the apoptotic pathway (Wenzel et al., 2005), and we evaluated the TUNEL-positive cells in the expression of the outer nuclear layer. The number of TUNEL-positive cells increased at 6 h to 48 h after light exposure in the saline-treated group, and edaravone decreased the number of TUNEL-positive cells at 48 h after exposure to light. Previous reports demonstrated that light exposure triggered apoptosis in the outer nuclear layer (Abler et al., 1996), and the apoptosis was protected by using antioxidants, such as dimethylthiourea, phenyl-*N*-*tert* butylnitrone 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl, and so on (Organisciak et al., 1992; Ranchon et al., 2001; Tomita et al., 2005; Tanito et al., 2007).

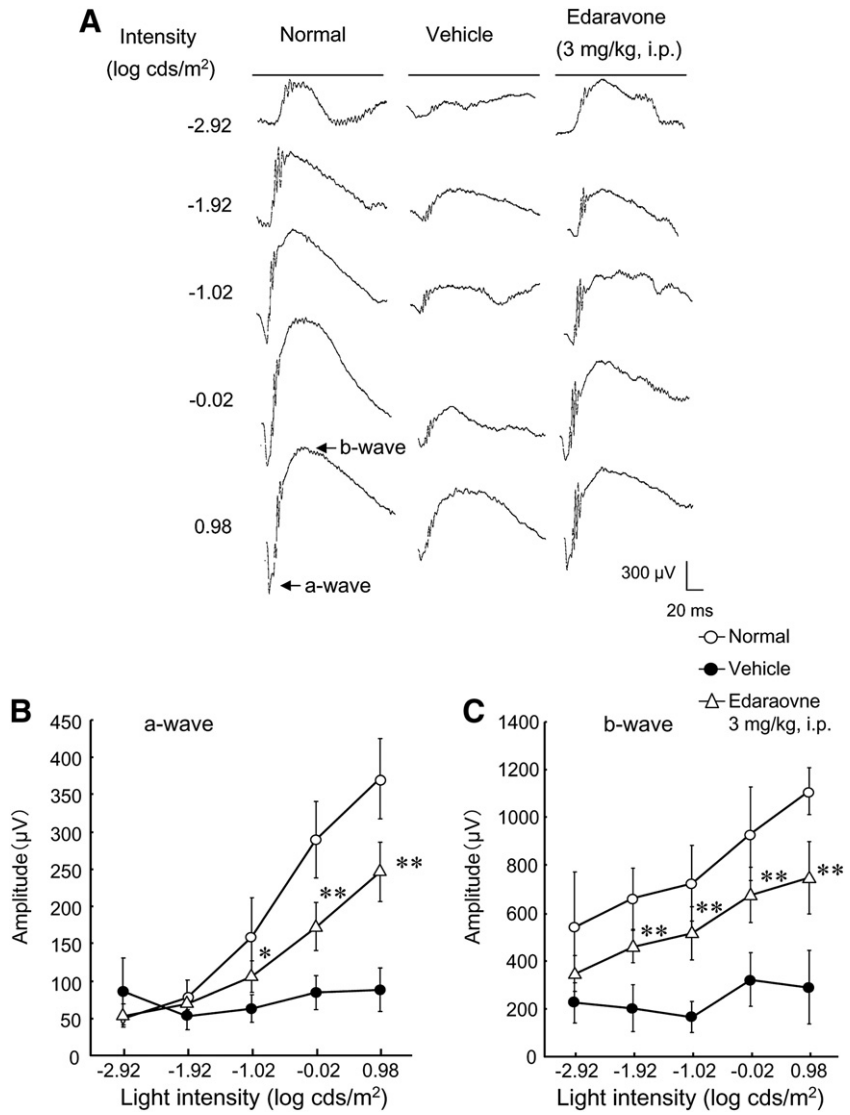

Fig. 5. Measurement of the dark-adapted ERG amplitudes at 5 days after exposure to light in the mouse retina. (A) Typical traces of dark-adapted ERG responses measured at 5 days after exposure to light. Stimulus flashes were used from −2.92 to 0.98 log cds/m^2. (B and C) Amplitudes of a- and b-waves of light exposure (8000 lx) plus the vehicle-treated group versus light exposure plus the edaravone-treated group (3 mg/kg, i.p.). Data are shown as means ± S.E.M., $n = 6$ or 8. $^*P<0.05$, $^{**}P<0.01$ vs light exposure plus the vehicle-treated group (vehicle).

Since excessive light exposure induces retinal dysfunction (Noell et al., 1966), we investigated the involvement of free radicals in light-induced retinal dysfunction by using an electroretinogram. The a-wave shows the function of the photoreceptors, and the b-wave reflects bipolar cells and Müller cell function. Both the a- and b-wave amplitudes were significantly decreased by the exposure to light. Edaravone prevented the decrease of both a- and b-wave amplitudes of flash ERG. These findings indicate that edaravone can attenuate photoreceptor and inner retinal dysfunction induced by light exposure, and ameliorate the visual function.

As mentioned above, light-induced retinal cell death was protected against by treatment with edaravone (3 mg/kg, i.p.), and we investigated the expression of 8-OHdG as a marker for oxidative stress. At 24 h after light exposure, 8-OHdG-positive cells were expressed in the outer nuclear layer, and the number of 8-OHdG-positive cells was significantly lower in the edaravone-treated mice compared with the saline-treated mice. Our previous study reported that edaravone scavenges hydroxyl radicals (•OH) more potently than the other radicals using RGC-5 (Inokuchi et al., 2009). It is known that 8-OHdG is produced by •OH. Furthermore, previous investigations have shown that expression of 8-OHdG was increased in retinal DNA after light exposure, and upregulation of 8-OHdG was observed in the outer nuclear layer after light exposure using immunohistochemistry (Organisciak et al., 1999; Tanito et al., 2002). Taken together, these findings indicate that oxidative stress was induced by excessive light stimulation in the retina and that edaravone can scavenge light-induced free radicals, including •OH, resulting in a protective effect in photoreceptor cell death.

MAPKs are stress-related kinases, and members of the MAPKs subfamily (JNK, p38, and ERK1/2) have been implicated in neuronal injury and diseases (Miloso et al., 2008). Activation of JNK and p38 MAPK, which are stimulated by various stresses, including ischemia, ultraviolet (UV) exposure, and oxidative stress, are involved in cell differentiation and apoptosis (Xia et al., 1995). ERK1/2 is activated by oxidative stress, mitogens, and survival factors, and that regulates cell proliferation and differentiation (Luo and DeFranco, 2006). Although the exact roles played by ERK1/2 were not clear, ERK1/2 may act in some cases to promote cell survival while also participating in neuronal cell death and neurodegeneration (Luo and DeFranco, 2006). Yang et al. (2007) have reported that light-induced photoreceptor apoptosis was related to MAPKs activation *in vitro*, but the authors indicated several limitations; for example, the 661 W cells were a

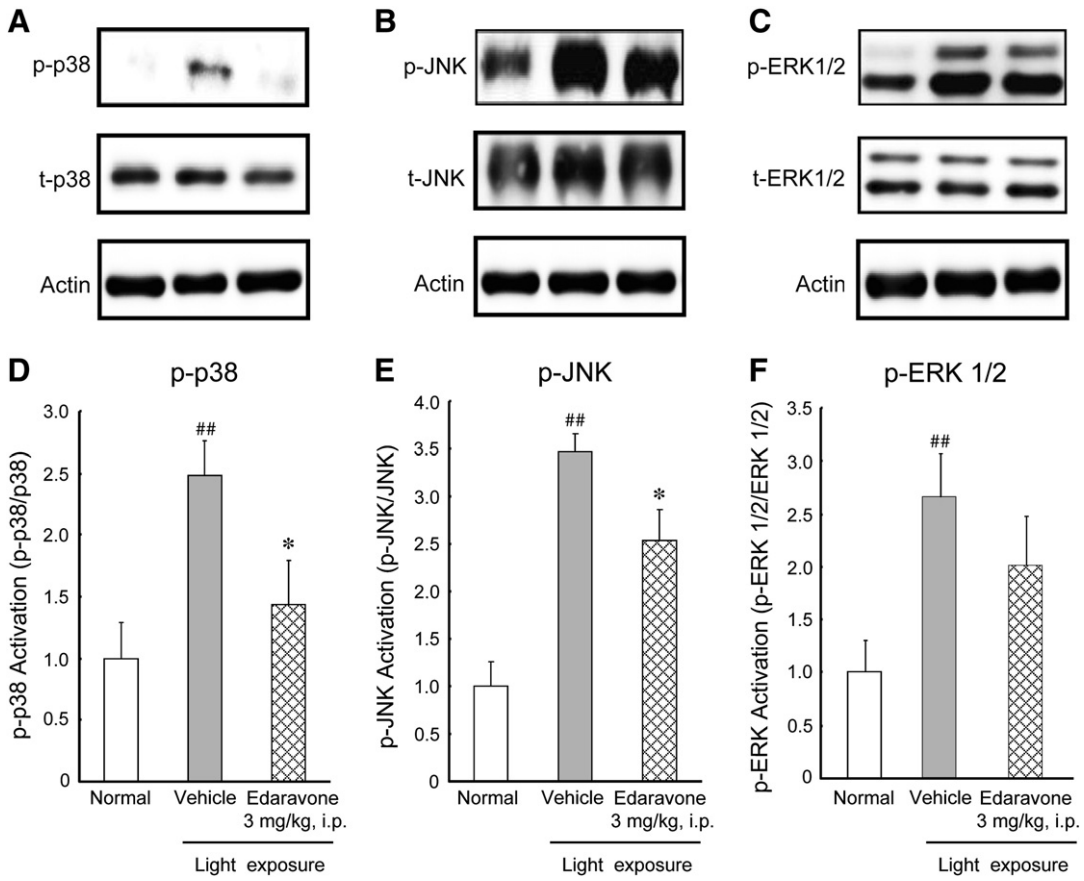

Fig. 6. Effects of edaravone on light-induced expression of phosphorylated p38, JNK, and ERK in the mouse retina. Representative band images showing activation of p38 (A), JNK (B), and ERK (C) in the nontreated, light exposure (8000 lx) plus vehicle-treated, and light exposure plus edaravone-treated (3 mg/kg, i.p.) retina. Quantitative analysis of the band density of p38 (D), JNK (E), and ERK (F) at 6 h after exposure to light plus vehicle-treated and the edaravone-treated (3 mg/kg, i.p.) group in mice. Data are shown as means ± S.E.M., $n=6$ or 7. $^{*}P<0.05$ vs light exposure plus vehicle-treated retina. $^{\#\#}P<0.01$ vs the nontreated retina (normal).

cone photoreceptor cell line, and they did not express RPE65, an important factor in rhodopsin regeneration kinetics. Therefore, we investigated the phosphorylation of MAPKs and the effect of edaravone on MAPKs activation *in vivo*. MAPKs activation achieved a peak at 6 h after light exposure, and edaravone significantly inhibited p38 and JNK activation but not ERK1/2 activation. These results indicate that light-induced reactive oxygen species may act as an upstream of the activation of the p38 and JNK signaling cascade, and edaravone can reduce light-induced retinal damage by an antioxidative mechanism. In contrast, edaravone did not reduce the increase in p-ERK1/2 seen after light exposure, suggesting that ERK1/2 activation is related to pathways other than reactive oxygen species signaling pathways. Although MAPKs (p38, JNK, and ERK) are activated by the light exposure (Samardzija et al., 2006; Sun et al., 2007), the effects of their inhibitors on light-induced retinal damages *in vivo* are still unknown. On the other hand, Yang et al. (2007) have reported that SB203850, a p38 inhibitor, protects from light-induced photoreceptor cell death *in vitro*, but does not curcumin, a JNK inhibitor. In contrast, pretreatment with U0126, a specific inhibitor of MEK1/2, exacerbated the light-induced photoreceptor apoptosis (Yang et al., 2007). Accordingly, these reports suggest that p38 activation may be involved in the light-induced photoreceptor cell death, and that the activation of ERK1/2 may play a protective role in photoreceptor cell death.

Activator protein-1 (AP-1) is an important factor for light-induced retinal degeneration (Grimm et al., 2000). AP-1 is a complex that consists either of heterodimers of members of the Fos (c-Fos, FosB, Fra-1, Fra-2) and the Jun (c-Jun, JunB, JunD) family of proteins or of homodimers of members of the Jun family of proteins (Hai and Curran, 1991). Light exposure leads to activation of AP-1 in mouse retina, and their complexes are mainly composed of c-Fos, c-Jun and JunD proteins (Hafezi et al., 1999). Hafezi et al. (1997) have reported that c-fos knockout mice were resistant to light-induced photoreceotor apoptosis. Furthermore, AP-1 is activated through phosphorylations of c-Jun and c-Fos, induced by JNK and p38 in retinal pigment epithelium ARPE19 cells (Roduit and Schorderet, 2008). These reports suggest that AP-1 activation through p38 and/or JNK is essential for light-induced photoreceptor apoptosis in mouse retina. Therefore, there is a possibility that edaravone may inhibit AP-1 activation by inhibiting p38 and/or JNK activation. However, further studies will be needed to clarify the precise mechanisms in light-induced retinal degeneration *in vivo*.

In conclusion, we demonstrated that edaravone protected retinal cells against light-induced photoreceptor degeneration *in vivo*, and oxidative stress plays a pivotal role in light-induced retinal damage. Although the excessive light exposure related to progression of age-related macular degeneration and retinitis pigmentosa, the systemic administration of edaravone may reduce the progression of retinal degeneration.

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