Edaravone, a free radical scavenger, protects against retinal damage in vitro and in vivo

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Abbreviations: Edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one; ROS, reactive oxygen species; RGC-5, retinal ganglion cell line; CM-H2DCFDA, 5-(and-6)-chloromethyl-2’, 7’-dichlorodihydrofluorescein diacetate, acetyl ester; APF, 2-[6-(4’-amino) phenoxy-3H-xanthen-3-on-9-Y] benzoic acid; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling; OGD, oxygen-glucose deprivation; IPL, inner plexiform layer; OPL, outer plexiform layer; OLM, outer limiting membrane; CFP, cyan fluorescent protein; 8-OHdG, 8-hydroxy-2-deoxyguanosine; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase
Abstract

Edaravone, a free radical scavenger, is used for the treatment of acute cerebral infarction. In this study, we investigated whether edaravone is neuroprotective against retinal damage. 

*In vitro*, we employed a radical scavenging-capacity assay using reactive oxygen species (ROS)-sensitive probes to investigate the effects of edaravone on hydrogen peroxide ($\text{H}_2\text{O}_2$), superoxide anion ($\text{O}_2^-\text{-}$), and hydroxyl radical ($\cdot\text{OH}$) production in a rat retinal ganglion cell-line (RGC-5). The effect of edaravone on oxygen-glucose deprivation (OGD)-induced RGC-5 damage was evaluated using a WST-8 assay of cell viability. Edaravone significantly decreased radical-generation, and reduced the cell death induced by OGD-stress.

*In vivo*, retinal damage was induced by intravitreous injection of $N$-methyl-$d$-aspartate (NMDA; 5 nmol), and was evaluated by examining ganglion cell layer (GCL) cell loss, TUNEL staining, and the expressions of two oxidant-stress markers [4-hydroxy-2-nonenal (4-HNE) and 8-hydroxy-2-deoxyguanosine (8-OHdG)]. In addition, activations of MAPKs [extracellular signal regulated protein kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK], as down stream signal pathways after NMDA-receptor activation, were measured using immunoblotting and immunostaining. Edaravone at 5 and 50 nmol (intravitreously) or at 1 and 3 mg/kg (i.v.) significantly protected against NMDA-induced retinal cell death. At 50 nmol (intravitreously) it: (a) decreased the retinal expressions of TUNEL-positive cells, 4-HNE, and 8-OHdG and (b) reduced the retinal expressions of
NMDA-induced phosphorylated-JNK and phosphorylated-p38, but not that of phosphorylated-ERK. These findings suggest that oxidative stress plays a pivotal role in retinal damage and that edaravone may be a candidate for the effective treatment of retinal diseases.
Introduction

Retinal ganglion cell (RGC) death, a common feature of many ophthalmic disorders (such as glaucoma, and central retinal artery or vein occlusion), may occur via a variety of mechanisms involving, for example, oxidative stress (Bonne et al., 1998), excitatory amino acid (glutamate) (Atlante et al., 2001), nitric oxide (NO) (Nucci et al., 2005), or reduced retinal perfusion (Flammer and Mozaffarieh, 2008). Of these, oxidative stress, which may occur because of an imbalance between the production and removal of reactive oxygen species (ROS), is considered to be a critical mediator in RGC injury of various etiologies and has been implicated in RGC death in the eye (Maher and Hanneken, 2005). Thus, oxidative stress is associated with various retinal diseases such as diabetic retinopathy (Abu el-Asrar et al., 1995), glaucoma (Chalasani et al., 2007), age-related macular degeneration (Kindzelskii et al., 2004), and retinitis pigmentosa (Doonan et al., 2005). In addition, RGC are exquisitely sensitive to the effects of both glutamate and its analog N-methyl-D-aspartate (NMDA), which produces a dose-dependent cell loss both in vivo and in vitro, and indeed glutamate toxicity has been implicated in the pathophysiology of glaucoma (Dreyer, 1998). NMDA-induced calcium entry and ROS production are well recognized to be mediators of ischemic neuronal damage, and activation of NMDA receptors reportedly generates free radicals and reduces antioxidant ability in the rat hippocampus (Ueda et al., 2007). Further, NMDA receptor-mediated neurotoxicity has been reported to depend, in part, on the...
generation of NO and superoxide anions (O\textsuperscript{2-})
which react to form peroxynitrite (ONOO\textsuperscript{-})
(Bonfoco et al., 1995). \textit{O}_2\textsuperscript{•-} radical is also converted either H\textsubscript{2}O\textsubscript{2}
by cellular superoxide dismutase (SOD) or to the more reactive hydroxyl radical (·OH) via the Fenton reaction.
Thus, oxidative stress, leading to the formation of free radicals, has been implicated as part of
the final common pathway for neurotoxicity in a wide variety of acute and chronic
neurodegenerative diseases (Bastianetto and Quirion, 2004).

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 indeed it has been prescribed
clinically in Japan for the treatment of acute brain infarction since 2001 (Toyoda et al., 2004).

In an experimental study on neonatal rat cerebrocortical slices, edaravone was found to
reduce NMDA-induced cytochrome c release and apoptosis (Nakano-Okuda et al., 2006).
Moreover, edaravone has been shown to inhibit (a) accumulations of 4-hydroxy-2-nonenal
(4-HNE) and 8-hydroxy-deoxyguanosine (8-OHdG), which are oxidative by-products, and
(b) neuronal cell death after transient focal ischemia in the murine brain (Zhang et al., 2005).
These protective effects of edaravone are thought to be attributable to its scavenging of ROS.

More recently, Song et al. reported that systemic administration of edaravone attenuated the
increase of malondialdehyde levels, the reduction of SOD activity, and further it suppressed
the retinal dysfunctions after retinal ischemia/reperfusion in rats (Song et al., 2008).

However, they did not determine any quantitative evaluation in RGC death after retinal injury,
so it remains unclear whether edaravone protects against RGC death with apoptosis through
ROS-scavenging effects.

In the present study, our aims were: (i) to evaluate the radical-scavenging ability of
edaravone in the retina, (ii) to observe the effects of edaravone on oxygen-glucose
deprivation (OGD)-induced retinal cell death, and (iii) to evaluate the effects of edaravone on
NMDA-induced retinal damage, we also hoped to elucidate the underlying mechanism(s).
Methods

Materials. NMDA was purchased from Sigma-Aldrich (St. Louis, MO, USA). The drugs used and their sources were as follows: edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one, hydrogen peroxide (H₂O₂), N-acetylcysteine (NAC), D-(+)-Glucose and iron (II) perchlorate hexahydrate were from Wako (Osaka, Japan). Isoflurane was from Nissan Kagaku (Tokyo, Japan), while fetal bovine serum (FBS) was from Valeant (Costa Mesa, CA, USA). Dulbecco’s modified Eagles’s medium (DMEM) and trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, were from Sigma-Aldrich (St Louis, MO, USA). Glucose-free DMEM, Hanks’ balanced salt solution, and Hepes were from Gibco (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO) was from Nacarai Tesque Inc. (Kyoto, Japan). CM-H₂DCFDA, 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, was from Molecular Probes (Eugene, OR, USA). APF, 2-[6-(4′-amino) phenoxy-3H-xanthen-3-on-9-yl] benzoic acid, was from Daiichi pure chemicals (Tokyo, Japan). Cell counting Kit-8 (WST-8) was from Dojin Kagaku (Kumamoto, Japan). Potassium dioxide (KO₂) was from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). While 2’-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride (Hoechst 33342) and propidium iodide (PI) were from Molecular Probes (Eugene, OR, USA).
**RGC-5 Culture.** RGC-5 were gifted by Dr. Agarwal (Department of Pathology and Anatomy, UNT Health Science Center Fort Worth, TX, USA). RGC-5 cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin (Meiji Seika Kaisha Ltd, Tokyo, Japan), and 100 μg/ml streptomycin (Meiji Seika Kaisha Ltd.) under a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The RGC-5 cells were passaged by trypsinization every 3 to 4 days.

**Radical Scavenging-capacity Assay.** RGC-5 cells were seeded at a density of 2×10³ cells per well into 96-well plates, and then incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Twenty-four hours later, the cell-culture medium was replaced, before treatment with edaravone or its vehicle (DMEM containing 1% FBS). Radical species (H₂O₂ and O₂⁻) oxidize nonfluorescent dichlorofluorescein (DCFH) to fluorescent dichlorofluorescein (DCF). The cells were loaded with radical probe CM-H₂DCFDA (10 μM) by incubation for 20 min at 37°C. Then, the cell-culture medium was replaced to remove the extra probe. To generate the radical species, we added H₂O₂ at 1mM or KO₂ at 1 mM (O₂⁻) as the radical probe loading-medium. Fluorescence was measured, after the ROS-generating compounds had been present for various time-periods, using SkanIt RE for Varioskan Flash 2.4 (Thermo Fisher Scientific, Waltham, MA, USA) at excitation/emission wavelengths of 485/535 nm. To try to detect the ·OH formed in the Fenton reaction, we
used APF. Briefly, the cells were loaded with APF by incubation for 20 min at 37°C in Hanks/Hepes buffer solution. To perform the Fenton reaction, H₂O₂ was added to the Hanks/Hepes buffer solution of APF, containing APF (10 μM) and then iron (II) perchlorate hexahydrate was added. Fluorescence was measured at excitation/emission wavelengths of 490/515 nm.

**OGD-induced Cell Death in RGC-5 Cell Cultures.** Cells were seeded at a density of 2×10³ cells per well into 96-well plates, and the cells were then incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C for 24 h. To induce OGD stress, the cells were washed with glucose-free DMEM, and then placed in the same medium in a hypoxic-incubator (94% N₂, 5% CO₂, and 1% O₂) for 4 h. At the end of the OGD period, glucose solution and FBS were added to final concentrations of 4.5 mg/ml and 1%, respectively, and the cultures were put back in the incubator for an additional 18 h at the regular atmospheric oxygen level (reoxygenation). Edaravone or trolox was added to the culture immediately after the above replacement of the culture medium. At the end of the reoxygenation period, cell viability was measured.

**Cell Viability.** To evaluate cell survival, we examined the change in fluorescence intensity following the cellular reduction of WST-8 to formazan. All experiments were performed in
DMEM medium at 37°C. Cell viability was assessed by culturing cells in a culture medium containing 10% WST-8 (Cell Counting Kit-8) for 2-3 h at 37°C, and obtained by scanning with a microplate reader at 492 nm. This absorbance was expressed as a percentage of that in control cells (which were in DMEM containing 1% FBS), after subtraction of background absorbance.

**Cell Viability (Hoechst 33342 and PI Staining).** Cell death was observed by employing combination staining with two fluorescent dyes, Hoechst 33342 and PI. To examine the effects of edaravone on the cell death induced by OGD stress, RGC-5 cells were seeded at a low density of 700 cells per well into 96-well plates. After pretreatment with edaravone, cells were exposed to OGD treatment (see above). At the end of this culture period, Hoechst 33342 (excitation/emission wavelengths, 360/490 nm) or PI (excitation/emission wavelength, 535/617 nm) was added to the culture medium for 15 min at final concentrations of 8 μM and 1.5 μM, respectively. Images were collected using an epifluorescence microscope (IX70; Olympus, Tokyo, Japan) fitted with a CCD camera (DP30BW; Olympus Co., Tokyo, Japan) and with fluorescence filters for Hoechst 33342 (U-MWU; Olympus) and PI (U-MWIG; Olympus)

**Animals.** Male adult ddY mice (Japan SLC, Hamamatsu, Japan), male adult Thy-1-Cyan
Fluorescent Protein (CFP) transgenic mice (The Jackson Laboratory, Maine, USA) (Feng et al., 2000), and C57BL/6J mice (the background strain of the transgenic mice) were used in the present study. They were kept under controlled lighting conditions (12 h:12 h light/dark). The mouse genotype was determined by applying standard polymerase chain reaction (PCR) methodology to tail DNA.

**NMDA-induced Retinal Damage.** Mice were anesthetized with 3.0% isoflurane and maintained with 1.5% isoflurane in 70% N₂O and 30% O₂ via an animal general anesthesia machine (Soft Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). The body temperature was maintained between 37.0 and 37.5°C with the aid of a heating pad. Retinal damage was induced by the injection (2 µl/eye) of NMDA (Sigma-Aldrich) at 2.5 mM dissolved in 0.01M phosphate-buffered saline (PBS). This was injected into the vitreous body of the left eye under the above anesthesia. One drop of 0.01% levofloxacin ophthalmic solution (Santen Pharmaceuticals Co. Ltd., Osaka, Japan) was applied topically to the treated eye immediately after the intravitreous injection. Seven days after the injection, eyeballs were enucleated for histological analysis. For comparative purposes, non-treated retinas from each mouse strain were also investigated. Edaravone (5 or 50 nmol), trolox (0.1 or 1 nmol), NAC (10, 100 or 1000 nmol), or vehicles (PBS with or without 5% DMSO) was co-administered (by intravitreous injection) with NMDA at 5 nmol/eye. Alternatively, edaravone (at 1 or 3
mg/kg) or vehicle (PBS) was intravenously injected just before intravitreous injection of NMDA (5 nmol/eye).

**Histological Analysis.** In mice under anesthesia produced by an intraperitoneal injection of sodium pentobarbital (80 mg/kg), each eye was enucleated. Eyes were kept immersed for at least 24 h at 4°C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (thickness, 4 µm) were cut parallel with the maximum circumference of the eye ball through the optic disc [as we extracted the eye ball, we identified the dorsal-most point (using the landmark of the optic axis)]. These sections were then stained with hematoxylin and eosin. The damage induced by NMDA was evaluated as follows, three sections from each eye being used for the morphometric analysis. Light-microscope images were photographed, using a digital camera (COOLPIX 4500; Nikon, Tokyo), and the cell counts in the ganglion cell layer (GCL) at a distance between 375 and 625 µm from the optic disc (nasal and temporal portions), were measured (three sites per section) on the photographs in a masked fashion by a single observer (Y.I.), then averaged to give a single value. Data from three sections (selected randomly from the six sections) were averaged for each eye, and used to evaluate the cell count in the GCL.

**Retinal Flatmounts and Analysis in Thy-1-CFP Transgenic Mice.** Transgenic mice
were given an anesthetic overdose, and retinas were dissected out and fixed for 3 h in 4% paraformaldehyde diluted in 0.1 M phosphate-buffer (PB) at pH 7.4. Retinas were subsequently washed with PBS at room temperature. Retinas were then flat-mounted on clean glass slides with fluorescent mounting medium (Dako Corp., CA, USA) and stored in the dark at 4°C. In each retina, the damage induced by NMDA at 5 nmol/eye was evaluated within three areas [a, b, and c; medial borders distances of 1, 1.5, and 2 mm from the center of the optic disc in each of four sectors (dorsal, ventral, temporal, and nasal); see Fig. 4 A-C]. In total, therefore, 12 areas per retina were used for the morphometric analysis described below. At 7 days after the intravitreous injections (NMDA 5 nmol, or NMDA 5 nmol plus edaravone 50 nmol), fluorescent images of the 12 areas (each, 0.144 mm²) were obtained in each mouse using an epifluorescence microscope (BX50; Olympus, Tokyo, Japan) fitted with a CCD camera (DP30VW; Olympus, Tokyo, Japan). The Thy-1-CFP-positive cells on the photographs were counted in a masked fashion by a single observer (Y.I.) in Thy-1-CFP transgenic mice. For comparative purposes, non-treated retinas were also investigated. In each eye, counts from the four areas designated “a” (total area, 0.576 mm²) were summed, and the value so obtained was used as the cell count for “Area a” (see Fig. 4D). Counts for “Area b” and “Area c” were obtained in similar fashions.

**Immunostaining.** Eyes were enucleated as described in “Histological Analysis”, 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 onto 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 0.01M PBS for 10 min, and then endogenous peroxidase was quenched by treating the sections with 3% hydrogen peroxide in absolute methanol for 10 min, followed by a pre-incubation with 10% normal goat serum. We used the following antibodies: phosphorylated-p38 mouse monoclonal antibody (Promega, CA, USA), phosphorylated-JNK rabbit polyclonal antibody (Cell Signaling, MA, USA), phosphorylated-ERK rabbit polyclonal antibody (Cell Signaling), 4-HNE mouse monoclonal antibody (Japan Institute for the Control of Aging, Shizuoka, Japan), and 8-OHdG mouse monoclonal antibody (Japan Institute for the Control of Aging). These were diluted in PBS (1:1000), then incubated with the tissue sections primary antibodies overnight at 4°C. 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 1 min. We confirmed the staining by comparison with the negative control. Images were obtained using a digital camera (COOLPIX 4500; Nikon, Tokyo, Japan).
Density of 4-HNE-like Immunostaining. We evaluated samples in the DAB-labeled area (for anti-4-HNE) in the GCL and inner plexiform layer (IPL) at a distance between 475 and 525 µm (50 x 50 µm) from the optic disc. Retinal DAB-labeled cell density was evaluated by appropriately calibrated computerized image analysis, using “median density” as an analysis tool [Image Processing and Analysis in Java (Image J); National Institute of Mental Health, Bethesda, MD, USA] and averaged for two areas. Briefly, light-microscope photographed of the above-mentioned areas were inverted in a gradation sequence using Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, CA, USA). Then optical intensity was evaluated using Image J. Finally, from the obtained score we subtracted the score obtained for the negative control (non-treated with the first antibody) as the background value.

TUNEL Staining. 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 NMDA. The mice were anesthetized with pentobarbital sodium at 80 mg/kg, i.p., 24 h after intravitreous injection of NMDA at 5 nmol/eye. The eyes were enucleated, fixed overnight in 4% paraformaldehyde, and immersed for 2 days in 25% sucrose with PBS. The eyes were then embedded in a
supporting medium for frozen-tissue specimens (OCT compound; Tissue-Tek, IL, USA).

Retinal sections were at 10-µm thick cut on a cryostat at -25°C, and stored at -80°C until staining. After twice washing with PBS, sections were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme at 37°C for 1 h. The sections were washed 3 times in PBS for 1 min at room temperature. Sections were subsequently incubated with an anti-fluorescein antibody-peroxidase (POD) conjugate at room temperature in a humidified chamber for 30 min, and then developed using DAB tetrahydrochloride peroxidase substrate. Light-microscope images were photographed, and the labeled cell counts in the ganglion cell layer (GCL) at a distance between 375 and 625 µm from the optic disc were obtained in two areas of the retina. The number of TUNEL-positive cells was averaged for these two areas, and the value plotted as the number of TUNEL-positive cells.

**Western Blot Analysis.** *In vivo*, mice were euthanized using sodium pentobarbital at 80 mg/kg, i.p., and their eyeballs were quickly removed. The retinas were carefully separated from the eyeballs and quickly frozen in dry ice. For protein extraction, the tissue was homogenized in cell-lysis buffer using a Phycotron (MICROTEC Co. Ltd., Chiba, Japan). The lysate was centrifuged at 12,000×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,
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), phosphorylated-JNK rabbit polyclonal antibody (Cell Signaling) (1:1000), phosphorylated-ERK rabbit polyclonal antibody (Cell Signaling) (1:1000), p38 mouse monoclonal antibody (Santa Cruz) (1:1000), JNK rabbit polyclonal antibody (Santa Cruz) (1:1000), ERK rabbit polyclonal antibody (Cell Signaling) (1:1000), β-actin mouse monoclonal antibody (Sigma-Aldrich) (1:4000), and a rabbit anti-Green Fluorescent Protein (GFP) polyclonal antibody (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) (1:1000). 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 (Toyobo, Osaka, Japan) and Gel Pro Analyzer (Media Cybernetics, Atlanta, GA, USA).

**Statistical Analysis.** Data are presented as the means ± SEM. Statistical comparisons were made using Dunnett’s test or a Student’s t-test [using STAT VIEW version 5.0 (SAS]
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Institute, Cary, NC). P < 0.05 was considered to indicate statistical significance.
Results

Intracellular Oxidation of DCFH or APF Induced by Various Types of ROS

To investigate the effect of edaravone on hydrogen peroxide (H$_2$O$_2$), superoxide anion (O$_2^\cdot$), and hydroxyl radical (-OH) production, we employed a radical scavenging-capacity assay using the ROS-sensitive probes, CM-H$_2$DCFDA and APF. The kinetics of ROS reactivity (monitored as fluorescence generation) are illustrated in Figure 1A-C.

H$_2$O$_2$-induced intracellular radicals were generated by treatment with H$_2$O$_2$ at 1 mM, and edaravone at 0.1-10 μM scavenged the H$_2$O$_2$ radical (Fig. 1D). O$_2^\cdot$ radicals, which were generated following treatment with KO$_2$ at 1 mM, were scavenged by edaravone at 1 and 10 μM (Fig. 1E). -OH radicals, generated by treatment with H$_2$O$_2$ at 1 mM plus Fe perchlorate (II) at 100 μM, were scavenged by edaravone at 1 and 10 μM (Fig. 1F). The IC$_{50}$ values (the concentrations causing 50% inhibition, with 95% confidence limits) for the action of edaravone against the H$_2$O$_2$, O$_2^\cdot$, and -OH radicals were 13.2 (5.3-72.4) μM, 8.34 (3.94-29.3) μM, and 1.86 (1.18-3.06) μM, respectively. For comparison, the IC$_{50}$ values for the action of trolox against the H$_2$O$_2$, O$_2^\cdot$, and -OH radicals were 0.29 (0.11-0.59) μM, 0.36 (0.13-0.75) μM, and 1.30 (0.97-1.77) μM, respectively.

Cell Damage Induced by OGD in RGC-5 Culture

Representative photographs of Hoechst 33342 and PI staining are shown in Fig. 2A.
Hoechst 33342 stains all cells (live and dead cells), whereas PI stains only dead cells.

Edaravone (1, 5, and 50 µM) added to the culture medium immediately after the replacement of the culture medium decreased the number of cells showing PI staining following OGD (versus vehicle treatment). In the WST assay, cell viability was found to be reduced by 83-87% after OGD (4 h) plus reoxygenation (18 h), and treatment with edaravone at 5 and 50 µM reduced this cell damage (Fig. 2B). On the other hand, trolox (0.1, 1, and 10 µM; added as the same way of edaravone) had no protective effect (Fig. 2B).

Retinal Damage Induced by Intravitreous Injection of NMDA

Intravitreous injection of NMDA decreased the cell number in GCL in the mouse retina (versus the non-treated normal retina) (Fig. 3A, 3B, 3E, 3F and 3G). Edaravone (5 and 50 nmol), when co-administered with NMDA, significantly reduced the cell loss in GCL (as compared with vehicle). On the other hand, trolox (0.1 and 1 nmol) and NAC (10, 100 and 1000 nmol) had no protective effects (Fig. 3B to 3F). Moreover, edaravone, when administered intravenously at 1 and 3 mg/kg immediately before NMDA injection, significantly inhibited the cell loss in GCL, as compared with vehicle (Fig. 3G). DMSO has been reported to show some radical scavenging and neuroprotective effects (Turan et al., 2008). In general, to avoid their effects we use 0.5% DMSO or less to be up to 0.1% as a final concentration in vitreous body. However, edaravone at 25 mM (for 50 nmol/eye) could
not be dissolved in saline or 0.5% DMSO and, therefore, 5% DMSO was used in this study. Accordingly, we compared the effect of 5% DMSO with PBS against normal retina and NMDA-induced retinal damage. As a result, there was no statistical difference between non-treated and 5% DMSO-treated groups or between PBS-treated and 5% DMSO-treated groups in NMDA-induced retinal damage. Therefore, we regarded that intravitreal injection of 5% DMSO contributed little to normal retina or retinal damage.

**NMDA-induced Retinal Damage in Thy-1-CFP Transgenic Mice**

To examine the effect of edaravone across a large retinal area, we used Thy-1-CFP (cyan fluorescence protein, a spectral variant of GFP)-transgenic mice (Feng et al., 2000). The relevant transgene contains a CFP gene under the direction of regulatory elements derived from the mouse Thy-1 gene, and the CFP protein is expressed only in RGC, not in other cells, indicating that the CFP-positive cells observed in Thy1-CFP transgenic mice are RGCs. A representative photograph of a vehicle-treated normal flat-mounted retina from a Thy-1-CFP transgenic mouse is shown in Figure 4A. Intravitreous injection of NMDA decreased the Thy-1-CFP-positive cell numbers (at 7 days after the injection) in flat-mounts of mouse retina (versus the vehicle-treated normal retina) (Fig. 4A and 4B). Edaravone (50 nmol), when co-administered with the NMDA, significantly inhibited the cell loss in each Area analyzed (a, b, and c) (Fig. 4C and 4D).
Immunoblotting revealed that intravitreous injection of NMDA decreased the expression of Thy-1-CFP protein (at 7 days after the injection) in whole retina (versus the vehicle-treated normal retina), and that edaravone (50 nmol), co-administered with the NMDA, significantly inhibited this reduction in Thy-1-CFP protein (Fig. 4E).

**NMDA-induced Expression of TUNEL-positive Cells**

TUNEL-positive cells were observed in both GCL and the inner part of the inner plexiform layer (INL) at 24 h after intravitreous NMDA injection (Fig. 5B), but almost none were seen in the untreated retina (Fig. 5A). Edaravone (50 nmol), co-administered with the NMDA, significantly reduced the number of TUNEL-positive cells (versus the vehicle-treated retina) (Fig. 5B to 5D).

**NMDA-induced Expression of 4-HNE and 8-OHdG-positive Cells**

To confirm the anti-oxidative effects of edaravone on the mouse retina after NMDA injection, 4-HNE and 8-OHdG, which are oxidative by-products, were measured using immunostaining techniques. Rather “blurry” immunostaining for 4-HNE was observed in both GCL and IPL at 12 h after intravitreous NMDA injection (Fig. 5B), but almost none were seen in the untreated retina (Fig. 6A). Edaravone (50 nmol), co-administered with the NMDA, significantly reduced the expression of 4-HNE (versus the vehicle-treated retina)
8-OHdG-positive cells were observed in both GCL and the upper part of INL at 12 h after intravitreous NMDA injection (Fig. 6F), but almost none were seen in the untreated retina (Fig. 6E). Edaravone (50 nmol), co-administered with the NMDA, significantly reduced the number of 8-OHdG-positive cells (versus the vehicle-treated retina) (Fig. 6F to 6H).

**NMDA-induced Phosphorylations of p38, JNK, and ERK**

To clarify the mechanism(s) of action of edaravone, the activities of mitogen-activated protein kinases (MAPK), which are signals down stream of NMDA-receptor activation, were measured using immunoblotting. This revealed that phosphorylated-p38 MAPK, phosphorylated-c-Jun N-terminal kinase (JNK), and phosphorylated-extracellular signal regulated protein kinase 1/2 (ERK1/2) were all markedly increased (versus the untreated retina) in the retina at 6 h after intravitreous NMDA injection, against total p38, JNK, and ERK1/2, respectively (Fig. 7A to 7C). Edaravone (50 nmol) significantly reduced the NMDA-induced phosphorylation of p38 and JNK, but not that of ERK1/2 (versus vehicle-treated retina) (Fig. 7A to 7C).

Immunostaining of phosphorylated-p38 was observed in both GCL and the inner part of INL at 6 h after intravitreous NMDA injection, but none was seen in the untreated retina, and the expression of phosphorylated-p38 was significantly reduced in the NMDA plus
edaravone (50 nmol)-treated retina (versus that in the NMDA-treated retina) (Fig. 7D).

Dark immunostaining of phosphorylated-JNK was observed in both GCL and IPL at 6 h after NMDA injection, but none was seen in the untreated retina, and the expression of phosphorylated-JNK was significantly reduced in the NMDA plus edaravone (50 nmol)-treated retina (versus that in the NMDA-treated retina) (Fig. 7E). Strong immunostaining of phosphorylated-ERK1/2 was observed in GCL, IPL, and INL at 6 h after NMDA injection, but none was seen in the untreated retina, and the expression of phosphorylated-ERK1/2 was not different between the NMDA plus edaravone (50 nmol)-treated retina and the NMDA-treated retina (Fig. 7F).
Discussion

Here, we found (a) that in RGC-5 in vitro, edaravone significantly decreased intracellular radical-generation (H$_2$O$_2$, O$_2^\cdot$-, and ·OH), and reduced the cell death induced by OGD-stress, and (b) that in mice in vivo, edaravone significantly protected against NMDA-induced retinal cell death and decreased the numbers of TUNEL-positive cells and cells positive for 4-HNE or 8-OHdG. Edaravone also reduced the expressions of NMDA-induced phosphorylated-JNK and phosphorylated-p38, but not that of phosphorylated-ERK, in the mouse retina.

In the present study, edaravone scavenged the intracellular ·OH radical more strongly than the other radicals examined (O$_2^\cdot$, H$_2$O$_2$), whereas trolox, a water-soluble vitamin E analogue, scavenged O$_2^\cdot$ and H$_2$O$_2$ more strongly than the ·OH radical. Mizuno et al. (1998) reported that in the rat, the protective effect of edaravone against brain damage after middle cerebral artery occlusion derives from its marked scavenging activity against the ·OH radical. The ·OH radical is a highly reactive oxygen species thought to attack cellular components and to cause irreversible cellular damage (such as microsomal or lysosomal disruption) after peroxidation of polyunsaturated fatty acids (Berman, 1991), fragmentation of DNA (Shao et al., 2005), and inactivation of enzymes (Shinar et al., 1983). Ophir et al. (1994) observed that the ·OH radical increases in the feline retina and optic nerve head during reperfusion after the ischemia induced by an elevation of intraocular pressure, and that a
Zn-desferrioxamine complex, which inhibits the generation of ·OH radical, protects against the retinal dysfunction seen after such ischemia/reperfusion. Taken together, the above findings indicate that the ·OH radical may play a pivotal role in retinal cell injury. Hence, edaravone, a potent ·OH-radical scavenger, would be expected to have neuroprotective effects against the retinal damage induced by ischemia or oxidative stress.

Oxidative stress can contribute to neuronal toxicity and has been implicated in both acute injury and chronic neuropathological conditions (Pettmann and Henderson, 1998). Many in vitro models have been employed in attempts to clarify the precise mechanisms responsible for the neuronal cell death induced by oxidative stress. In vitro, damage mimicking that seen after ischemic-mimicked stress is usually induced by OGD in neurons, and oxidative stress is considered to be involved in OGD-induced neuronal cell death (Iijima et al., 2003). In the present study, edaravone protected against OGD stress-induced cell death in RGC-5. On the other hand, there was little effect of trolox against this cell death, even though trolox displayed more powerful radical-scavenging activities than edaravone. Recently, we reported that trolox at 10 µM significantly protected against the reduction in cell viability induced by H₂O₂ in RGC-5 (Nakajima et al., 2008). Although in the present study the radical-scavenging activities of trolox towards the H₂O₂ and O₂⁻ radicals were approximately 47- and 23-fold, respectively, more potent than those of edaravone, scavenging activity towards the ·OH radical was similar between these two agents. However, in the OGD
experiment (Fig. 2) we used these agents at different concentrations: trolox at up to 10 µM and edaravone at up to 50 µM. Accordingly, the observed lack of a protective effect of trolox against OGD-induced cell death may be due to it being present at too low a concentration to scavenge the ·OH radical and so reduce its for lower penetration into cells.

On the other hand, Cui et al. reported a correlation between the anti-apoptotic effects and free radical-scavenging abilities of anti-oxidants (edaravone, trolox, and NAC) was not observed in a study performed to elucidate the mechanism underlying the involvement of free radicals in the apoptosis induced by X-rays or hyperthermia (Cui et al., 2004). In our results, apparent correlations between anti-apoptotic effects and the rate constants of the free radical-scavenging actions of antioxidants was not observed. Usami et al. (2004), who evaluated antioxidant activities in the water-soluble and lipid-soluble phases, found that edaravone exhibited approximately ten-times stronger activity in the water-soluble phase than trolox. These results indicate that the prevention of apoptosis by an antioxidant is not simply related to its scavenging ability or concentration. Therefore, further detailed studies (including use of trolox) will be needed if we are significantly to advance our understanding of this issue.

Activation of the NMDA receptor leads to an intracellular Ca\textsuperscript{2+} influx, and the subsequent ROS increase may be detrimental to cell viability (Choi, 1992). Furthermore, activation of this receptor depletes intracellular glutathione, which could decrease the intracellular capacity.
for ROS inactivation (Wallin et al., 1999). Thus, oxidative stress, leading to the formation of ROS, has been implicated as a stage in the final common pathway for neurotoxicity in a wide variety of acute and chronic neurologic diseases such as glaucoma (Tezel, 2006), age-related macular degeneration (Kindzelskii et al., 2004), and retinitis pigmentosa (Doonan et al., 2005). In the present study, edaravone displayed significant protective effects against both RGC loss and the expression of TUNEL-positivity within retinal cells after an intravitreal injection of NMDA in mice in vivo. The pathologic mechanisms underlying various CNS diseases are closely related to the neuronal glutamate excitotoxicity that follows NMDA-receptor activation and the NMDA-induced generation of free radicals, such as lipid radicals and nitroxide radicals, and these events lead to cell death (Ueda et al., 2007). In the present study, to investigate the effects of edaravone on free radical generation, we evaluated the expressions of 4-HNE and 8-OHdG as markers of oxidative stress. Both of these markers were expressed mainly in the GCL and IPL of the NMDA-injected retina, as also were TUNEL-positive cells, and edaravone reduced the expressions of both markers. These results indicate that the protective effect of edaravone against NMDA-induced retinal damage may be mediated by a reduction in oxidative stress. This is the first report to demonstrate that in experimental retinal injury, edaravone reduces the RGC death associated with apoptosis through ROS-scavenging effects. On the other hand, neither of the other antioxidants tested (trolox at 0.1 and 1 nmol/eye or NAC at 10, 100 and 1000 nmol/eye)
attenuated the NMDA-induced retinal damage in this experiment, even though those concentrations used in the present study were chosen with reference to previous in vitro studies (Tian et al., 2003; Chalasani et al., 2007). In our previous study, alpha-tocopherol at 10 mg/kg was administered orally (p.o.) each day for 14 days reduced the NMDA-induced retinal damage (Nakajima et al., 2008). Accordingly, such lack of effect may be due to their being in insufficient concentration within the retina and/or penetrating poorly into the retina.

MAPK are key kinases in signal-transduction pathways, and members of each major MAPK subfamily (JNK, p38, and ERK1/2) have been implicated in neuronal injury and diseases (Harper and Wilkie, 2003). JNK and p38 MAPK -- which are stimulated by various stresses, including ischemia 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 regulate cell proliferation and differentiation (Luo and DeFranco, 2006), although the precise roles played by ERK1/2 in neuronal cell death or survival remain controversial (Chu et al., 2004). Briefly, ERK1/2 plays disparate roles in neurons, acting in some cases to promote cell survival while also participating in neuronal cell death and the pathogenesis of neurodegeneration. In the present study, we found that activations of p38, JNK, and ERK in the retina were significantly increased after intravitreous NMDA injection. Likewise, Munemasa et al. found that intravitreous injection of NMDA resulted in significant activations of MAPKs such as p38, JNK, and ERK in the rat retina (Munemasa et al., 2005),
and furthermore that p38 and JNK are involved as proapoptotic molecules in NMDA-induced RGC death. In this study, edaravone significantly reduced the activations of both p38 and JNK, but not that of ERK1/2, after NMDA injection. These results indicate that ROS production occurs upstream of the activations of the p38 and JNK cascades. On the other hand, edaravone did not reduce the increase in ERK1/2 seen after NMDA injection, suggesting that ERK1/2 may be activated by ROS-independent signaling pathways. Taken together, our results provide direct evidence that inhibitions of p38 and JNK can ameliorate the retinal damage occurring after NMDA injection.

In conclusion, we have demonstrated that edaravone, a free radical scavenger, inhibits (a) the in vitro neuronal cell death induced by OGD stress in RGC-5 cells and (b) the in vivo RGC death induced by NMDA. Hence, oxidative stress plays a pivotal role in such retinal damage, and edaravone may become a major therapeutic candidates for the treatment of retinal diseases.
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References


Footnotes

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Legends for Figures

**Fig. 1.** Time-kinetic and concentration-response data for antioxidant activities of edaravone against the production of various radical species (H$_2$O$_2$, O$_2^-$, ·OH) in RGC-5.

(A-C) RGC-5 cultures were treated edaravone with for 1 h, and then with either CM-H$_2$DCFDA at 10 μM (H$_2$O$_2$, O$_2^-$) or APF at 10 μM (·OH) for 20 min. ROS production was stimulated with H$_2$O$_2$ at 1 mM, or with KO$_2$ at 1 mM, or with H$_2$O$_2$ at 1 mM plus ferrous perchlorate (II) at 100 μM, and fluorecesence was measured for various time periods. (A) H$_2$O$_2$-induced oxidation of DCFH in RGC-5. (B) O$_2^-$-induced oxidation of DCFH in RGC-5. (C) ·OH-induced oxidation of APF in RGC-5. (D-F) Integrals of ROS production from time-kinetics graphs. Radical species were (D) H$_2$O$_2$, (E) O$_2^-$, and (F) ·OH. Data are shown as mean ± S.E.M., n = 6. **p < 0.01 vs. vehicle-treatment (black column).

**Fig. 2.** Effects of edaravone on retinal cell damage induced by oxygen-glucose deprivation (OGD) in RGC-5 culture.

Representative fluorescence microscopy of (A-C) Hoechst 33342 and (D-F) propidium iodide (PI) staining (added at the end of OGD plus reoxygenation treatment). (A and D) Non-treated cells showed normal nuclear morphology. (B and E) OGD-induced neurotoxicity, with cells showing condensation of their nuclei, including PI-positive cells. (C and F) Pretreatment with edaravone at 50 μM reduced the nuclear condensation and
number of PI-positive cells seen after OGD plus reoxygenation. (G) Cell viability was assessed by immersing cells in WST-8 solution for 2 h at 37°C, with absorbance being recorded at 492 nm. Edaravone at 1, 5 and 50 μM or trolox at 0.1, 1 and 10 μM added to the culture medium immediately after the replacement of the culture medium. Data are shown as mean ± S.E.M., n = 8. **p < 0.01 vs. OGD-treatment alone. Scale bar represents 25 μm.

**Fig. 3.** Effects of edaravone on retinal damage induced by intravitreous injection of N-methyl-D-aspartate (NMDA) in mice.

(A) Non-treated, (B) NMDA-treated, (C and D) NMDA (5 nmol) plus edaravone (5 or 50 nmol)-treated retinal cross sections at 7 days after with or without NMDA alone or NMDA plus with edaravone in mice. (E) Retinal damage was evaluated by counting cell numbers in GCL at 7 days after intravitreous injection of NMDA (5 nmol/eye). Intravitreous injection of edaravone (5 or 50 nmol), trolox (0.1 or 1 nmol), or vehicle (5% DMSO in PBS). (F) Retinal damage was evaluated by counting cell numbers in GCL at 7 days after intravitreous injection of NMDA (5 nmol/eye). Intravitreous injection of NAC (10,100, or 1000 nmol), edaravone (5 nmol), or vehicle (PBS). (G) *Intravenous* injection of edaravone (1 or 3 mg/kg) or vehicle (5% DMSO in PBS) immediately before intravitreous injection of NMDA (5 nmol). Retinal damage was evaluated by counting cell numbers in GCL at 7 days after
intravitreous injection of NMDA. Data are shown as mean ± S.E.M., n = 9 or 10. *P < 0.05, **P < 0.01 vs. vehicle plus NMDA-treated group. Scale bar represents 25 µm.

**Fig. 4.** Effects of edaravone on retinal damage induced by intravitreous injection of NMDA in Thy-1-CFP transgenic mice.

(A) Non-treated, (B) vehicle plus NMDA-treated, and (C) edaravone plus NMDA-treated retinas in flat-mounts, together with (inset) enlargements, at 7 days after intravitreous co-injection of vehicle plus NMDA (5 nmol) or edaravone (50 nmol) plus NMDA. Scale bars represents 1 mm (50 µm in insets). (D) Retinal damage at 7 days after intravitreous injection was evaluated by counting Thy-1-CFP-positive cell numbers in the 12 regions indicated in A (each square represents 0.144 mm²), and analyzing the data separately for Areas a, b, and c [each summed Area = 0.576 mm² (viz. 0.144×4 mm²); see Materials and Methods for further details]. (E) Western immunoblots of Thy-1-CFP and GAPDH proteins in retina at 7 days after intravitreous co-injection of vehicle plus NMDA (5 nmol) or edaravone (50 nmol) plus NMDA. Data are shown as mean ± S.E.M., n = 8 to 10. **P < 0.01 vs. vehicle plus NMDA-treated group.

**Fig. 5.** Effect of edaravone on NMDA-induced expression of TUNEL-positive cells at 1 day after intravitreous injection of vehicle plus NMDA (5 nmol) or edaravone (50 nmol) plus...
NMDA in mice.

(A) Non-treated normal retina, (B) vehicle plus NMDA-treated treated retina, and (C) edaravone plus NMDA-treated treated retina.  (D) Number of TUNEL-positive cells in GCL.  Arrows in B and C indicate TUNEL-positive cells.  Data are shown as mean ± S.E.M., n = 9 or 10.  **P < 0.01 vs. vehicle plus NMDA-treated group.  Scale bar represents 25 μm.

**Fig. 6.** Effect of edaravone on NMDA-induced expressions of 4-HNE and 8-OHdG-positivity cells at 12 h after intravitreous injection of vehicle plus NMDA (5 nmol) or edaravone (50 nmol) plus NMDA in mice.

4-HNE expression in (A) non-treated normal retina, (B) vehicle plus NMDA-treated treated retina, and (C) edaravone plus NMDA-treated treated retina.  (D) Optical-density analysis of the levels of 4-HNE protein expression in GCL and IPL at 12 h after intravitreous injection.  Data are shown as mean ± S.E.M. (n = 8 to 10).  Expression of 8-OHdG-positivity in (E) non-treated normal retina, (F) vehicle plus NMDA-treated treated retina, and (G) edaravone plus NMDA-treated treated retina.  (H) Number of 8-OHdG-positive cells in GCL.  Data are shown as mean ± S.E.M., n = 8 or 10.  **P < 0.01 vs. vehicle plus NMDA-treated group.  Scale bar represents 25 μm.

**Fig. 7.** Effect of edaravone on NMDA-induced expression of phosphorylated-p38, JNK,
and ERK at 6 h after intravitreous injection of vehicle plus NMDA (5 nmol) or edaravone (50 nmol) plus NMDA in mice.

Representative Western blots showing activation of (A) p38, (B) JNK, and (C) ERK in the normal, vehicle, and edaravone groups. Representative photographs showing immunostaining of (D) phosphorylated-p38, (E) JNK, and (F) ERK in the normal, vehicle, and edaravone groups. Data are shown as mean ± S.E.M., n = 6. *P < 0.05 vs. vehicle plus NMDA-treated group. Scale bar represents 25 μm.
Fig. 4

D

Thy-1-CFP-positive cell number / 0.576 mm²

**

Area a | Area b | Area c

E

Relative expression (arb. Units) (Thy-1-CFP/GAPDH)

**

Normal | Vehicle | Edaravone

NMDA

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