Edaravone-Loaded Liposome Eyedrops Protect against Light-Induced Retinal Damage in Mice

Hiroki Shimazaki,1,2 Kobe Hironaka,2,3 Takuya Fujisawa,5 Kazuhiro Tsuruma,1 Yuichi Tozuka,5 Masamitsu Shimazawa,1 Hirofumi Takeuchi,3 and Hideaki Hara1

Purpose. To investigate the pharmacologic effects of eyedrops containing liposomes loaded with edaravone (3-methyl-1-phenyl-2-pyrazolin-5-1) against light-induced retinal damage in mice.

Methods. Edaravone was incorporated into submicron-sized liposomes (ssLips) by the calcium acetate gradient method. Retinal damage in mice was induced in dark-adapted mice by exposure to white light at 8000 lux for 3 hours. Edaravone-loaded ssLips were dropped into the left eye just before and after light exposure and then three times daily for 5 days after light exposure. Retinal damage was evaluated by recording the scotopic electroretinogram (ERG) and measuring the thickness of the outer nuclear layer (ONL) and by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. The scavenging capacity of reactive oxygen species (ROS) of edaravone-loaded ssLips was determined using a murine cone photoreceptor cell line (661W). The human corneal and conjunctival cell lines were exposed to edaravone-loaded ssLips to determine cytotoxicity.

Results. Eyedrop administration of edaravone-loaded ssLips significantly prevented both the decrease in a- and b-wave amplitudes of flash ERG and the shrinkage of the ONL compared with the control group (treated with empty ssLips) after 5 days of light exposure. The edaravone-loaded ssLips prevented the increase in the numbers of TUNEL-positive cells after 48 hours of light exposure. This marked protection was not found in the group treated with free edaravone. Edaravone-loaded ssLips showed a stronger inhibition of in vitro light-induced ROS production and cell death than did free edaravone. The ssLips showed little cytotoxicity toward ocular cell lines.

Conclusions. Edaravone-loaded ssLips protected against light-induced retinal dysfunction by eyedrop administration. Liposomal eyedrops may become one of the therapeutic candidates for drug delivery to posterior eye segments. (Invest Ophthalmol Vis Sci. 2011;52:7289–7297) DOI:10.1167/iovs.11-7983

Age-related macular degeneration (AMD) is a disease associated with aging that gradually destroys central vision through damage to the retinal pigment epithelium and photoreceptor. The macula is located in the center of the retina and the light-sensitive tissue at the back of the eye. Two main types of AMD are recognized: wet AMD occurs as a result of the formation of pathologic choroidal neovascularization, and dry AMD occurs when the light-sensitive cells in the macula slowly break down. Dry AMD usually takes a number of years to reach its final degenerative stage (geographic atrophy). New drugs for wet AMD, such as antivascular endothelial growth factor (VEGF) agents ranibizumab and pegaptanib, can cause regression of the abnormal blood vessels and improvement of vision. However, potential treatments with these new drugs are hampered by the problems of drug delivery to the retina.

At present, intravitreal injection is the most efficient clinical method for the delivery of drugs to the retina. Although direct intravitreal injection provides high concentrations of drugs in the retina, repeated injections are associated with potential risks of complications such as endophthalmitis, cataracts, vitreous hemorrhages, and retinal detachment. Systemic administration is one possible way to circumvent intravitreal injection. However, the doses needed for a therapeutic effect by this route can lead to considerable side effects. Consequently, a pressing need exists for noninvasive and harmless delivery systems that can target the posterior segment of the eye.

Eyedrop administration is one attractive alternative for minimizing drug delivery side effects. However, conventional eyedrops usually cannot deliver drugs to the retina because of the ocular barriers presented by structures such as the cornea and the conjunctiva. One way to enhance the bioavailability of drugs administered by eyedrops is through the use of liposomes, which are artificially prepared vesicles made of phospholipid bilayers. A lipophilic drug will bind within the vesicle membranes, whereas a hydrophilic drug will become encapsulated within the aqueous phase in the interior of the liposome. In our previous study, we were able to follow the appearance within the retina of the fluorescence of coumarin-6 that had been formulated into ssLips and administered as eye drops in mice, rabbits, and monkeys. The present study extends this finding to examine the effectiveness of ssLips in the delivery of pharmacologically active ingredients to the retina.

Excessive light exposure leads to photoreceptor degeneration in many animals and can be a risk factor for onset and progression of AMD. The human retina consumes significant amounts of oxygen and readily produces reactive oxygen species (ROS) such as superoxide (O2•−) and hydrogen peroxide (H2O2). Photoreceptor apoptosis due to ROS production is a common feature of AMD and can be mimicked by excessive light exposure. Therefore, animal models of retinal light damage are widely used as AMD models.
Edaravone is a potent free radical scavenger and has been prescribed clinically in Japan since 2001 for the treatment of acute brain infarction. It also has protective effects against cerebral ischemia and reperfusion injuries in a variety of experimental animal models. We have previously shown the protective effects of intraperitoneal or intravitreal edaravone treatments against light-induced retinal damage in mice.

In the present study, we evaluated the potential protective effects of eyedrop-administered edaravone-loaded ssLips against light-induced retinal damage. We examined histologic and electrophysiological responses in an in vivo murine model and determined cell viability and ROS production using an in vitro murine photoreceptor cell line. The cytotoxicity of the ssLips was also tested in vitro using corneal and conjunctival cell lines.

**Materials and Methods**

**Materials**

Egg phosphatidylcholine was purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). 3-Methyl-1-phenyl-2-pyrazolin-5-one (edaravone; MW, 174.2) and H2O2 were purchased from Wako Pure Chemicals (Osaka, Japan). Cholesterol was obtained from Sigma-Aldrich (St. Louis, MO). 2-Morpholinoethanesulfonic acid monohydrate (Mes) was purchased from Nakalai Tesque (Kyoto, Japan). Hank’s balanced salt solution (HBSS) was purchased from Gibco Brl (Grand Island, NY). A cell counting kit-8 (WST-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Hoechst 33342 was purchased from Molecular Probes (Eugene, OR). 5-(and-6)-Chloromethyl-2',7'-dichlorodihydrofluoresceindiacetate (CM-H2DCFDA) was purchased from Invitrogen (Carlsbad, CA). All other chemicals were commercial products of reagent grade.

**Remote Loading of Edaravone into ssLips**

Remote loading of edaravone was performed by the calcium acetate gradient method described previously. The transmembrane concentration gradient of calcium acetate (liposome inner phase > liposome outer phase) was used as a driving force for the remote loading of the edaravone. Leakage of protonated acetic acid from the liposome increases the liposome internal pH. This pH imbalance (inside basic, outside acidic) leads to the accumulation of weak acids within the liposome. Weakly acidic molecules, such as edaravone in its protonated (uncharged) membrane-permeable form, are pumped into the slightly alkaline liposome internal phase by diffusion through the lipid bilayers. Edaravone then becomes deprotonated and assumes its charged, membrane-impermeable form.

**Preparation of Edaravone-Loaded ssLips**

Egg phosphatidylcholine and cholesterol (8:1 molar ratio) were dissolved in a small amount of chloroform and dried in a rotary evaporator to form a thin lipid film. The film was dried in a vacuum oven overnight and was hydrated with calcium acetate solution (120 mM). Resultant multilamellar vesicles were frozen and thawed four times. ssLips were prepared using an extruder (LipoFast-Pneumatic; Avestin, Inc., Ottawa, Canada) with a 0.1-μm polycarbonate membrane (Whatman Japan KK, Tokyo, Japan). A calcium acetate concentration gradient was created across the liposomal membrane by replacing the calcium acetate of the external liposome medium with HBSS-Mes buffer (pH 6.0) at 4°C in two dialysis steps. Edaravone was dissolved in HBSS-Mes buffer and was mixed with the ssLips. Remote loading was achieved by incubation of the ssLips at 37°C for 10 minutes. The final phospholipid concentration in the resultant liposomal suspension was 40.8 μmol/mL.

Conventional liposomes were prepared as a reference by a hydration method. The lipid film was hydrated with edaravone dissolved in HBSS-Mes buffer. The resultant multilamellar vesicles were freeze-thawed and then downsized using the extruder.

**Characterization of ssLips**

The particle size and zeta potential of the ssLips were measured (Zetasizer Nano ZS; Malvern, Worcestershire, UK). The entrapment efficiency of edaravone into ssLips was determined by the method described in the previous report. Release of edaravone from the ssLips was determined in phosphate-buffered saline (PBS) at pH 7.4. A 0.5-mL volume of ssLips containing edaravone was transferred to a dialysis bag. The bag was then soaked in PBS at 37°C (49.5 mL). At a predetermined time, 0.2 mL PBS was removed and replaced with fresh PBS of equal volume. The released edaravone was quantified with HPLC.

**Animals**

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the study was approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. Male albino ddY mice (Japan SLC, Hamamatsu, Japan), aged 5 to 6 weeks, were used in this study. They were kept under controlled lighting conditions (12 hours light/12 hours dark).

**Exposure to Light**

After dark adaptation for 24 hours, the pupils were dilated with 1% cyclopentolate hydrochloride eye drops (Santen, Osaka, Japan) 30 minutes before light exposure. Nonanesthetized mice were exposed to 8000 lux of white fluorescent light (Toshiba, Tokyo, Japan) for 5 hours in cages with reflective interiors. Temperature during the exposure to light was maintained at 25°C ± 1.5°C. After light exposure, all mice were placed in the normal light/dark cycle.

**Treatment with Edaravone-Loaded ssLips**

Five doses of edaravone-loaded ssLips, free edaravone, or empty ssLips were dropped (3 μL) onto the left eye every 5 minutes. This eye drop administration was performed just before and after light exposure and was then repeated three times daily for 5 days after light exposure. The concentration of edaravone was 0.15%. The contralateral eye was used as the control and received no treatment.

**Electroretinograms**

Electroretinograms (ERGs) were recorded at 5 days after light exposure (Mayo, Aichi, Japan). Mice were maintained in a completely dark room for 24 hours and 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). Their pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen). Flash ERGs were 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.

**Histologic Analysis**

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (80 mg/kg; NakalaiTesque, Kyoto, Japan). Each eye was then enucleated and kept immersed for at least 24 hours at 4°C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (thickness, 5 μm) cut through the optic disc of each eye were prepared in the standard manner and were 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 (ONL) from the optic disc was measured at 240-μm intervals on the photographs in a blinded fashion by a single observer (HS). Data from three sections (selected randomly from the six sections) were averaged for each eye.

### 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 light-induced retinal cell death. The mice were anesthetized with 80 mg/kg pentobarbital sodium administered intraperitoneally 48 hours after light exposure for 5 hours. Their eyes were enucleated, fixed overnight in 4% paraformaldehyde, immersed for 2 days in 25% sucrose with PBS, and were then embedded in a supporting medium for frozen-tissue specimens (OCT compound; Tissue-Tek, Naperville, IL). Retinal sections 10 μm thick were cut on a cryostat at −25°C and stored at −80°C until staining. After two washes with PBS, the sections were incubated with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 hour and were then washed three times in PBS for 1 minute at room temperature. After that, the sections were incubated with an anti–fluorescein antibody-peroxidase conjugate at room temperature in a humidified chamber for 30 minutes followed by three times in PBS for 1 minute. After that, the sections were incubated with DAB tetrahydrochloride peroxidase substrate. Light microscope images were photographed, and labeled cell counts in the ONL 480 to 720 μm from the optic disc were obtained in both the superior and the inferior areas of the retina. The number of TUNEL-positive cells was averaged for the superior and inferior areas.

### Cell Cultures

The murine photoreceptor cell line (661W) was a gift from Muayad R. Al-Ubaidai (University of Oklahoma Health Sciences Center, Oklahoma City, OK). The 661W cells were cultured in DMEM (Sigma-Aldrich) containing 10% FBS (Gibco), 100 IU/mL streptomycin, and 100 IU/mL penicillin (Gibco). Immortalized human corneal epithelial cells (RIKEN Cell Bank, Tsukasa, Japan) were cultured in DMEM/Ham’s F12 (1:1) supplemented with 10% FBS, 5 μg/mL insulin (Sigma), 0.1 μg/mL cholera toxin (List Biological Laboratories, Campbell, CA), 0.5% DMSO, 10 ng/mL epidermal growth factor (Sigma), 100 IU/mL streptomycin, and 100 IU/mL penicillin, as described by Araki-Sasaki. Human corneal and conjunctival cells were seeded. The corneal and conjunctival cells were seeded at 5 × 10^5 cells per well in 96-well plates and then incubated for 24 hours, at which time the cells had reached 80% to 90% confluence. The medium was replaced with fresh 1% FBS-DMEM. After 37°C for 1-hour incubation, edaravone-loaded ssLips at 100 μM, empty ssLips, or a mixture (edaravone solution at 100 μM plus empty ssLips) were added. The cells were then exposed to 2500 lux of white fluorescent light (Nikon, Tokyo, Japan) for 24 hours at 37°C. Viable cell numbers were measured using a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(4-sulfophenyl)-2H tetrazolium monosodium salt (WST-8). Briefly, 10 μL WST-8 was added to each well, incubated at 37°C for 3 hours. Absorbance was measured at 492 nm (reference wavelength, 660 nm) using a spectrophotometer (Varioskan; Thermo Electron Corporation, Vantaa, Finland).

### Measurement of ROS Production in 661W Cells

Intracellular radical activation within 661W cells was determined using CM-H2DCFDA. CM-H2DCFDA taken up into the cell is converted to dichlorodihydrofluorescein (DCFH) by an intracellular esterase. The ROS then oxidizes DCFH (nonfluorescent) to DCFH (fluorescent). At the end of the light exposure period, CM-H2DCFDA was added to the culture medium and was incubated at 37°C for 1 hour at a final concentration of 10 μM. The 96-well plate was loaded onto a plate holder in a fluorescence spectrophotometer, and the reaction was carried out at 37°C. Cell fluorescence was determined by Hoechst 33342 staining and was used to calculate ROS production per cell.

### Hoechst 33342 Staining

At the end of the ROS measurement, Hoechst 33342 (excitation, 360 nm; emission, 490 nm) was added to the culture medium (final concentration, 8.1 μM) and incubated for 15 minutes. Microscopic images through fluorescence filters for Hoechst 33342 (U-MWU; Olympus, Tokyo, Japan) were captured by a CCD camera (DP50BW; Olympus).

### Light-Induced Cell Death in 661W Cell Cultures

The 661W cells were seeded at 1 × 10^5 cells per well in 96-well plates and then incubated for 24 hours, at which time the cells had reached 80% to 90% confluence. The medium was replaced with fresh 1% FBS-DMEM. After 37°C for 1-hour incubation, edaravone-loaded ssLips at 100 μM, empty ssLips, or a mixture (edaravone solution at 100 μM plus empty ssLips) were added. The cells were then exposed to 2500 lux of white fluorescent light (Nikon, Tokyo, Japan) for 24 hours at 37°C. Viable cell numbers were measured using a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(4-sulfophenyl)-2H tetrazolium monosodium salt (WST-8). Briefly, 10 μL WST-8 was added to each well, incubated at 37°C for 3 hours. Absorbance was measured at 492 nm (reference wavelength, 660 nm) using a spectrophotometer (Varioskan; Thermo Electron Corporation, Vantaa, Finland).

### Cytotoxicity of Edaravone-Loaded ssLips on the Ocular Surface

The cytotoxicity of ssLips was evaluated using human corneal and conjunctival cell lines. The corneal and conjunctival cells were seeded at 5 × 10^5 cells per well in 96-well plates and then incubated for 24 hours. At the end of the ROS measurement, Hoechst 33342 (excitation, 360 nm; emission, 490 nm) was added to the culture medium (final concentration, 8.1 μM) and incubated for 15 minutes. Microscopic images through fluorescence filters for Hoechst 33342 (U-MWU; Olympus, Tokyo, Japan) were captured by a CCD camera (DP50BW; Olympus).
hours. The medium was removed and washed twice with 200 μL HBSS-Mes buffer. The cells were then exposed to 100 μL of each test solution (edaravone-loaded ssLips at 1.5 mg/mL, empty ssLips, or free edaravone at 1.5 mg/mL). A solution of 0.01% of benzalkonium chloride in HBSS-Mes buffer served as a positive control. After 180 minutes of incubation, the cells were washed three times with 150 μL HBSS-Mes buffer and replaced with fresh 1% FBS-DMEM. The viable cell numbers were measured using WST-8, as described.

**Statistical Analysis**

Data are presented as the mean ± SEM. Statistical comparisons were made using Student’s t-test (StatView, version 5.0; SAS Institute, Cary, NC). P < 0.05 was considered to indicate statistical significance.

**RESULTS**

**Characterization of Edaravone-Loaded ssLips**

Table 1 shows the physicochemical properties of edaravone-loaded ssLips. The average particle size of edaravone-loaded ssLips was 92.6 ± 1.5 nm. Edaravone-loaded ssLips showed almost neutral surface charge. Entrapment efficiency using a conventional hydration method was <20% in this system, whereas entrapment efficiency using the calcium acetate gradient method was 41.4% ± 0.3%. We confirmed that the entrapment efficiency of edaravone was almost unchanged (40.7% ± 1.0%) when edaravone-loaded ssLips were stored at 4°C for 12 hours.

The release of edaravone from edaravone-loaded ssLips was investigated in PBS at pH 7.4 (Fig. 1). For free edaravone, approximately 80% was released from the dialysis bag at 30 minutes, and the amount released reached a maximum at 1 hour. In the case of ssLips prepared by the hydration method, the release profile was unchanged compared with that of free edaravone. This burst release profile indicated that most of the edaravone molecules had not been encapsulated inside liposomes but had been adsorbed or had physically interacted with the surface of liposomes prepared by the hydration method. A significantly slower edaravone release rate was found for the ssLips prepared by the calcium acetate gradient method than for ssLips prepared by the hydration method. This slow release profile suggests that an inner to outer penetration of edaravone molecules across the liposome membrane was occurring. We

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**FIGURE 2.** Measurement of the dark-adapted ERG amplitudes in the mouse retina five days after light exposure. (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 cd·s/m² at the ipsilateral retina. Amplitudes of a- and b-waves of light exposure (8000 lux) in the empty ssLips-treated group, the edaravone-ssLips-treated group; and the free edaravone-treated group (B) at the ipsilateral retina and (C) at the contralateral retina. Data are shown as mean ± SEM; n = 6–11. *P < 0.05 versus light exposure plus the empty ssLips treated group. Eda, edaravone.
confirmed that the particle size of each edaravone-loaded ssLip remained unchanged throughout the release experiment.

**Electroretinograms**

Because excessive light exposure induces retinal dysfunction, we used ERGs to investigate the effects of edaravone-loaded ssLips on light-induced photoreceptor degeneration.

Figure 2 shows the measurement of the dark-adapted ERG amplitudes at 5 days after light exposure in the mouse retina. The a-wave shows the function of the photoreceptors, and the b-wave reflects bipolar cell and Müller cell function. In the normal mouse retinas, the amplitudes of the a- and b-waves increased as the light intensity increased, but they were attenuated 5 days after 8000 lux white light exposure for 3 hours. A significant reduction in the a- and b-wave amplitudes was found in both ipsilateral and contralateral retinas of the group treated with empty ssLips compared with the nontreated group. Treatment with edaravone-loaded ssLips resulted in a significantly lower reduction in the a- and b-wave amplitudes (57% and 35% reduction, respectively) compared with the group treated with empty ssLips on the ipsilateral side, at 0.98 log cd·s/m² (Fig. 2B). Edaravone-loaded ssLips significantly protected against light-induced retinal dysfunction from −0.02 to 0.98 log cd·s/m² for the a- and b-wave amplitudes. On the other hand, the treatment with free edaravone showed no protective effects against light-induced retinal damage. On the contralateral side, no significant differences were noted between the amplitudes of groups treated with empty ssLips or edaravone-loaded ssLips (Fig. 2C).

**Light-Induced Photoreceptor Degeneration**

Representative retinal images show the ipsilateral retina 5 days after light exposure (see Figs. 3A–D). ONL was markedly thinner in the group treated with empty ssLips (Fig. 3B) than in the nontreated group (Fig. 3A), but the group treated with edaravone-loaded ssLips showed significantly less photic damage (Fig. 3C) than the group treated with empty ssLips (Fig. 3B). No marked suppression was seen in response to treatment with free edaravone (Fig. 3D). ONL thickness images between 480 µm and 640 µm from the optic disc on the ipsilateral side at 5 days after light exposure are shown in Figure 3E. ONL thickness of the group treated with empty ssLips was reduced by 50% (average of points 240–1920 µm from the optic nerve) compared with the normal retina. Treatment with edaravone-

![Figure 3. Effects of eyedrop administration of edaravone-loaded ssLips on light-induced retinal damage in mice. (A) Nontreated. (B) Light exposure (8000 lux) plus empty ssLips. (C) Light exposure plus edaravone-loaded ssLips. (D) Retinal cross-sections at 5 days after light exposure plus free edaravone. ONL thickness at 5 days after light exposure in the (E) ipsilateral retina and the (F) contralateral retina. Data are shown as mean ± SEM; n = 5–11. *P < 0.05, **P < 0.01 versus light exposure plus empty ssLips dropped group. Scale bar, 25 µm. Eda, edaravone.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933248/)
loaded ssLips resulted in significant protection of both the superior and the inferior areas from 240 μm to 1680 μm compared with the group treated with empty ssLips. ONL size was reduced by only 48% (average of points 240–1920 μm from the optic nerve) of that seen for the empty-ssLip treatment group. ONL thickness of the free edaravone-treated group was not different from the thickness of the empty-ssLip treatment group.

In the contralateral eye, treatment with empty ssLips or with free edaravone had no effect on ONL thickness (Fig. 3F). ONL shrinkage was slightly suppressed in the retinal inferior area from 240 μm to 960 μm after treatment with edaravone-loaded ssLips, but no protection was observed in the superior retinal area. Liposomes themselves had no effect against light-induced retinal damage because there was no difference in ONL thickness between the ipsilateral eye and the contralateral eye.

**Light-Induced Expression of TUNEL-Positive Cells**

Figure 4 shows the light-induced apoptotic cell death and the effects of edaravone-loaded ssLips, as determined by the expression of TUNEL-positive cells at 48 hours after light exposure. TUNEL-positive cells were observed only at the ONL, not in any other retinal areas. No TUNEL-positive cells were observed in any retinal areas in nontreated mouse retinas (Fig. 4A). Quantitative analysis showed that light exposure significantly increased the number of TUNEL-positive cells in the ONL at 48 hours (compared with the nontreated normal retina) (Fig. 4E). Edaravone-loaded ssLips significantly reduced the number of TUNEL-positive cells in the ipsilateral eye (45% reduction vs. the empty-ssLip–treated group), but free edaravone had no effect on the numbers of TUNEL-positive cells (Fig. 4E).

**Effects of Edaravone-Loaded ssLips on Light-Induced ROS Production in 661W Cultures**

Intracellular ROS production was detected with the ROS-sensitive probes of CM-H$_2$DCFDA. Edaravone-loaded ssLips and a mixture (free edaravone plus empty ssLips) significantly inhib-
Effects of Edaravone-Loaded ssLips on Light-Induced 661W Cell Death

Cell viability was reduced by 42% after light irradiation in cells treated with empty ssLips. Treatment with edaravone-loaded ssLips and the mixture (free edaravone plus empty ssLips) significantly inhibited light-induced cell death (Fig. 6). The edaravone-loaded ssLips were more effective at reducing cell death than was the mixture.

Cytotoxicity of Edaravone-Loaded ssLips on Ocular Surface

The cytotoxicity of edaravone formulations was determined in ocular surface cell lines, cornea, and conjunctiva. Figure 7 shows changes in the viability of corneal and conjunctival cells in the presence of liposomes. The viability of the cells remained unchanged when the cells came into contact with empty ssLips, edaravone-loaded ssLips, and free edaravone. In contrast, the viability of benzalkonium chloride (0.01%)-treated cells was significantly lower than that of control, although this concentration of benzalkonium chloride is commonly used in eyedrops. The edaravone-loaded ssLips used in this study were confirmed to be highly biocompatible and showed low toxicity in ocular cells.

**DISCUSSION**

One of the greatest challenges in ophthalmic drug delivery is the determination of effective and safe administration of drugs to the posterior segment of the eye to treat diseases of the retina, choroids, and vitreous body. In recent years, new ocular drug delivery systems using nanocarriers have been developed to improve the ocular bioavailability of eyedrop-administered drugs. We have reported the potential of ssLips as a drug delivery system for the retina based on fluorometric investigations using coumarin-6. However, no actual pharmacologic investigations have yet explored the use of liposomal carriers by eyedrops in the retina. For this purpose, the protective effects of edaravone-loaded ssLip eyedrops against retinal damage were evaluated using in vitro and in vivo light-induced animal models.

In the present study, eyedrop administration of edaravone-loaded ssLips successfully suppressed the decrease in both a- and b-wave amplitudes of flash electroretinography and in ONL thickness, and it prevented the expression of TUNEL-positive cells at the ipsilateral retina. These protective effects were not observed in response to free edaravone and or empty ssLips. These results suggest that the liposomal carriers effectively delivered edaravone to the posterior segment, leading to a strong pharmacologic effect of edaravone.

Liposomes, composed of phospholipid, are known to come into intimate contact with the ocular barriers. An improvement in drug affinity for the ocular barriers may improve the effectiveness of drug delivery into the retina, resulting in significant retinal protection by liposomal eyedrops. Several investigators have demonstrated that drug-containing liposomes delay precorneal clearance, thereby increasing the ocular availability of drugs. The longer retention of liposomes on the ocular surface may increase the association with the ocular surface tissues, the cornea, and the conjunctiva.

The in vitro study presented here showed that edaravone-loaded ssLips reduced light-induced intracellular ROS-generation and cell death to a greater extent than was seen for free edaravone treatment. Onyeji et al. reported that liposome entrapment of ofloxacin and clarithromycin markedly enhanced their uptake into human macrophages. We have also reported that liposomes provide efficient intracellular delivery of edaravone into RGC-5 cells, a neuronal precursor cell line that can be differentiated to resemble retinal ganglion cells.

Thus, a high antioxidant activity may be induced by an increased uptake of encapsulated edaravone into 661W cells compared with the uptake of free edaravone.

**FIGURE 6.** Effects of edaravone-loaded ssLips on light-induced cell damage in 661W culture. Cell viability was assessed by WST-8 for 3 hours at 37°C, with fluorescence recorded at 492/660 nm. Data are shown as mean ± SEM; n = 6. **P < 0.01. Eda, edaravone; Eda+Empty ssLips, free edaravone plus empty ssLips.

**FIGURE 5.** Effects of edaravone-loaded ssLips on light-induced ROS production in 661W culture. Intracellular ROS levels were determined by measuring the fluorescence of CM-H$_2$DCFDA (at excitation 488 nm/emission 525 nm) after light exposure at 24 hours in 661W. Data are shown as mean ± SEM; n = 6. **P < 0.01. Eda, edaravone; Eda+Empty ssLips, free edaravone plus empty ssLips.
Komatsu et al.\textsuperscript{22} reported that plasma levels of edaravone decreased rapidly, with a half-life of 5.4 minutes, after intravenous administration in rats.\textsuperscript{22} Although the reaction rates of edaravone in ocular tissues are unknown, edaravone might rapidly lose its antioxidant activity after administration. Liposomes can be used to protect drug molecules from metabolic enzymes present at the tear and corneal epithelium interfaces.\textsuperscript{23} The prevention of edaravone inactivation by ssLip entrapment may partially contribute to significant retinal protection.

Although determination of the concentration of edaravone in the ipsilateral retina is difficult, we confirmed in the present study the pharmacologic effects of edaravone against light-induced retinal damage. Other studies have shown that edaravone at 1 \( \mu \text{M} \) had a protective effect on 15-hydroperoxyeicosatetraenoic acid-induced oxidative injury in cultured bovine aortic endothelial cells.\textsuperscript{24} We previously reported that edaravone at 0.1 to 10 \( \mu \text{M} \) scavenged the \( \text{H}_2\text{O}_2 \) radicals generated in RGC-5 cells.\textsuperscript{25} The concentrations of edaravone may be sufficient to show a radical-scavenging effect at the ipsilateral retina after eyedrop administration, and this concentration range may be similar to the concentrations in these previous reports.

Absorption of liposomes after eyedrop administration from the ocular surface to the retina may occur mainly by three routes: systemic, corneal, and noncorneal pathways.\textsuperscript{26} A portion of topically applied drugs enters the systemic circulation through nasolacrimal drainage and can contribute to drug accumulation in the retina.\textsuperscript{27,28} In this study, ONL shrinkage in the contralateral retina in the edaravone-loaded ssLip-treated group was less than that in the empty ssLip-treated group. The weak effect in the contralateral retina was assumed to be due to systemically absorbed edaravone because we had reported previously that systemic administration of edaravone also protects against light-induced retinal damage in mice.\textsuperscript{10} However, the suppression of light-induced retinal damage was more potent in the ipsilateral retina than in the contralateral retina in the edaravone-loaded ssLip-treated group.

We cannot rule out the possibility of the periorcular or transscleral route for retinal delivery because long-term eye drop therapy might have induced a large accumulation of drugs in tenon capsules, providing an access to the posterior segment for instilled drugs.\textsuperscript{29} We previously focused on the role of noncorneal pathways in providing access through the tissues involving the trabecular meshwork, iris root, and pars plana for retinal delivery.\textsuperscript{3} Therefore, in the present study, the accumulation of edaravone in periocular tissue might have made a small contribution to the overall retinal protective effect.

In previous studies, the pharmacokinetics of liposomes were evaluated using liposomes labeled with coumarin-6 and 5(6)-carboxyfluorescein as hydrophobic and hydrophilic fluorescent markers, respectively.\textsuperscript{3,30} The highest fluorescence in the inner plexiform layer was observed at 30 minutes with both fluorescence markers used, and most fluorescence disappeared at 60 minutes after eyelid administration. Therefore, we assume that the pharmacokinetic profile of edaravone may be similar to the fluorescence marker-involved liposomes. Based on the release profile of edaravone-loaded ssLips in Figure 1, approximately 50% of edaravone was released at PBS at pH 7.4. The release rate of edaravone might be insufficient to protect the retina. Although the accurate amount of edaravone distributed in the retina could not be estimated in this study, we found the positive findings of the edaravone-loaded liposomal formulation to protect against retinal damage. A plausible explanation of the effective role of edaravone in retinal protection is the intracellular degradation of liposomes owing to lysosomal enzyme.\textsuperscript{31} During the penetration of edaravone-loaded ssLips into the retina, edaravone molecules were released in the retina not only by diffusion across the liposomal membrane but also by the collapse of the liposomal structure.

Edaravone-loaded ssLips provided stronger protection to the inferior retina than the superior retina against light-induced ONL shrinkage. On the other hand, the retinal flat-mount images reported in the previous study indicated that liposome-mediated fluorescence was distributed homogeneously in the dorsal, ventral, temporal, and nasal retina after eyelid administration.\textsuperscript{4} The retinal damage induced by light exposure was more severe in the superior retina than in the inferior retina in rats.\textsuperscript{5} Therefore, differences in protective effects between superior and inferior retinas may not be attributable to the differences in the distribution of edaravone. Retinal sensitivity to light exposure may be responsible for the differences in light-induced retinal damage.

Most oxidative stress-related retinal diseases require long-term therapy. Our in vitro experiments using the human corneal and conjunctival cell line showed that edaravone formulations were not toxic to these cell lines, indicating an adequate safety range for the application of edaravone-loaded ssLips as a therapeutic ocular drug delivery system.

In conclusion, we demonstrated that the eyedrop administration of edaravone-loaded ssLips had marked neuroprotective...
effects against light-induced photoreceptor degeneration in mice. Comparison between ipsilateral and contralateral retinas revealed that edaravone may reduce the progression of dry AMD. These findings indicate that liposomal eyedrops containing edaravone may reduce the progression of dry AMD.

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References