Ligation of the Pterygopalatine and External Carotid Arteries Induces Ischemic Damage in the Murine Retina

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PURPOSE. This study aimed to characterize the functional and morphologic changes in a murine model of ocular ischemic disease caused by vascular occlusion.

METHODS. Retinal ischemia was induced by unilateral ligation of the pterygopalatine artery (PPA) and the external carotid artery (ECA) in anesthetized mice. Changes in ocular blood flow and retinal circulation were evaluated by three different methods: laser speckle blood flow imaging, fundus imaging, and fluorescein isothiocyanate angiography. Five days after reperfusion following 3- or 5-hour ischemia, an electroretinogram (ERG) was recorded, and the retinal histology was examined and quantified. The effects of a free radical scavenger, edaravone, using the model were evaluated by ERG and histologic analysis.

RESULTS. The ligation of both the PPA and the ECA significantly reduced ocular blood flow and narrowed the blood vessels. Five hours of ischemia reduced the a-wave, b-wave, and oscillatory potential amplitudes of the ERG. The number of cells in the ganglion cell layer and the thickness of both the inner plexiform layer and the inner nuclear layer were reduced in the ischemic group. Retinal ischemia caused an increase in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in the inner layer after 21-hour reperfusion following 3-hour ischemia and 19-hour reperfusion following 5-hour ischemia. Edaravone (1 mg/kg, administered intraperitoneally) significantly reduced the retinal ischemic damage.

CONCLUSIONS. These findings indicate that the murine model in which both the PPA and the ECA are ligated may be useful to clarify the pathologic mechanisms of retinal ischemic diseases and to evaluate neuroprotective drugs that target retinal ischemic injury. (Invest Ophthalmol Vis Sci. 2011;52:9710–9720) DOI:10.1167/iovs.11-8160

Carotid artery stenosis is a leading cause of ocular ischemic syndrome, a devastating disease induced by chronic ischemia of the eye.1 Amaurosis fugax is the most frequent type of ocular ischemic syndrome, occurring in approximately one-third of patients with carotid artery disease.2,3 Chronic retinal ischemia resulting from carotid artery stenosis has been receiving increasing attention in the literature.4 Retinal ischemia is involved in several retinal disorders such as ischemic optic neuropathies, diabetic retinopathy, and rubectic glaucoma.5,6 To investigate the pathogenesis of retinal cell death and to determine the efficacy of novel neuroprotective therapies targeting ischemic retinal diseases, several animal models have been used. These experimental neurodegenerative models include those that use techniques such as high intraocular pressure (IOP) induction, optic nerve bundle ligation, and middle cerebral artery occlusion (MCAO).6–8 In the most commonly used retinal ischemic model, high IOP induces retinal ischemia, however, the retinal injury achieved in this model may be caused by both ischemia and pressure. Optic nerve bundle ligation also constricts the optic nerve, elevates IOP by pressing on the globe, and damages axons.6 In the MCAO model, cerebral ischemia is induced in addition to retinal ischemia.8,9 In the MCAO model, severe brain injury induces the exacerbation of systemic conditions and, in some cases, death in mice. Such neurologic or systemic deteriorations could affect ocular circulation or retinal susceptibility. Moreover, brain infarction in the visual center, including the superior colliculus, lateral geniculate nucleus, and visual cortex, may affect retinal function or susceptibility to ocular ischemia through reduced retrograde transport.

Most of the aforementioned retinal ischemic models show irreversible morphologic changes. Recent studies6,10 have revealed that apoptosis is involved in neuronal cell death in animal models of transient retinal ischemia. Lelong et al.11 created a novel murine model of global and transient retinal ischemia resulting in amaurosis fugax by occluding both the pterygopalatine artery (PPA) and the external carotid artery (ECA). The PPA supplies blood to the ophthalmic artery, and the ECA contributes to the vascular network between the PPA and the ophthalmic artery.11,12 This group also investigated gene expression profiles and residual functional deficits but did not investigate the long ischemic effects >30 minutes. Briefly, the 30-minute unilateral occlusions of PPA and ECA in C57BL/6J mice were slightly reduced retinal functions measured by scotopic or photopic ERGs at 4 weeks after the reperfusion, but there was no histologic damage to the retina. Amaurosis fugax is transient episodic blindness caused by decreased blood flow to the retina. The visual loss typically lasts from a few seconds to several minutes but can persist for a few hours before vision returns to normal. On the other hand, retinal dysfunction and histologic damage in prolonged ischemia, which is characterized as an ocular ischemic syndrome, are still unclear, and it is crucial to establish the standard model for evaluating novel neuroprotective drugs targeting ocular ischemic injury and for elucidating the pathologic mechanisms of ocular ischemic diseases.

To confirm the establishment of the model, we must evaluate the protective effects of edaravone, a free radical scavenger, that reduce retinal ischemic damage.
ger, which is used in stroke patients and has some protective effects without affecting the circulation in various animal models. Hence, the present study investigated how the murine PPA and ECA ligation model induces changes in the retinal circulation and whether a free radical scavenger prevents retinal damage.

**METHODS**

**Animals**

Experiments were performed on 8- to 10-week-old male ddY mice (Japan SLC, Inc., Hamamatsu, Japan). All investigations were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

**Ocular Ischemic Model in Mice**

Retinal ischemia was induced as previously described. Anesthesia was induced by 2.0% to 3.0% isoflurane and was maintained using 1.0% to 1.5% isoflurane (both in 70% N2O/30% O2) by means of an animal anesthesia machine (Soft Lander; Sin-Ei Industry Co. Ltd., Saitama, Japan). Body temperature was maintained at 37.0°C to 37.5°C with the aid of a temperature control system N8-TC 10; Neuroscience Inc., Tokyo, Japan). Briefly, after a midline skin incision, the left common carotid artery was exposed, and the ECA was ligated. The internal carotid artery and its first branch were dissected, then the PPA was ligated. Ischemia was maintained for 3 hours, 5 hours, or 5 days, and then ligatures were removed. At 24 hours (3-hour ischemia and 21-hour reperfusion, or 5-hour ischemia and 19-hour reperfusion) or 5 days after the beginning of ischemia, each eye was removed for histologic analyses.

**Laser Speckle Blood Flow Imaging**

Changes in ocular blood flow were monitored using a laser speckle blood flow imaging system (Omega Zone; Omegawave Inc., Tokyo, Japan). Laser speckle imaging can measure ocular blood flow, including retina and choroidal vessel flow, but is not specific to only the retinal circulation. Animals were placed in the prone position, the ipsilateral eye was exposed under anesthesia using 2.0% to 3.0% isoflurane, and body temperature was maintained at 36°C to 37°C. The pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Santen Pharmaceutical Co., Ltd, Osaka, Japan). The eye was diffusely illuminated by semiconductor laser (780 nm), and scattered light was filtered using a hybrid filter and detected by a CCD camera positioned above the head. The hybrid filter was used to detect only scattered light that had perpendicular polarization to the incident laser light so that stable and specific measurements were achieved. The raw speckle images were recorded by video capture and were used to compute speckle contrast, which corresponds to the number and velocity of moving red blood cells. A color-coded blood flow image was obtained with the high-resolution mode (638 × 480 pixels; one image). One blood flow image was generated by averaging numbers obtained from 20 consecutive raw speckle images. To avoid influence on laser scattering at the margin of a pupil, blood flow in a region of interest at the center (approximately 0.5 × 0.5 mm) of a pupil was obtained by using the software installed in a laser speckle blood flow imaging system (Omega Zone; Omegawave); seven images acquired at each time point were averaged as the ocular blood flow. The color map images showed relative blood flow, and the color-coded bar shown vertically at the right end in Figure 1B was defined by an arbitrary unit between 6 and 34. It is known that laser speckle values are not zero, even when there is no blood flow. Therefore, we measured the ocular blood flow of four dead mice as offset, and its average (4.55) was subtracted from each laser speckle value (Figs. 1C, 1D). Ocular blood flow was measured before ischemia, at 5 minutes, 30 minutes, 1 hour, 3 hours, and 5 hours after ischemia, and at 30 minutes and 5 days after reperfusion.

**Ocular Fundus Photography**

Ocular fundus images were obtained using a retinal imaging microscope (Micron III; Phoenix Research Laboratories Inc., Pleasanton, CA). Ocular fundus photography with this microscope is a simple, noninvasive technique for retinal imaging that can be used to evaluate the adaptive changes in retinal vascularization. Three microliters of ophthalmic solution containing 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydrin-P; Santen Pharmaceutical Co. Ltd, Osaka, Japan) was applied topically 10 minutes before anesthesia to dilate the pupil, and then mice were anesthetized using 2.0% to 3.0% isoflurane. A few minutes later, hydroxyethyl cellulose gel (Scopisol; Senju Pharmaceutical Co. Ltd., Osaka, Japan) was applied topically to prevent desiccation and to keep the surface smooth. Fundus images were captured before and at 5 minutes, 5 hours, and 5 days after ischemia and at 5 minutes and 5 days after reperfusion of the left eye (ipsilateral to lesion). To evaluate the changes in retinal vessels after ischemia, we binarized fundus images and set the threshold for defining the outline of vessel walls. Then we chose three major arterial and venous vessels, respectively, from each binarized image (1024 × 768 pixels), and measured the vessel diameter at the point of 100 pixels from the optic nerve. Measurement of vessel diameter was performed using the straight tool of ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Normal artery and venous diameters measured approximately 11 and 15 pixels. These measurements were conducted in a masked fashion by a researcher. At each time point, we took the fundus images from three or five mice, and data were averaged from them.

**FITC-Dextran Angiography**

Flat-mounted retinas were visualized according to our previous report. Mice were deeply anesthetized intraperitoneally with sodium pentobarbital (Nacarai Tesque, Inc, Kyoto, Japan) at 30 mg/kg. Through a median sternotomy, they were perfused through the left ventricle with fluorescein-conjugated dextran (Sigma-Aldrich, St. Louis, MO) dissolved in PBS. For each animal, both the ischemic left eye and the contralateral right eye were then enucleated and placed in 4% paraformaldehyde. Under a microscope, the cornea and lens were removed from each eye, and the retinas were dissected, flat-mounted, and covered with a coverslip after a few drops of aqueous mounting medium (Fluoromount; Diagnostic BioSystems, Pleasanton, CA) for fluorescent staining had been placed on the slide. To quantify the retinal blood supply, we evaluated the fluorescence intensity of FITC dextran, which was perfused in flat-mounted retinas. The images of flat-mounted retinas were taken by using Metamorph (Universal Imaging Corp., Downingtown, PA). The right eyes served as controls.

**Electroretinogram Recording**

Electroretinogram (ERG) recordings were performed according to our previous report. ERG was recorded at 5 days after the ischemic surgery. Scotopic ERG was used to evaluate retinal function in the animals. The a-wave provides information about photoreceptor function, whereas the b-wave provides information about the functional activity of Müller cells, bipolar cells, or both. The oscillatory potentials (OPs), which originate from inner retinal neurons, including the amacrine and ganglion cells, were also evaluated. At our preliminary study, it was confirmed that there was no difference in the clarity of the cornea or lens between unilateral PPA and ECA ligation mice and sham-operated mice. Mice were maintained in a completely dark room for 24 hours. They were anesthetized intraperitoneally 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 Pharmaceutical Co., Ltd.). Flash ERG was recorded in the left eyes of the dark-adapted mice by placing a golden-ring electrode (Mayo, Aichi, Japan) 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 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. OP amplitudes were measured in the time domain between the a-wave peak and the major b-wave peak.

Histology

Mice were euthanized after ERG recordings. Left eyes were enucleated and kept immersed for at least 24 hours at 4°C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (thickness, 4 μm) were cut parallel with the maximum circle of the eyeball through the optic disc. When we extracted the eyeball, we checked the top of the dorsal point (using the optic axis as the landmark) and stained it with hematoxylin and eosin. The damage induced by retinal ischemia was evaluated as described; two sections from each eye were used for the morphometric analysis. Light microscope images were photographed, and the cell counts in the ganglion cell layer (GCL) between 375 and 625 μm from the optic disc (nasal and temporal portions) and the thickness of the inner plexiform layer (IPL) and inner nuclear layer (INL) were measured at three points per section on the photographs; values were then averaged. Data from two sections were averaged for each eye and were used to evaluate the cell count in the GCL and the thickness of the IPL and the INL.

Terminal Deoxynucleotidyl Transferase UTP Nick End Labeling Staining

Terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) was performed according to the manufacturer’s instructions (In Situ Cell Death Detection Kit; Roche Diagnostics, Mannheim, Germany) to detect retinal cell death. The mice were euthanized, and the eyes were enucleated, kept immersed for at least 24 hours at 4°C in a fixative solution containing 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 (optimum cutting temperature compound; Tissue-Tek; Miles Laboratories, Naperville, IL). Retinal sections were cut at 10-μm thickness with a cryostat at −25°C and were stored at −80°C until staining. After two washes with PBS, sections were incubated with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 hour. Light microscope images were photographed, and the labels cell counts in the GCL, INL, and ONL 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 was plotted as the number of TUNEL-positive cells.
Effect of Edaravone

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; Sigma-Aldrich) was dissolved in saline. The edaravone, or an identical volume of saline, was administered intraperitoneally at a dose of 1 mg/kg just before ischemia induction, immediately after reperfusion, and twice daily the following 4 days. In addition, we examined the effects of edaravone on apoptosis using TUNEL staining. Saline-dissolved edaravone, or an identical volume of edaravone, was administered intraperitoneally at the same dose, just before 5-hour ischemia induction, and immediately after reperfusion. Left eyes were euthanized after 19 hours of reperfusion.

Statistical Analysis

Data are presented as mean ± SEM. Statistical comparisons were made by one-way ANOVA followed by Tukey’s test (Fig. 1C), t-test (Figs. 1D, 2–5, 7), or Mann-Whitney U test (Figs. 6, 8). Statistical software (StatView, version 5.0; SAS Institute Inc., Cary, NC) was used. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

RESULTS


Temporal changes in ocular blood flow of the ischemic retina were examined (Figs. 1A–D). Ocular blood flow was reduced to 39% and 61% at 5 minutes and 5 hours after occlusion, respectively, compared with normal levels (Figs. 1A, 1C). Five days after reperfusion following 5-hour ischemia, ocular blood flow increased to 87% of the control values (Figs. 1A, 1C). However, in a permanent occlusion group, the blood flow, which reduced remarkably at 5 minutes after occlusion, showed incomplete recovery (50% of control) at 5 days after reperfusion (Figs. 1B, 1D).

Fundus Photography of Ischemic Retinas

At 5 minutes after ligation of both the PPA and the ECA, blood vessels were significantly narrower and the fundus was clearly paler than those of nontreated mice (Fig. 2A). After 5 hours of ischemia, blood perfusion was still obstructed, and arterial blood flow was barely observed (Fig. 2A). Five days after ischemia, the blood vessels were still narrower than those of the control (Fig. 2A). After either 5 minutes or 5 days of reperfusion following 5-hour ischemia, blood perfusion could not be distinguished from that of normal mice (Fig. 2A). Figure 2B shows quantitative analysis of the changes in the diameters of both the arteries and veins in experimental mice. Both were significantly narrower at 5 minutes and 5 hours after ischemia than in controls (P < 0.01), and they were normalized for time (Fig. 2B). There was variability between the mice with respect to venous vessel diameter at 5 minutes after reperfusion following 5 hours of ischemia (Fig. 2B).

Fluorescein Isothiocyanate-Dextran Angiography

We compared the blood supply among the retinas in both the PPA and the ECA ligation models, other models (in which the ECA, common carotid artery [CCA], or PPA was ligated individually), and the nontreated group (Fig. 3A). There was complete perfusion of FITC-dextran in the main retinal artery, vein, and capillary vessels in all contralateral and control retinas (Fig. 3A).
3B). In contrast, animals with either a ligated ECA, CCA, or PPA (which may supply anterograde blood flow to the eye) showed relatively low intensity but not complete obstruction of eye vascularization and blood flow (Fig. 3B). After 2 hours of ligation of both the PPA and the ECA, no FITC intensity could be observed in the ischemic eye (Figs. 3B, 3C). Five days after ligation of both the PPA and the ECA, mild blood flow recovery was observed in 1 of the 4 investigated mice, whereas the other 3 retinas revealed scant levels of FITC intensity (Figs. 3B, 3C).

**Functional Analysis Using Electroretinography**

In the nontreated group, the amplitudes of both the a- and b-waves increased in a light intensity-dependent manner. In contrast, both waves were significantly attenuated in the group that had 5 days of permanent ischemia and the groups that had 5 days of reperfusion following either 3 or 5 hours of ischemia (Figs. 4B, 4C). The mean amplitude of isolated OPs, especially OP3 and OP4 (Fig. 4E), and the sum of all isolated OPs (Fig. 4F) were significantly reduced in an ischemic time-dependent manner.

**Histologic Damage in the Inner Retinal Layer**

Three and 5 hours of ischemia after 5 days’ reperfusion and 5 days’ permanent ischemia induced significant neuronal losses in the GCL and INL (Figs. 5A, 5B, 5D). IPL thickness was significantly decreased in mice with 5 days of reperfu-
Following 5 hours of ischemia and those with 5 days of permanent ischemia (Figs. 5A, 5C). In our preliminary study, we demonstrated that ligation of the PPA alone reduced the number of RGCs to 84.5% ± 3.2% (n = 8), thickness of the IPL to 88.2% ± 7.0% (n = 8), and thickness of the INL to 91.3% ± 5.7% (n = 8), all with respect to control mice, and these changes were not significant. Furthermore, there were no significant differences in ERG data between the PPA individual ligation group and the control group (data not shown).

### Apoptosis in Ischemic Reperfusion Retinas

In nontreated control retinas, TUNEL-positive cells could not be found in the GCL, INL, or ONL (Figs. 6A–D). On the other hand, in both the 3-hour and the 5-hour ischemia groups there were significant increases in the number of TUNEL-positive nuclei in the GCL, INL, and ONL compared with control (Figs. 6B–D).

### Assessment of Brain Injury

To investigate whether the ligation of PPA or ECA has an effect on the brain, we sampled the brains of PPA and ECA ligation model mice and performed histologic analyses (Supplementary Methods, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8160/-/DCSupplemental). Supplementary Figure S1A (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8160/-/DCSupplemental) shows a representative brain section. PPA and ECA ligation did not induce brain infarction or edema. Quantitative data (Supplementary Fig. S1B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8160/-/DCSupplemental) also indicate there was no histologic damage in the brain tissues (cerebral cortex, hippocampal CA1,
and striatum) at 5 days after PPA and ECA ligation compared with control brain.

Effects of Edaravone on Retinal Ischemic Damage

Ischemia was induced by 5-hour ligation of the PPA and ECA after 5 days of reperfusion. The B-wave in a vehicle-treated group with ischemia/reperfusion was significantly reduced compared with a control group without ischemia/reperfusion. The edaravone-treated group showed significantly less reduction in b-wave amplitudes compared with the vehicle-treated group (Figs. 7A, 7B). On the other hand, the edaravone-treated group did not affect the reduction in a-wave amplitude compared with the vehicle-treated group. Figure 7C shows representative retinal images at 5 days after ischemia/reperfusion.

![Figure 5](image_url)

**Figure 5.** Morphologic analysis of ischemic retinas in model mice. (A) Representative photomicrographs of hematoxylin and eosin-stained retinal sections; control, 5 hours of ischemia and 5 days of reperfusion, and permanent ischemia. (B) Number of retinal ganglion cells. Measurements of (C) IPL and (D) INL thicknesses. Data are presented as mean ± SE (n = 9–11). *P < 0.05, **P < 0.01 versus control (t-test). Scale bar, 50 μm. 3h-I, 3 h of ischemia and 5 days of reperfusion; 5h-I, 5 h of ischemia and 5 days of reperfusion; Permanent, 5 days of permanent ischemia.

![Figure 6](image_url)

**Figure 6.** Quantitative analysis of the number of TUNEL-positive cells in retinal ischemic mice. (A) Representative photomicrographs of TUNEL-stained retinal sections. In the 3-hour ischemia followed by 21-hour reperfusion retina and the 5-hour ischemia and 19-hour reperfusion retina, there was a marked increase in the number of TUNEL-positive nuclei in the GCL (B), INL (C), and ONL (D) compared with each control. Scale bar, 50 μm. *P < 0.05, **P < 0.01 versus control (Mann-Whitney U test). 3 h/I/21 h, 3-hour ischemia and 21-hour reperfusion; 5 h/I/19 h, 5-hour ischemia and 19-hour reperfusion.
Edaravone significantly suppressed the reduction in cell number of the GCL and the thickness of the IPL and INL induced by retinal ischemia (Fig. 7D). In addition, we investigated the effects of edaravone against apoptosis (Fig. 8). Edaravone significantly decreased TUNEL-positive cells induced by 5 hours of PPA and ECA ligation (Figs. 8A–D). However, edaravone had no effects against retinal vessel diameters (Fig. 8E).

**DISCUSSION**

The present study characterized retinal damage and functional deficits in a retinal ischemic animal model subsequent to ligation of both the PPA and the ECA. Histologic changes in the retina at different time points following permanent ischemia or ischemia and reperfusion were also characterized. We found that combined ligation of both the PPA and the ECA induced histologic damage and a functional deficit in the retina but did not affect the brain, implying that retinal cell death in this model was partially induced by the apoptotic pathway.

Laser speckle imaging is a simple and suitable method to image blood flow in vivo. In the reperfusion group, ocular blood flow greatly decreased at 5 minutes after ligation and was restored up to control levels after 5 days. On the other hand, in the permanent ischemia group, blood flow was not...
completely restored even under ligation. Fundus camera imaging supported these data of laser speckle imaging. Under ligation, vasoconstriction was observed using retinal imaging. At 5 days permanent ligation of the PPA and ECA, slight restoration of retinal vasoconstriction was observed, but the fundus image still appeared pale.

Flat-mounted retinas revealed successful interruption of retinal blood supply after PPA and ECA ligation, consistent with the results of other studies. In FITC angiography, ligation of both the PPA and the ECA almost completely interrupted the retinal blood supply, but on laser speckle imaging measurement, approximately 50% of blood flow was apparent. In the present study, we assessed for presence of blood supply to the retina at each time point after vessel ligation using FITC angiography and, therefore, they could not reflect accurately retinal blood flow. Furthermore, FITC angiography was carried out under open-chest; therefore, it remains possible that perfusion pressure to the eye could be aggravated, to some extent, compared to the normal intact animals. Accordingly, it may be augmented compared with the results of ocular circulation or vessel diameter. Thus, these findings strongly suggest that retinal ischemia created using this model is not an isolated type of retinal ischemia but is ischemia of ocular tissues supplied by the ophthalmic artery.

In ERG analysis, we detected the reduction of the a- and b-waves amplitudes in the ischemic group, suggesting the functional deficit of photoreceptors and bipolar and/or Müller cells in the inner retinal layer. In addition, the present result of the marked reduction of OPs especially indicates the dysfunction of amacrine cells, which are interneurons in the retina.

Actually, we detected histologic cell damage in the inner retinal layers in PPA and ECA ligation–induced retinal ischemic mice. In the 5-day permanent model of ischemia, the retinal histologic insult was milder than expected considering the results of ERG analysis. Thus, the retinal damage in this model was mild compared with our previous data and other acute ischemic models, such as those induced by high IOP, optic nerve ligation, and occlusion of the central retinal artery.

**FIGURE 8.** Effects of edaravone against apoptotic cell death after retinal ischemic injury in mice. (A) Representative photomicrographs of TUNEL-stained retinal sections, vehicle-treated retina, and edaravone (1 mg/kg, administered intraperitoneally)–treated retina. Scale bar, 50 μm. (B–D) Edaravone significantly reduced the number of TUNEL-positive nuclei in the INL, induced by retinal ischemic surgery. Data are presented at mean ± SE (n = 7–9). (E) Edaravone did not effects against retinal vessel diameter. Arterial and venous diameters were measured by the same method stated in the Figure 2 legend. *P < 0.05, **P < 0.01 versus control; #P < 0.05 versus vehicle (Mann-Whitney U test).
using rose bengal.10 Taken together, these findings indicate that the present model induced mild retinal dysfunction similar to the ocular ischemic syndrome caused by carotid artery disease.

In the transient retinal ischemic animal models, apoptosis was observed in the GCL, INL, and ONL of the retina.8,10 In the present study, we detected TUNEL-positive cells in the GCL, INL, and ONL in the ischemic retina, suggesting that neuronal cell death in this model and the other retinal ischemic models might be a result of the apoptotic pathway. Although we examined whether the ligation of both the PPA and the ECA can induce brain cerebral infarction, no histologic damage was observed.

MCAO has been reported to induce retinal ischemia and has been used as a model for mild retinal ischemia.9,10 The primary drawback of the MCAO model is that it may produce cerebral infarction and it is difficult to distinguish between brain ischemia and retinal ischemia. According to our previous work, there are strain-related differences in brain vascular anatomy and in the susceptibility to brain damage after transient forebrain ischemia.22 Therefore, it is implied that the vascular anatomy that supplies blood to the eyes differs among different mouse strains. Our preliminary study revealed that there is no strain difference between C57/BL6 and ddY mice in retinal susceptibility (number of cells in the GCL and thickness of the INL) against ischemia with 3-hour ligation of both PPA and ECA and 5 days’ reperfusion (data not shown). However, work with other strains of mice may lead to different results and outcomes. Therefore, future studies may have to investigate the differences among the different mouse strains with respect to their susceptibility to retinal ischemia.

Edaravone, a potent free radical scavenger, has been used clinically in Japan since 2001 for the treatment of acute brain infarction,12 but there is no evidence of neuroprotective effect in ocular ischemic syndrome. On the other hand, a large number of patients with ocular ischemic syndrome present with a history of previous stroke or transient ischemic attack.7,12,24 Severe carotid artery stenosis or occlusion associated with atherosclerosis is a common cause of ocular ischemic syndrome and stroke. Thus, prolonged disturbance in the blood supply to the brain or retina commonly induces irreversible neuronal death. Furthermore, we have reported that edaravone can protect against brain infarction in a mouse model of cerebral ischemia25 and retinal cell death in a mouse model of excitatory amino acid–induced retinal injury.20 Mizuno et al.27 have reported that thrombotic occlusion of rat middle cerebral artery increases hydroxyl radical at the ischemic border zone, and its production is attenuated by edaravone. We investigated the expression of 8-hydroxy-2-deoxyguanosine (8-OHdG), which is produced by hydroxyl radical (data not shown). Five-hour ligation of PPA and ECA markedly increased 8-OHdG-positive cells in the GCL and INL at 7 and 19 hours after reperfusion. These data strongly suggest that oxidative stress is increased in the retina in the ocular ischemia model, as in the stroke model. In the present study we used edaravone for evaluating drugs targeting ocular ischemic diseases without affecting blood flow. The improvement of reduced blood flow using calcium channel blockers or antplatelet agents may be effective in this model, but edaravone did not affect normal vessel diameters of retinal central arteries or veins. Moreover, it has been reported that edaravone does not affect blood pressure, heart rate, or cerebral blood flow.13 In the present study, edaravone displayed significant protective effects against retinal ischemic damage induced by ligation of the PPA and the ECA. On the other hand, edaravone markedly protected the reduction of b-waves, but not a-waves, after ocular ischemia. In the present ocular ischemic model, 5-hour ischemia followed by 5 days of reperfusion strongly reduced the amplitude of the b-wave, but reduction of the a-wave amplitude was mild. These results can be explained by the fact that INL neurons, which reflect b-wave amplitudes, are more susceptible to ischemia than ONL neurons28,29 and that ONL cells, whose function was reflected by the a-wave, are less sensitive to ischemic damage. The present study also indicates that the inner retina was sensitively damaged by ligating PPA and ECA. Because there was a mild reduction of a-wave amplitude after retinal ischemia, the ameliorative effect of edaravone against a-wave amplitudes could not be detected statistically. Taken together, these findings indicate that oxidative stress plays a pivotal role in the mechanism of retinal damage in the retinal ischemia model.

Ocular ischemic syndrome is the constellation of a variable spectrum of ocular signs and symptoms that result from chronic ocular hypoperfusion, usually secondary to severe carotid artery obstruction, and it includes transient monocular blindness (amaurosis fugax), central retinal artery occlusion, or branch retinal artery occlusion.30,31 Severe carotid artery stenosis or occlusion associated with atherosclerosis is a common cause of ocular ischemic syndrome. Thus, prolonged disturbance in the blood supply to the retina commonly induces retinal dysfunction, depending on the extent of ischemia or target vessels. Accordingly, our animal model could reflect some clinical features of ocular ischemic syndrome.

In conclusion, we have demonstrated that ligation of both the PPA and the ECA reduces retinal blood flow and induces retinal damage in the form of cell death because of activation of the apoptotic pathway. These findings indicate that a retinal ischemic animal in which both the PPA and the ECA are ligated might be useful for investigating pathophysiological cell death mechanisms induced by retinal ischemia or for screening potential therapeutic agents.

References

12. Tamaki M, Kidoguchi K, Mizobe T, et al. Carotid artery occlusion and collateral circulation in c57black/6j mice detected by synchro-


