ELSEVIER

LABORATORY INVESTIGATIONS

Autoantibody Against Neuron-Specific Enolase Found in Glaucoma Patients Causes Retinal Dysfunction In Vivo

Ikuyo Maruyama*, Tadao Maeda†, Shigekuni Okisaka‡, Atsushi Mizukawa‡, Mitsuru Nakazawa* and Hiroshi Ohguro*

**Department of Ophthalmology, Hirosaki University School of Medicine, Hirosaki, Aomori Prefecture, Japan; †Department of Ophthalmology, University of Washington, Seattle, WA, USA; ‡Department of Ophthalmology, National Defense Medical College, Tokorozawa, Saitama Prefecture, Japan*

Purpose: In our recent paper, we have reported the presence of serum autoantibody against neuron-specific enolase (NSE) in patients with glaucoma. The purpose of the present study was to investigate further the pathological effects of anti-NSE antibody on retina by comparing them with the effects induced by N-methyl-D-aspartate (NMDA).

Methods: Either a glaucoma patient's serum or purified anti-NSE antibody, or 10–40 mM NMDA was intravitreously administered into Lewis rat eyes, and electrophysiological, histopathological, and biochemical evaluations were performed. In addition, the neuroprotective effects of anti-glaucoma drugs, such as timolol, betaxolol, nipradilol, and isopropyl unoprostone, and a calcium antagonist were also studied using these animal models.

Results: Electron microscopy revealed that intravitreal administration of a glaucoma patient's serum, which immunoreacted with retinal 50 kDa in Western blot analysis, and purified anti-NSE antibody induced retinal ganglion cell apoptosis in rat eyes. Functionally, these eyes showed a significant decrease in electroretinogram (ERG) responses and a remarkable decrease in rhodopsin phosphorylation reaction. These changes were comparable to the effects observed after the intravitreal administration of 20 mM NMDA. Co-administration of nipradilol, an α- and β-blocker, with anti-NSE antibody or 20 mM NMDA caused marked recovery of the affected ERG responses within 2 weeks. In contrast, administration of timolol or betaxolol showed no recovery effect on the ERG responses. Among these drugs, only betaxolol showed a recovery effect on NMDA-induced decrease of rhodopsin phosphorylation. Nilvadipine functioned beneficially on both impaired ERG and rhodopsin phosphorylation reactions observed in rat eyes injected intravitreously with anti-NSE antibody or NMDA. These effects of nilvadipine were not changed by the addition of endothelin-1. In contrast, isopropyl unoprostone had no effect on these functions.

Conclusion: These observations suggest that serum autoantibody against NSE found in some patients with glaucoma induces retinal dysfunction in vivo, similarly to NMDA. **Jpn J Ophthalmol 2002;46:1–12** © 2002 Japanese Ophthalmological Society

Key Words: Apoptosis, autoantibody, glaucoma, retinal ganglion cell.

Introduction

Glaucoma optic neuropathy is characterized by loss of retinal ganglion cells and their axons, an excavated appearance of the optic nerve head, and progressive loss of visual field sensitivity.[1] With regard to the etiology of glaucoma, retinal ganglion cell death by apoptosis has been suggested as a cause based upon histopathological studies of human eyes with primary open angle glaucoma (POAG),[2] neovascular glaucoma,[3] and experimental glaucoma models with elevated intraocular pressure (IOP).[4,5] In terms of the molecular mechanism triggering the apoptosis, several mechanisms, such as deprivation

Received: October 10, 2000

Correspondence and reprint requests to: Hiroshi OHGURO MD, Department of Ophthalmology, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan

Published by Elsevier Science Inc.

0021-5155/02/$–see front matter
PII S0021-5155(01)00453-1

Jpn J Ophthalmol
Vol 46: 1–12, 2002

of neurotrophic factors,[4] ischemia,[6] chronic elevation of glutamate,[7] and disorganized nitric oxide (NO) metabolism[8] have been proposed. Recently Wax et al and his associates reported that autoantibodies against rhodopsin,[9] 60-kDa heat shock protein (hsp 60),[10] 27-kDa heat shock protein (hsp 27), and α-crystallin[11] were identified in serum from glaucoma patients. Therefore, they suggested that these autoimmune reactions toward retinal components may be related to the apoptotic cell death process in some glaucoma patients, particularly in patients with normal tension glaucoma (NTG). We independently have found the presence of the serum autoantibody against neuron-specific enolase (NSE), detected by Western blot analysis in 20 of 79 patients with glaucoma (NTG, 23 cases; POAG, 56 cases). Clinically, maximum IOP in the serum antibody-positive patients was significantly lower than that in the serum antibody-negative patients. However, no statistical differences were observed in visual field loss, disc cupping, or other clinical factors. Injection of a patient's serum or purified anti-NSE antibody into the vitreous cavity of Lewis rats caused slight reduction of the b-wave in electroretinography (ERG) and increase of TUNEL (TdT-dUTP terminal nick-end labeling)-positive staining within the retinal ganglion cells.[12] Therefore, based upon these results, we suggested that the serum autoantibody against NSE might be one of the risk factors for retinal ganglion cell death in glaucoma.

Herein, to study further the effects of anti-NSE antibody on the retina, we injected either anti-NSE antibody-positive patient's serum or purified anti-NSE antibody into the vitreous cavity of the Lewis rat, and performed histopathological, electrophysiological, and biochemical characterizations of the retina. These results were compared with those from an experimental retinal ganglion cell death model in which N-methyl-D-aspartate (NMDA) was administered intravitreously.[13] In addition, using rat models treated with anti-NSE antibody or NMDA, we evaluated the neuroprotective effects of several kinds of β-blocker, including timolol, betaxolol, and nipradilol, and calcium antagonist and prostaglandin derivative, which have been used clinically as anti-glaucoma drugs.

Materials and Methods

The studies were performed in accordance with the guidelines set by Hirosaki University and the Declaration of Helsinki on Biomedical Research Involving Human Subjects. The protocols were approved by the Clinical Research Ethics Board of the University of Hirosaki General Hospital and this institution's Committee for the Protection of Human Subjects. All experimental procedures were designed to conform to both the ARVO statements governing the Use of Animals in Ophthalmic and Vision Research and our own institution's guidelines.

Vitreous Injection of Antibodies

Six-week-old Lewis rats (approximately 180 g) reared in cyclic light conditions (12 hours on/12 hours off) were used. Anesthesia of rats was performed by intramuscular injection with a mixture of ketamine (80–125 mg/kg) and xylazine (9–12 mg/kg) as described by Ohguro et al.[14] To anesthetized rats, a total of 10 μL phosphate-buffered saline (PBS) solution containing anti-NSE antibody (5 μL), 10–40 mM NMDA (5 μL), and/or anti-glaucoma drug (5 μL) (betaxolol, 1 pM–1 nM; nipradilol, 2.5 pM–1 nM; desnitro-nipradilol, 2.5 pM; timolol, 0.1–10 nM) were injected into the vitreous cavity of the eyes as described by Ohguro et al.[14] Two weeks after the treatment, ERG analysis and rhodopsin phosphorylation were carried out as described below. In each experimental condition, ERG was separately measured in 8 eyes and rhodopsin phosphorylation was performed in triplicate. Animals showing apparent traumatic changes following vitreous injection, such as cataract, vitreous hemorrhage, and/or retinal detachment were excluded from the present study. After the treatment, a drop of ofloxacin was administered to avoid infection.

Purified anti-NSE antibody was purchased from UltraClone (Wellow, UK). Betaxolol, nipradilol, and its inactive compound (desnitro-nipradilol) were obtained from Alcon (Ft. Worth, TX, USA) and Kowa (Nagoya), respectively. Timolol was purchased from Sigma (St Louis, MO, USA). Nilvadipine [5-isopropyl 3-methyl 2-cyano-6-methyl-4-(3-nytrophenol)-1, 4-dihydro-3,5-pyridinedicarboxylate] (Fujisawa, Osaka) was dissolved in a mixture of ethanol:polyethylene glycol 400:distilled water (2:1:7) at a concentration of 0.1 mg/mL, diluted twice with physiological saline before use, and injected intraperitoneally (0.5 mL/kg) into anesthetized rats twice a week for 2 weeks. Isopropyl unoprostone or its vehicle solution (Fujisawa) was administered topically into rat eyes twice a day for 2 weeks.

Light and Electron Microscopy

Two weeks after administration of 5 μL of anti-NSE antibody-positive glaucoma patient's serum

into Lewis rat eyes, animals were transcardially perfused under anesthesia with a total of 100 mL of 82 mM sodium phosphate buffer (pH 7.2) containing 4% paraformaldehyde. Enucleated eyes were fixed in 10% glutaraldehyde and 2.5% formalin in 0.15 M phosphate buffer solution (pH 7.2) for several days. The retinochoroidal tissues adjacent to the pupil-optic nerve block at the posterior region were cut in five to seven small pieces, postfixed in 1.0% osmic acid and embedded in epoxy resin. These sections were stained with Azur for light microscopy. Ultrathin sections double-stained with uranium-lead were examined by electron microscopy. After obtaining informed consent, a blood sample was collected from a 74-year-old woman with POAG, and immediately subjected to serum separation. Her detailed clinical data were described in our previous report.[15]

Electroretinography

The amplitudes of a- and b-waves on ERG measurement of rats were determined as described by Ohguro et al.[14] Briefly, the anesthetized animals were kept in dark adaptation for at least 1 hour in an electrically shielded room. The pupils were dilated with drops of 0.5% tropicamide. The scotopic ERG response was recorded with a contact electrode equipped with a suction apparatus to fit on the rat cornea (Kyoto Contact Lens, Kyoto). A grounding electrode was placed on an ear. Responses evoked by white flashes (3.5×10^2 lux, 200 ms duration) were recorded by an ERG recording instrument (Neuropack, MES-3102, Nihon Kohden, Tokyo). The a-wave amplitude was determined from the baseline to the bottom of the a-wave. The b-wave amplitude was determined from the bottom of the a-wave to the top of the b-wave.

Rhodopsin Phosphorylation

Rhodopsin phosphorylation was studied as a retinal photoreceptor function using isolated rod outer segments from rat eyes as described by Ohguro et al[16] with some modifications. Briefly, after dark adaptation of enucleated eyeballs (4–6 eyes for each condition) for 1 hour on ice, retinas were dissected and homogenized in 0.5 mL of 45% sucrose in buffer A (100 mM Na-phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$). After centrifugation at 10,000 *g* for 5 minutes, the supernatant was diluted twice with buffer A and centrifuged again at 10,000 *g* for 5 minutes. The pellet was dissolved in 200 μL of buffer A containing 0.5 mM [γ-^{32}P] ATP (100 cpm/nmol) and incubated at 30°C for 5 minutes under a 100-W lamp from a distance of 10 cm. The reaction was terminated by the addition of buffer B (200 mM Na-phosphate buffer, pH 7.2, containing 5 mM adenosine, 100 mM KF, and 200 mM EDTA) and the mixture

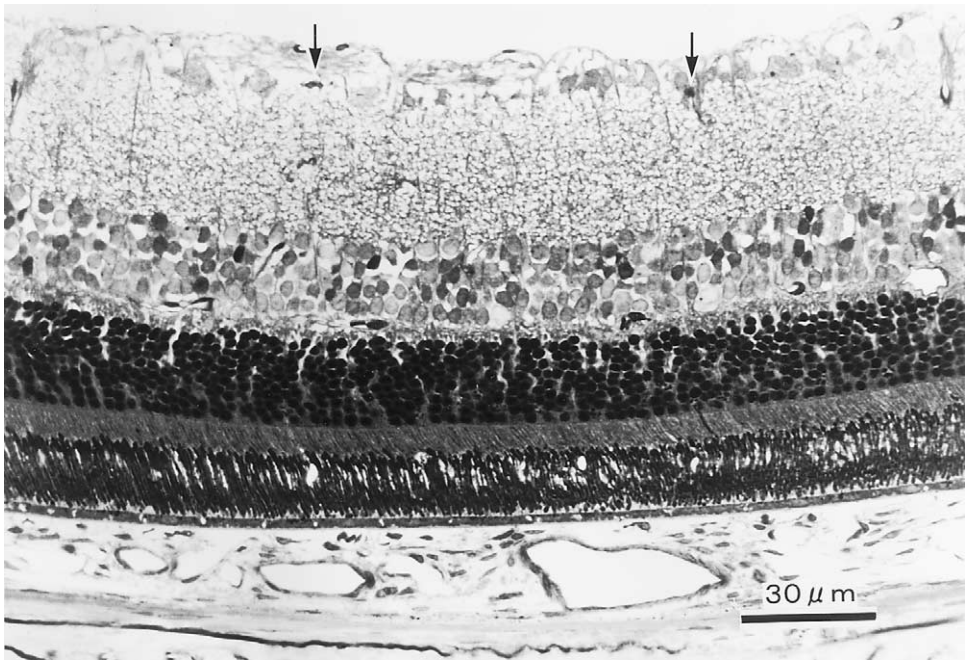

Figure 1. Light micrograph (Azur staining) of rat retina after intravitreal administration of glaucoma patient's serum. Two weeks after administration of 5 μL of anti-neuron-specific enolase (NSE) antibody-positive glaucoma patient's serum into Lewis rat eyes (4 rats, 8 eyes). Pyknosis of retinal ganglion cell (arrows) is seen in ganglion cell layer.

Jpn J Ophthalmol
Vol 46: 1–12, 2002

was centrifuged at 10,000 *g* for 5 minutes. The pellet was dissolved in 50 μL of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed by SDS-PAGE using a 12.5% gel. The gel was stained and de-stained with Coommassie Blue and dried, followed by autoradiography. The band corresponding to rhodopsin was cut out, dissolved with 0.5 mL of H_2O_2, and the radioactivity was counted in a scintillation cocktail.

Statistical Analysis

The data are shown as mean ± SD. *P* values < .05 were considered significantly different from controls as assessed by a two-paired Student *t* test.

Results

Specimens of retina and choroid after intravitreal administration of glaucoma patient's serum or purified anti-NSE antibody showed almost the same findings in light micrographs and electron micrographs. Pyknosis of retinal ganglion cells was found by Azur staining at the posterior region in all eyes (Figures 1 and 3). Electron microscopic examination of the pyknotic retinal ganglion cells showed two types of apoptotic findings. In the first type, the nucleus was malformed and segmented; chromatin was still concentrated in the nucleus. There was less cytoplasm and the organelles were significantly reduced (Figure 2). In the second type, a fragmented nucleus, the apoptotic body, was surrounded by Müller cells and axons (Figure 4).

Next, to study the pathologic effect of anti-NSE antibody on retinal function, its effect on retina was compared with NMDA-treated rat retina. Five microliters of PBS containing purified anti-NSE antibody or 10–40 mM NMDA was injected intravitreously in Lewis rat eyes, and the retinal functions were then evaluated. In ERG responses, eyes injected with NMDA showed a significant decrease in the amplitude of b-waves in a concentration-dependent manner when compared with eyes injected with PBS (Figure 5, upper graph). Amplitude of a-waves showed a greater decrease than that of b-waves, data not shown. In addition, anti-NSE antibody also caused a significant decrease in amplitude of the ERG b-wave, and this effect was almost identical with that of 20 mM NMDA. To further study the effects of NMDA and anti-NSE antibody on retina, rhodopsin phosphorylation, which is known to be the most critical reaction controlling the phototransduction pathway in retinal photoreceptors,[17] was examined as a func-

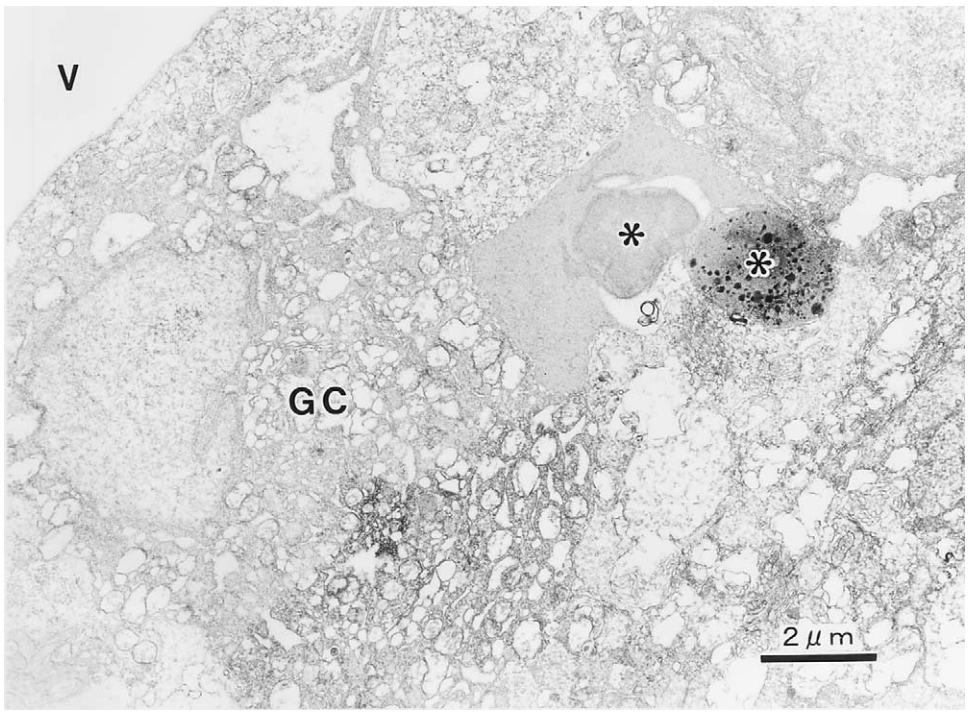

Figure 2. Electron micrograph of inner layer of retina after intravitreal administration of glaucoma patient's serum. Two weeks after administration of 5 μL of anti-NSE antibody-positive glaucoma patient's serum into Lewis rat eyes (4 rats, 8 eyes). Ganglion cell (GC) on left is intact; nucleus (*) of ganglion cell on right is malformed and segmented. V: vitreous.

tion of retinal photoreceptors. The levels of rhodopsin phosphorylation were significantly decreased in NMDA-treated eyes, in a dose-dependent manner. Such inhibition of rhodopsin phosphorylation was also observed in anti-NSE antibody-treated eyes and this effect corresponded to the effect with 20 mM NMDA administration (Figure 5, lower graph). Therefore, these data demonstrate that the effect of anti-NSE antibody on rat retina was functionally identical with that of NMDA.

Using these animal models, we evaluated the neuroprotective effects of anti-glaucoma drugs such as timolol, betaxolol, and nipradilol co-administered with either 20 mM NMDA or anti-NSE antibody, and their effects on the retina were examined. In ERG measurement, retinal dysfunction obtained by 20 mM NMDA or anti-NSE serum was not affected by co-injection with 0.1–10 nM of timolol (Figure 6) or 1 pM–1 nM of betaxolol (Figure 7) during the 2 weeks following injection. In contrast, co-injection with 2.5 pM of nipradilol showed significant recovery of the ERG amplitude ($P < .01$), but this effect was diminished by increasing the concentrations of the drug (0.1–1 nM) (Figure 8). Nipradilol was identified as having an NO effect in addition to α and β blocker function,[18] suggesting that the NO effect may be important for the recovery of ERG responses. To test this hypothesis, a biologically NO-inactive derivative, desnitro-nipradilol, was examined. As shown in Figure 8, desnitro-nipradilol (2.5 pM) had no effect on the ERG responses. Rhodopsin phosphorylation was also examined in terms of its retinal outer segment function. Rhodopsin phosphorylation was not altered by the co-administration of timolol (1 nM) with 20 mM NMDA or anti-NSE serum, while the NMDA- or anti-NSE serum-induced suppression of rhodopsin phosphorylation was slightly enhanced or significantly diminished ($P < .01$) by the co-administration of nipradilol (2.5 pM) or betaxolol (1 nM), respectively (Figure 9).

To evaluate the neuroprotective effects of drugs which increase ocular blood flow, such as calcium antagonists and prostaglandin derivatives, the effects of these drugs on retinal functions were evaluated using rat models intravitreously injected with anti-NSE antibody or 20 mM NMDA. Administration of nilvadipine, a calcium antagonist, caused effective recovery from both ERG and rhodopsin phosphorylation impairment after intravitreal injection of 20 mM NMDA (open columns) or anti-NSE antibody (dotted columns). In contrast, isopropyl unoprostone had no effect on these retinal functions (Figures 10 and 11). Based upon these data, we speculated that the recovery-producing effects of nilvadipine

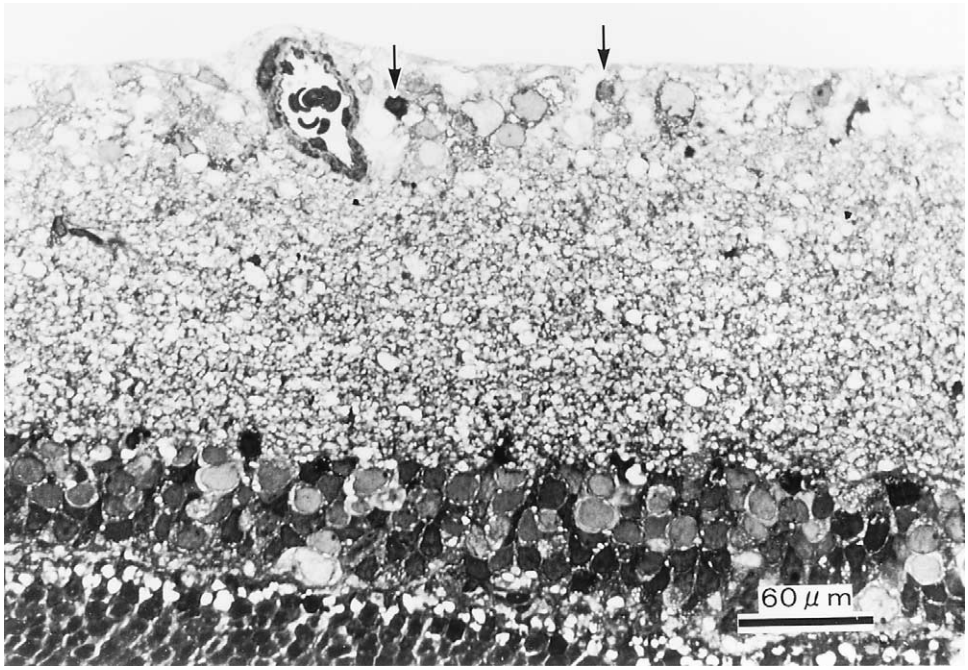

Figure 3. Light micrograph (Azur staining) of retina and choroid after intravitreal administration of anti-NSE antibody. Two weeks after administration of 5 μL of purified anti-NSE antibody into Lewis rat eyes (4 rats, 8 eyes). Pyknosis of retinal ganglion cell (arrows) is seen in ganglion cell layer.

Jpn J Ophthalmol
Vol 46: 1–12, 2002

may not be related to its vasodilative effects because the other drug increasing intraocular blood flow, isopropyl unoprostone, had no effect. To test our hypothesis, 5 μL of 10 mM endothelin-1, which has been known to cause marked constriction of retinal vessels and pallor of the optic nerve head persisting for 10 days after injection,[19] was co-administered with nilvadipine to anti-NSE antibody-treated rats, and their retinal functions were evaluated. As shown in Figures 10 and 11, endothelin-1 had no influence on the recovery producing effect of nilvadipine.

Discussion

In our recent study, we found that 20% of glaucoma patients have the autoantibody toward NSE, and that intravitreal administration of anti-NSE antibody induced a decrease of ERG responses and an increase of TUNEL-positive staining of the retinal ganglion cells in the rat in vivo.[12] Therefore, we suggested that the autoantibody toward NSE may be related to the retinal ganglion cell death found in glaucoma. Here, we confirmed by electron microscopic examination that retinal ganglion cell death was induced by either serum from a glaucoma patient or purified anti-NSE antibody. Functionally, anti-NSE antibody induced a significant reduction in both ERG amplitude and rhodopsin phosphorylation. Interestingly, this anti-NSE antibody-induced retinal dysfunction was comparable to the excitotoxic retinal dysfunction caused by NMDA. In our present data, we found that intravitreal injection of NMDA caused not only impairment of ERG responses but also significant suppression of rhodopsin phosphorylation in a dose-dependent manner. So far, several investigators have claimed that NMDA induced retinal damage, occurring mainly in retinal inner layers, retinal ganglion cell layer and inner nuclear layer, citing the thinning of retinal inner layers and TUNEL positivity in retinal ganglion cells and amacrine cells.[20] However, our present results showing (1) significant suppression of rhodopsin phosphorylation and (2) marked decrease in a- and b-waves of ERG in NMDA-treated retinas, strongly suggested that NMDA may affect both retinal inner and outer layers. This is not surprising since a glutamate-mediated synaptic connection is present between the photoreceptor and bipolar cells.[21] In fact, it is known that both flash and pattern ERG responses were affected in cat eyes with acute IOP elevation,[22] suggesting that damage to retinal outer segments is also involved in such models.

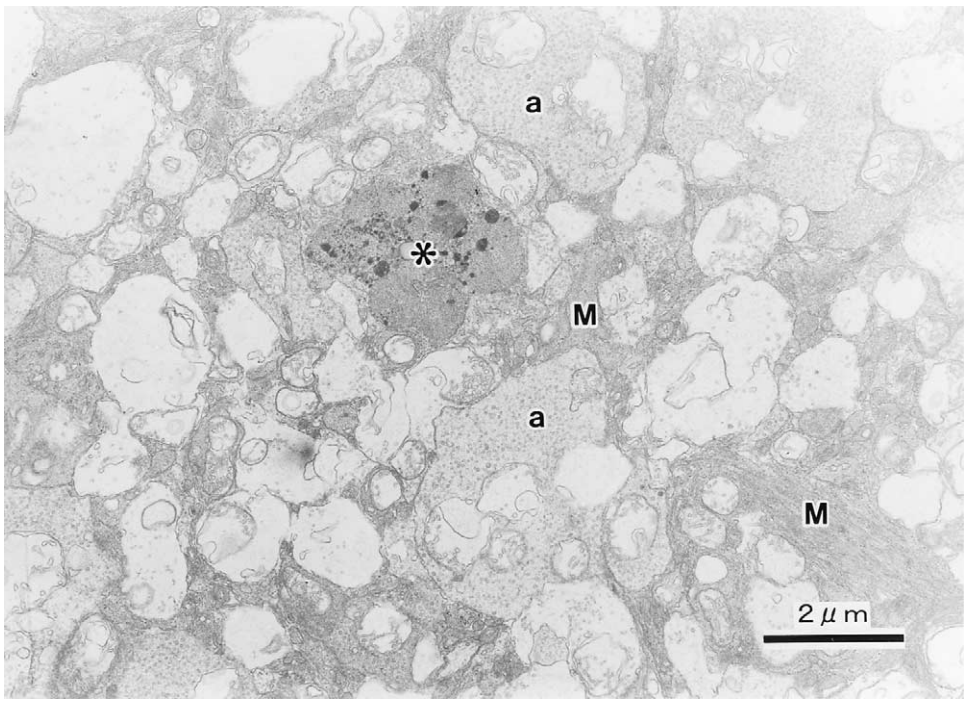

Figure 4. Electron micrograph of ganglion cell layer after intravitreal administration of anti-NSE antibody. Two weeks after administration of 5 μL of purified anti-NSE antibody into Lewis rat eyes (4 rats, 8 eyes). Apoptotic body (*) is surrounded by Müller cells (M) and axons (a).

In contrast with the knowledge we have about the excitotoxic neuronal damage by NMDA, we do not know, at present, the precise molecular mechanisms causing retinal ganglion cell apoptosis by anti-NSE antibody. However, it was suggested that antibody against α-enolase which is a homoenzyme with NSE is found in patients with cancer-associated retinopathy (CAR). CAR is characterized as progressive retinal degeneration similar to retinitis pigmentosa. In CAR, it was suggested that photoreceptor apoptosis is primarily caused by autoimmune responses against several kinds of retinal antigens, including α-enolase, recoverin, and hsc 70.[14] Because enolases are known to be important enzymes regulating glycolysis, we speculated that autoimmune responses toward enolases may block their enzyme activities and cause severe damage to target cells. In fact, in our recent paper,[12] we found that anti-NSE antibody was found not only in glaucoma patients (approximately 20%), but also in normal control subjects (approximately 10%). However, serum from glaucoma patients specifically reacted with the retinal ganglion cell layer, while serum from control subjects did not react with the retinal ganglion cell layer in immunocytochemical studies.[12] Therefore, we suggested that antibodies in glaucoma patients may differ from anti-enolase antibodies found in normal individuals, in their affinity, avidity, specificity, or class.

To explain the molecular mechanism of retinal ganglion cell death in glaucoma, an increase in intra-

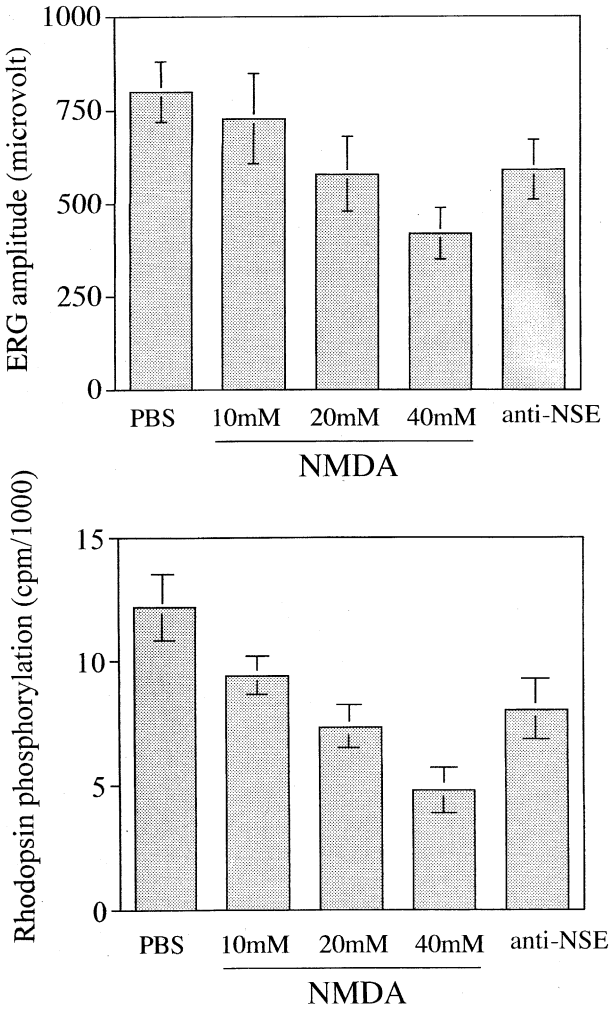

Figure 5. Comparison of electroretinogram (ERG) amplitudes of b-wave and rhodopsin phosphorylation of retinas intravitreously treated by phosphate buffered saline (PBS), or 10–40 mM N-methyl D-aspartate (NMDA) or anti-neuron-specific enolase (NSE) antibody. Two weeks after administration of 5 μL of PBS, 10-40 mM NMDA, or anti-NSE antibody into Lewis rat eyes (4 rats, 8 eyes in each experimental condition), ERG measurement (upper graph) and rhodopsin phosphorylation (lower graph) were performed as described in Materials and Methods. Numbers of rats showing similar changes in ERG waves were 8/8 (PBS), 7/8 (10 mM NMDA), 8/8 (20 mM NMDA), 8/8 (40 mM NMDA), and 7/8 (anti-NSE serum). Data represent mean ± SD. Experiments of rhodopsin phosphorylation were performed in triplicate.

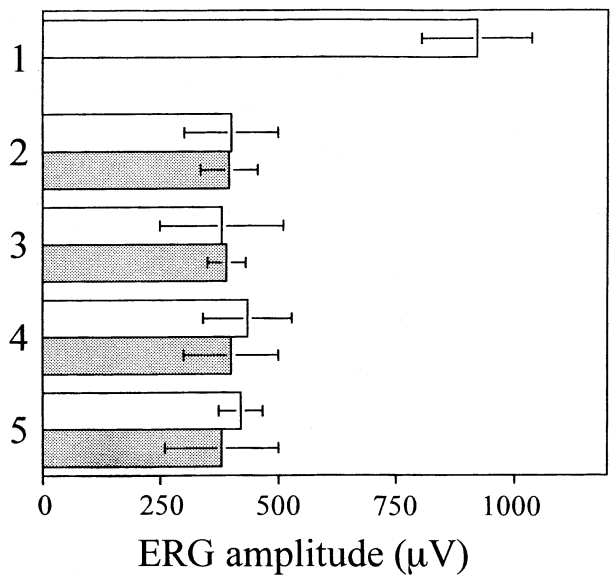

Figure 6. Effects of timolol on ERG amplitudes of b-wave of retinas intravitreously treated by 20 mM NMDA or anti-NSE antibody. Two weeks after administration of 5 μL of PBS, 20 mM NMDA (open column), or anti-NSE antibody (dotted column) in presence or absence of 0.1–10 nM of timolol into Lewis rat eyes (4 rats, 8 eyes in each experimental condition), ERG measurement was performed as described in Materials and Methods. Data represent mean ± SD. (1) PBS only, (2) 20 mM NMDA, or anti-NSE antibody, (3) (2) + 0.1 nM timolol, (4) (2) + 1 nM timolol, (5) (2) + 10 nM timolol.

Jpn J Ophthalmol
Vol 46: 1–12, 2002

cellular Ca^{2+} concentrations resulting from activation of NMDA and kainate/AMPA receptor channels following ischemic changes in retina and optic nerve are thought to be responsible.[23,24] The NMDA receptor activation causes an influx of calcium, $[Ca^{2+}]_i$, through the NMDA receptor channels as well as through voltage-gated calcium channels in retinal ischemia/reperfusion injury models.[23–25] It is known that the kainate/AMPA receptors are generally regarded as low Ca^{2+}-permeable receptors. Therefore, the activation of these receptors is thought to increase $[Ca^{2+}]_i$ mainly via a depolarization-induced opening of voltage-gated permeable calcium channels.[23–25] In addition, activation of other receptors linked to phospholipase C causes a release of calcium from internal stores,[26] and this can theoretically lead to an additional influx of calcium by activation of an unidentified channel in plasma membranes.[27] Based upon the above observations, so-called "neuroprotection" is required for effective therapy in glaucoma patients in addition to decreasing IOP levels. Because it is known that betaxolol reduces voltage-gated calcium entry in ciliary and retinal blood vessels, this compound may act neuroprotectively by reducing calcium influx into neurons.[28,29] Recently, Osborne et al[30] reported that betaxolol is a retinal neuroprotective compound based on the following experimental observations: (1) administration of betaxolol caused significant recovery of ERG b-wave in rat eyes after ischemia/reperfusion; (2) betaxolol remarkably reduced kainite-induced $[Ca^{2+}]_i$ in retinal cultured cells; (3) betaxolol antagonized hypoxyia/reoxygenation effects on the lactose dehydrogenase released from rat cortical cultures and the ischemia-induced effect on the γ-aminobutyric acid immunoreactivity in rabbit retina. In addition, Gross et al[31] reported that betaxolol functioned as a neuroprotective agent for retinal ganglion cells by single-unit extracellular and whole-cell voltage clamp re-

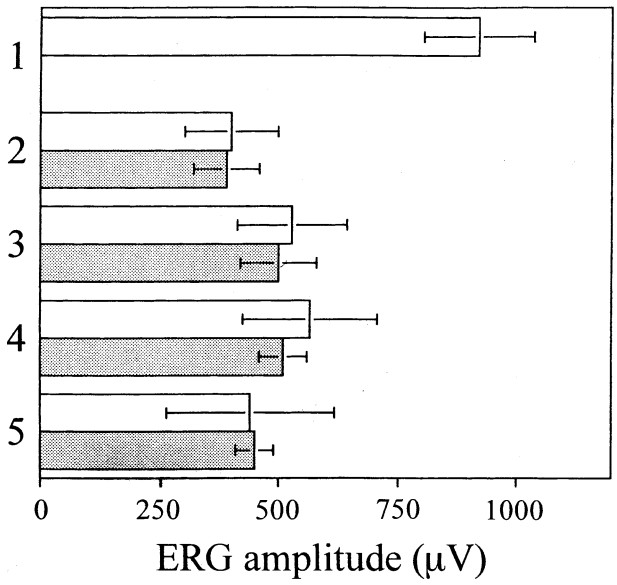

Figure 7. Effects of betaxolol on ERG amplitudes of b-wave of retinas intravitreously treated by 20 mM NMDA or anti-NSE antibody. Two weeks after administration of 5 μL of PBS, 20 mM NMDA (open column) or anti-NSE antibody (dotted column) in presence or absence of 1 pM–1 nM of betaxolol into Lewis rat eyes (4 rats, 8 eyes in each experimental condition), ERG measurement was performed as described in Materials and Methods. Data represent mean ± SD. (1) PBS only, (2) 20 mM NMDA or anti-NSE antibody, (3) (2) + 1 pM betaxolol, (4) (2) + 0.1 nM betaxolol, (5) (2) + 1 nM betaxolol.

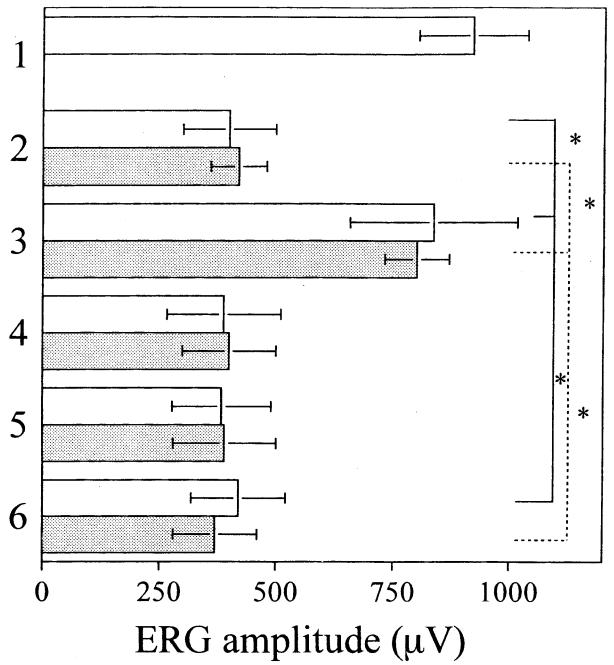

Figure 8. Effects of nipradilol on ERG amplitudes of b-wave of retinas intravitreously treated by 20 mM NMDA or anti-NSE antibody. Two weeks after administration of 5 μL of PBS, 20 mM NMDA (open column) or anti-NSE antibody (dotted column) in presence or absence of 2.5 pM–1 nM of nipradilol or NMDA and 2.5 pM of desnitro-nipradilol into Lewis rat eyes (4 rats, 8 eyes in each experimental condition), ERG measurement was performed as described in Materials and Methods. Data represent mean ± SD. *$P < .01$ (Mann-Whitney test). (1) PBS only, (2) 20 mM NMDA or anti-NSE antibody, (3) (2) + 2.5 pM nipradilol, (4) (2) + 0.1 nM nipradilol, (5) (2) + 1 nM nipradilol, (6) (2) + 2.5 pM desnitro-nipradilol.

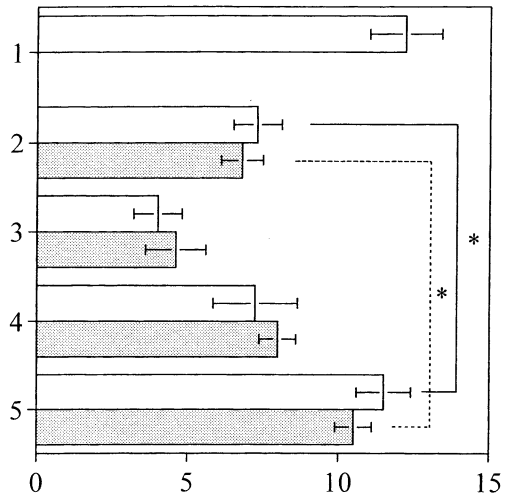

Figure 9. Effects of anti-glaucoma drugs (timolol, betaxolol, or nipradilol) on rhodopsin phosphorylation of rat retinas treated with anti-NSE antibody or 20 mM NMDA. Either 5 μL of PBS, 20 mM NMDA (open column), or anti-NSE antibody (dotted column) in presence or absence of timolol (1 nM), betaxolol (1 nM), or nipradilol (2.5 pM) were administered intravitreously in Lewis rat eyes. Two weeks after administration, rod outer segment was prepared and light-dependent phosphorylation by [γ-^{32}P] ATP was examined as described in Materials and Methods. Experiments were performed in triplicate. *$P <$ .01 (Mann-Whitney test). (1) PBS only, (2) 20 mM NMDA, or anti-NSE antibody, (3) (2) + 2.5 pM nipradilol, (4) (2) + 1 nM timolol, (5) (2) + 1 nM betaxolol.

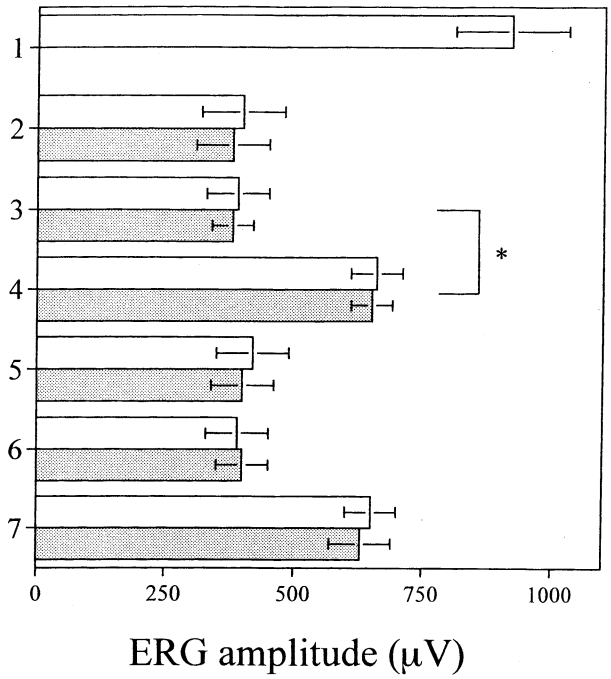

Figure 10. Effects of nilvadipine and isopropyl unoprostone on ERG amplitude of rat retinas treated with anti-NSE antibody. Two weeks after administration of 5 μL of PBS, 5 μL of 20 mM NMDA (open column) or 5 μL of purified anti-NSE antibody (dotted column) in presence or absence of 5 μL of 10 mM ET-1 into Lewis rat eyes. These rats were administered nilvadipine (0.05 mg/kg) or its vehicle solution twice a week intraperitoneally, or isopropyl unoprostone (0.12%) or its vehicle solution twice a day for 2 weeks topically (4 rats, 8 eyes in each experimental condition). ERG measurement was performed as described in Materials and Methods. Data represent mean ± SD. (1) PBS only, (2) 20 mM NMDA or anti-NSE antibody, (3) (2) + nilvadipine control, (4) (2) + nilvadipine, (5) (2) + isopropyl unoprostone control, (6) (2) + isopropyl unoprostone, (7) (2) + nilvadipine + ET-1.

cording methods using tiger salamander retina. Therefore, they suggested that in glaucoma patients betaxolol could act to lower IOP by the action of β-receptors in anterior uvea as well as by giving neuroprotection to retinal ganglion cells. However, our present data revealed betaxolol to be ineffective in remedying the NMDA-induced ERG impairment, whereas it produced significant recovery of rhodopsin phosphorylation, suggesting that it may be more effective in retinal outer layers than in retinal inner layers. In fact, it is known that Ca^{2+} regulation by Ca^{2+}-binding proteins, including recoverin,[32] GCAP,[33] and calmodulin,[34] is pivotal for signal transduction mechanisms in photoreceptors. Thus, it seems a likely possibility that NMDA may cause photoreceptor dysfunction by misregulation of these Ca^{2+}-binding proteins induced by an influx of Ca^{2+} into the photoreceptor cells. If this is indeed the case, our present observations suggest that betaxolol may also function neuroprotectively in photoreceptor cells.

Another neuroprotective mechanism was suggested to be NO serving to protect retinal ganglion cells against glutamate-mediated toxicity.[35] Recently, nipradilol, an α- and β-adrenergic receptor antagonist possessing NO function, which was initially produced as a drug for hypertension, has been used as an anti-glaucoma drug. Therefore, this drug may be expected to be effective not only in decreasing IOP by the action of its α- and β-adrenergic receptor antagonists but also for neuroprotection because of its intrinsic NO function.[36] As expected, our present data showed that nipradilol causes significant recovery of the NMDA-induced ERG impairment at a relatively low dose. However, this effect on ERG was diminished by an increase in the concentration of the drug, and levels of rhodopsin phosphorylation were fur-

Jpn J Ophthalmol
Vol 46: 1–12, 2002

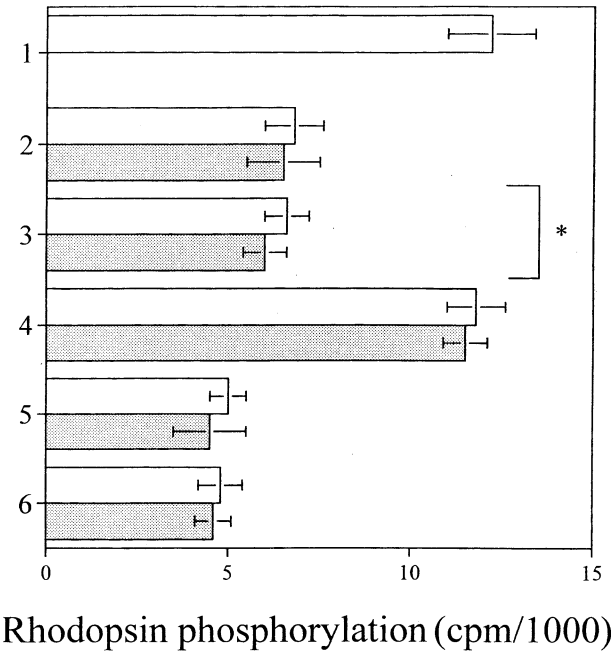

Figure 11. Effects of nilvadipine and isopropyl unoprostone on rhodopsin phosphorylation of rat retinas treated with anti-NSE antibody. Two weeks after administration of 5 μL of PBS, 5 μL of 20 mM NMDA (open column) or 5 μL of purified anti-NSE antibody (dotted column) in presence or absence of 5 μL of 10 mM ET-1 into Lewis rat eyes. Nilvadipine (0.05 mg/kg) or its vehicle solution was administered intraperitoneally to these rats twice a week; or topical isopropyl unoprostone (0.12%) or its vehicle solution was administered twice a day for 2 weeks (4 rats, 8 eyes in each experimental condition). Two weeks after this administration, rod outer segment was prepared and light-dependent phosphorylation by [γ-^{32}P] ATP was examined as described in Materials and Methods. Experiments were performed in triplicate. (1) PBS only, (2) 20 mM NMDA or anti-NSE antibody, (3) (2) + nilvadipine control, (4) (2) + nilvadipine, (5) (2) + isopropyl unoprostone control, (6) (2) + isopropyl unoprostone.

ther decreased by the increase in nipradilol. This is consistent with results demonstrating that increases in NO levels cause retinal ganglion cell and photoreceptor cell loss in in vitro and in vivo studies.[37,38]

Calcium antagonists, which have been widely used in treatment of systemic hypertension, inhibit the entry of calcium ion intracellularly, relax vascular smooth muscle cells, and increase regional blood flow in several organs.[39] It was suggested that some calcium antagonists effectively retarded the progression of visual field defects in some glaucoma patients,[40–43] especially in NTG patients, due to their vasodilating effects on intraocular blood flow. Hirano et al[44] reported that intravenous administration of nicardipine, one of the dihydropyridine calcium antagonists, increased blood flow in the optic nerve head, but not in retinal vessels when measured by laser Doppler flowmetry in the cat. More recently, it was reported that nilvadipine, another dihydropyridine calcium antagonist, with minimum effects on systemic blood pressure in subjects without hypertension,[45] increased vertebrate blood flow more effectively than nifedipine or nicardipine in the dog,[46] and increased blood velocity and blood flow in the optic nerve head as well as in the choroid and retina in the rabbit.[47] In addition, it also increased blood velocity in the optic nerve head of NTG patients.[47] In the present study, administration of nilvadipine caused significant recovery from impairment of ERG and rhodopsin phosphorylation in rat eyes treated with anti-NSE antibody or NMDA. It was reported that clinical oral administration of calcium antagonists produced beneficial effects on glaucomatous visual field loss in some POAG and NTG patients.[48] The mechanism of action of the calcium antagonist is not yet understood, but it may be related to its vasodilating action on vessels within the central nervous system as well as its effect in decreasing intracellular calcium levels,[49] which are known to trigger apoptotic cell death in neurons.

Isopropyl unoprostone, a prostaglandin derivative, is used mainly in Japan as an anti-glaucoma drug. As the mechanism for decreasing IOP, isopropyl unoprostone promotes aqueous outflow and has no effect on aqueous production.[50] In addition, it was suggested that isopropyl unoprostone increased intraocular blood flow.[51]

Therefore, similarly to calcium antagonists, such effects on increase of the intraocular blood flow may function as neuroprotection. However, in the present study, we could not find any effects on ERG or rhodopsin phosphorylation in rat eyes administered anti-NSE antibody or NMDA. In addition, suppression of the nilvadipine-dependent increase in the intraocular blood flow by co-administration of endothelin-1, produced no change in the recovery of rhodopsin phosphorylation and ERG amplitude, suggesting that the decrease of intracellular Ca^{2+} levels may be important for the recovery effect rather than the increase of the intraocular blood flow. Therefore, taken together, the combination of lowering intracellular Ca^{2+} and decreasing IOP may be suitable treatment for glaucomatous optic neuropathy.

In conclusion, our present study demonstrated that anti-NSE antibody found in serum autoantibody in a glaucoma patient caused retinal cell dysfunction similar to NMDA-mediated excitotoxic damage, and that rat models intravitreously administered either anti-

NSE antibody or NMDA may be useful for furthering our understanding of the molecular pathology of glaucoma as well as for evaluating anti-glaucoma drugs.

This work was supported by grants from the Japanese Ministry of Health, Naito Memorial Foundation, Ciba-Geigy Foundation for the Promotion of Science, The Mochida Memorial Foundation for Medical and Pharmaceutical Research, Uehara Memorial Foundation, and JRPS Research Foundation.

References

1. Shields MB, Ritch R, Krupin T. In: Ritch R, Shields MB, Krupin T, eds. The glaucomas. St. Louis: Mosby, 1996:717–25.
2. Kerrigan LA, Zack DJ, Quigley HA, Smith SD, Pease ME. TUNEL-positive ganglion cells in human primary open-angle glaucoma. Arch Ophthalmol 1997;115:1031–5.
3. Okisaka S, Murakami A, Mizukawa A, Ito J. Apoptosis in retinal ganglion cell decrease in human glaucomatous eyes. Jpn J Ophthalmol 1997;41:84–8.
4. Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. Invest Ophthalmol Vis Sci 1995;36:774–86.
5. Garcia-Valenzuela E, Shareef S, Walsh J, Sharma SC. Programmed cell death of retinal ganglion cells during experimental glaucoma. Exp Eye Res 1995;61:33–44.
6. Levin LA, Louhab A. Apoptosis of retinal ganglion cells in anterior ischemic optic neuropathy. Arch Ophthalmol 1996; 114:488–91.
7. Dreyer EB, Zurakowski D, Schumer RA, Podos SM, Lipton SA. Elevated glutamate levels in the vitreous body of humans and monkeys with glaucoma. Arch Ophthalmol 1996;114:299–305.
8. Neufeld AH, Hernandez R, Gonzalez M. Nitric oxide synthase in the human glaucomatous optic nerve head. Arch Ophthalmol 1997;115:497–503.
9. Romano C, Barrett DA, Li Z, Pestronk A. Anti-rhodopsin antibodies in sera from patients with normal-pressure glaucoma. Invest Ophthalmol Vis Sci 1995;36:1968–75.
10. Wax MB, Tezel G, Saito I, et al. Anti-Ro/SS-A positivity and heat shock protein antibodies in patients with normal-pressure glaucoma. Am J Ophthalmol 1998;125:145–57.
11. Tezel G, Seigel GM, Wax MB. Autoantibodies to small heat shock proteins in glaucoma. Invest Ophthalmol Vis Sci 1998; 39:2277–87.
12. Maruyama I, Ohguro H, Ikeda Y. Retinal ganglion cells recognized by serum autoantibody against γ-enolase found in glaucoma patients. Invest Ophthalmol Vis Sci 2000;41:1657–65.
13. Sabel BA, Sautter J, Stoehr T, Siliprandi R. A behavioral model of excitotoxicity: retinal degeneration, loss of vision, and subsequent recovery after intraocular NMDA administration in adult rats. Exp Brain Res 1995;106:93–105.
14. Ohguro H, Ogawa K, Maeda T, Maeda A, Maruyama I. Cancer-associated retinopathy induced by both anti-recoverin and anti-hsc 70 antibodies in vivo. Invest Ophthalmol Vis Sci 1999;40:3160–7.
15. Ikeda Y, Ohguro H, Maruyama I. Two cases of primary open angle glaucoma with serum autoantibody against retinal ganglion cells. Jpn J Ophthalmol 2000;44:648–52.
16. Ohguro H, Hooser JPV, Milam AH, Palczewski K. Rhodopsin phosphorylation and dephosphorylation in vivo. J Biol Chem 1995;270:14259–62.
17. Palczewski K, Sarri JC. Activation and inactivation steps in the visual transduction pathway. Curr Opin Neurobiol 1997; 7:500–4.
18. Uchida Y, Nakamura M, Shimizu S, Shirasawa Y, Fujii M. Vasoactive and β-adrenoreceptor blocking properties of 3,4-dihydro-8-(2-hydroxy-3-isopropylamino) propoxy-3-nitroxy-2H-1-benzopyran (K-351), a new antihypertensive agent. Arch Int Pharmacodyn 1983;262:132–49.
19. Oku H, Sugiyama T, Moriya S, Hamada J, Azuma I. Effects of intravitreal injection of endothelin on the visual system. Nippon Ganka Gakkai Zasshi (Acta Soc Ophthalmol Jpn) 1993;97:467–73.
20. Lam TT, Abler AS, Kwong JMK, Tso MOM. N-methyl-D-aspartate (NMDA)-induced apoptosis in rat retina. Invest Ophthalmol Vis Sci 1999;40:2391–7.
21. Koulen P, Kuhn R, Wassle H, Brandstatter JH. Modulation of the intracellular calcium concentration in photoreceptor terminals by a presynaptic metabotropic glutamate receptor. Proc Natl Acad Sci USA 1999;96:9909–14.
22. Siliprandi R, Bucci MG, Canella R, Carmignoto G. Flash and pattern electroretinograms during and after acute intraocular pressure elevation in cats. Invest Ophthalmol Vis Sci 1988; 29:558–65.
23. Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic ischaemic death. Annu Rev Neurosci 1990;13:171–82.
24. Olney JW. Excitotoxic amino acids and neuropsychiatric disorders. Annu Rev Pharmacol 1990;30:47–71.
25. Choi DW. Calcium-mediated neurotoxicity: relationship to specific channel types and role of ischaemic damage. Trends Neurosci 1988;11:465–69.
26. Duarte CB, Ferreira IL, Santos PF, Oliveira CR, Carvalho AP. Ca^{2+}-dependent release of ^{3}H-GABA in cultured chick retinal cells. Brain Res 1992;591:27–32.
27. Putney JW, Bird GSJ. The signal for capacitive calcium entry. Cell 1993;75:199–201.
28. Hester RK, Chen Z, Becker EJ, McLaughlin M, DeSantis L. The direct vascular relaxing action of betaxolol, carteolol and timolol in porcine long posterior ciliary artery. Surv Ophthalmol 1994;38:S125–34.
29. Hoste AM, Sys SU. The relaxant action of betaxolol on isolated bovine retinal microarteries. Curr Eye Res 1994;13:484–7.
30. Osborne NN, Cazevieille C, Carvalho AL, Larsen AK, DeSantis L. In vivo and in vitro experiments show that betaxolol is a retinal neuroprotective agent. Brain Res 1997;751:113–23.
31. Gross RL, Hensley SH, Gao F, Wu SM. Retinal ganglion cell dysfunction induced by hypoxia and glutamate: potential neuroprotective effects of β-blockers. Surv Ophthalmol 1999; 43:S162–70.
32. Kawamura S. Rhodopsin phosphorylation as a mechanism of cyclic GMP phosphodiesterase regulation by S-modulin. Nature 1991;62:855–7.
33. Palczewski K, Subbaraya I, Gorczyca WA, et al. Molecular cloning and characterization of retinal photoreceptor guanylyl cyclase-activating protein. Neuron 1994;3:395–404.
34. Chen T-Y, Illing M, Molday LL, Hsu Y-T, Yau K-W, Molday RS. Subunit 2 (or b) of retinal rod cGMP-gated cation channel is a component of the 240 kDa channel-associated protein and mediates Ca^{2+}-calmodulin modulation. Proc Natl Acad Sci USA 1994;91:11757–61.
35. Zurakowski D, Vorwerk CK, Gorla M, et al. Nitrate therapy may retard glaucomatous optic neuropathy, perhaps through modulation of glutamate receptors. Vision Res 1998;38:1489–94.
36. Kanno M, Araie M, Tomita K, Sawanobori K. Effects of topi-

cal nipradilol, a β-blocking agent with α-blocking and nitroglycerin-like activities, on aqueous humor dynamics and fundus circulation. Invest Ophthalmol Vis Sci 1998;39: 736–43.

37. Morgan J, Caprioli J, Koseki Y. Nitric oxide mediates excitotoxic and anoxic damage in rat retinal ganglion cells cocultured with astroglia. Arch Ophthalmol 1999;117:1524–9.
38. Oku H, Yamaguchi H, Sugiyama T, Ota M, Azuma I. Retinal toxicity of nitric oxide by administration of a nitric oxide donor in the albino rabbit. Invest Ophthalmol Vis Sci 1997; 38:2540–4.
39. Cohn JN. Calcium, vascular smooth muscle, and calcium entry blockers in hypertension. Ann Intern Med 1983;98:806–9.
40. Kitazawa Y, Shirai H, Go FJ. The effect of Ca^{2+}-antagonist on visual field in low-tension glaucoma. Graefes Arch Clin Exp Ophthalmol 1989;227:408–12.
41. Netland PA, Chaturvedi N, Dreyer EB. Calcium channel blockers in the management of low-tension and open-angle glaucoma. Am J Ophthalmol 1993;115:608–13.
42. Bose S, Piltz JR, Breton ME. Nimodipine, a centrally active calcium antagonist, exerts a beneficial effect on contrast sensitivity in patients with normal-tension glaucoma. Ophthalmology 1995;102:1236–41.
43. Sawada A, Kitazawa Y, Yamamoto T, Okabe I, Ichien K. Prevention of visual field defect progression with brovincamine in eyes with normal-tension glaucoma. Ophthalmology 1996; 103:283–8.
44. Hirano S, Riva CE, Petrig BL. Intravenous nicardipine in cats increases optic nerve head, but not retinal blood flow. Invest Ophthalmol Vis Sci 1992;33:2885–90.
45. Imai J, Omata K, Kamizuki M, et al. Antihypertensive effects of nilvadipine determined by ambulatory blood pressure monitoring. Ther Res 1996;17:1847–56.
46. Ohtsuka M, Ono T, Hiroi J, Esumi K, Kikuchi H, Kumada S. Comparison of the cardiovascular effect of FR34235, a new dihydropyridine, with other calcium antagonists. J Cardiovasc Pharmacol 1983;5:1074–82.
47. Tomita K, Araie M, Tamaki Y, Nagahara M, Sugiyama T. Effects of nilvadipine, a calcium antagonist, on rabbit ocular circulation and optic nerve head circulation in NTG subjects. Invest Ophthalmol Vis Sci 1999;40:1144–51.
48. Yamazaki Y, Hayamizu F, Tanaka C. Effects of systemic nilvadipine on preventing progression of visual field defect in normal-tension glaucoma. Rinsho Ganka (Jpn J Clin Ophthalmol) 1999;53:729–33.
49. Glossmann H, Striessnig J, Ferry DR, Goll A, Moosburger K, Schirmer M. Interaction between calcium channel ligands and calcium channels. Circ Res 1987;61:30–6.
50. Goh Y, Kishino J. Pharmacological characterization of prostaglandin-related ocular hypotensive agents. Jpn J Ophthalmol 1994;38:236–45.
51. Sugiyama T, Azuma I. The effect of topically applied unoprostone on optic nerve head blood flow in circulatory disorder model eyes. Atarashii Ganka (J of Eye) 1997;14:745–8.