Glaucoma

Soluble Nogo-66 Receptor Prevents Synaptic Dysfunction and Rescues Retinal Ganglion Cell Loss in Chronic Glaucoma

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PURPOSE. Myelin inhibitory proteins inhibit axon growth and synaptic function by binding to the Nogo-66 receptor (NgR1) in the central nervous system. Glaucoma is a progressive neuropathy characterized by loss of vision as a result of retinal ganglion cell (RGC) death. Synaptic degeneration is thought to be an early pathologic feature of glaucoma and precedes RGC loss. The authors aimed to examine whether the NgR1 antagonist promotes synaptic recovery and RGC survival in glaucoma.

METHODS. Experimental ocular hypertension model was induced in adult rats with laser coagulation of the episcleral and limbal veins. NgR1 antagonist, soluble NgR1 (sNgR-Fc) was administered to examine their effect on synaptic recovery and RGC survival. Expression of c-Fos, a neuronal connectivity marker, in the retinas was investigated using immunohistochemistry.

RESULTS. NgR1 was expressed in RGCs and upregulated after intraocular pressure elevation. Treatment with sNgR-Fc significantly reduced RGC loss at 2 and 4 weeks after the induction of ocular hypertension and also promoted RGC survival after optic nerve transection. There was no RGC loss at 5 days but there was significant synaptic degeneration as measured by c-Fos. Administration of sNgR-Fc attenuated synaptic degeneration at 5 days, and at 2 and 4 weeks.

CONCLUSIONS. These data suggest that synaptic degeneration may be an initial molecular mechanism for neurodegeneration in glaucoma and appropriate NgR1 antagonism may delay the progression of the disease. (Invest Ophthalmol Vis Sci. 2011; 52:8574–8580) DOI:10.1167/iovs.11-7667

Glaucoma is a neurodegenerative disease characterized by the degeneration of retinal ganglion cells (RGCs) and their axons. Increasing evidence also supports the presence of compartmentalized degeneration in the synapses1–5 and may underlie visual functional deficits in glaucoma. Early changes in RGC dendrites have critical consequences on synaptic efficacy and may underlie functional deficits in glaucoma.6–8 The loss of visual sensitivity in the early stages of experimentally-induced glaucoma could be attributed to a loss of connectivity secondary to synaptic degeneration.9 We previously identified that there is synaptic degeneration for RGCs, which may precede RGC death, in an ocular hypertension model.10 These findings suggest that the synapse destruction, even without the death of RGCs, could contribute significantly to the progressive nature of vision loss in glaucoma. Devising ways to induce such a reversal could represent an effective potential approach to neuroprotection.

The neuronal Nogo-66 receptor (NgR1) mediates the inhibition of axonal regeneration in the presence of three myelin proteins in the central nervous system (CNS) including Nogo-A,8 myelin-associated glycoprotein (MAG),9 and oligodendrocyte myelinating protein (OMgp).10 Much data demonstrates that Nogo-A is expressed by many populations of neurons in the CNS, and is not limited to oligodendrocytes. Nogo protein was detected in the retina of mouse embryos11 and in the neonatal rat.12 We previously found that Nogo-A was expressed in the RGCs and upregulated after the induction of ocular hypertension.13 NgR1 is expressed in the CNS neurons and in the RGCs of the retina.12,14 Soluble NgR1 fusion protein (sNgR-Fc) effectively blocked the myelin molecules’ interaction with NgR1 and promoted axon sprouting and functional recovery after CNS injuries15–17 and dorsal root rhizotomy,18 suggesting its applications in neurorepair and neuron regeneration. Inhibiting the function of NgR1 by RNA interference19 or transfecting the dominant-negative form of NgR20 can stimulate axons in optic nerves to regrow. Knockout of NgR1 is effective for enhancing axonal regeneration after optic nerve crush.21 sNgR-Fc promotes a gradual restoration of synaptic function after dorsal root crush in the rat.22 However, there are no reports about the effect of sNgR-Fc on neuron survival and synaptic function in the retina.

The c-Fos protein was transiently expressed in neurons after synaptic stimulation. c-Fos activation could serve as a func-
tionable tracer to map neuronal pathways with cellular resolution.23–27 The expression of c-Fos protein in immunohistochemically stained RGC indicates the intactness of the neural synaptic pathways from photoreceptors to RGCs.25,26 In this study, we examined whether sNgR-Fc rescues RGC loss and prevents synaptic dysfunction in a rat glaucoma model of ocular hypertension.

**Materials and Methods**

**Preparation of sNgR-Fc Protein**

Purified sNgR-Fc was as described previously.17 This protein comprises a 310 amino acid fragment of rat NgR1 fused to a rat IgG1-Fc fragment (Biogen Idec, Inc., Cambridge, MA). Rat IgG1 (control protein) was purchased from Protos Immunoresearch (San Francisco, CA).

**Animals**

Animal experiments were approved by the University of Hong Kong Faculty of Medicine Committee on the Use of Live Animals in Teaching and Research. The effect of Nogo receptor complex-related antagonists on the survival of retinal ganglion cells in a rat glaucoma model (No. 1120-05). All efforts were made to minimize the number of animals used and their suffering, in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult female Sprague-Dawley (SD) rats (starting weight, 250 to 280 g) were used for the study. A total of 62 rats were used in evaluating RGC loss in the study. A second identical laser treatment was performed 7 days before euthanization.28,29 Both superior and inferior optic nerve were exposed after removing a small piece of skull and dura mater. The vascular integrity of the retinas was verified by fundus examination, and animals whose retinas showed ischemic damage were excluded from the study. The optic nerve was completely transected at 1.5 mm from the optic disc at Day 0, and the left optic nerve at 2 days before euthanization as the control. Surviving RGCs were retrogradely labeled by placing a piece of absorbable gelatin sponge (Gelfoam; Pharmacia & Upjohn) soaked with 6% FG at the ocular stump 2 days before euthanization. Animals were euthanized 7 days after surgery. Six animals were used for each group.

**Drug Treatments**

After the first laser treatment or optic nerve transaction, the right eyes immediately received an intravitreal injection of 2 μg sNgR-Fc in 2 μL phosphate-buffered saline (PBS), using a 26-gauge sterile microsyringe (10 μL, Hamilton #80300; Reno, NV), in the 5-day, 2-week, 4-week ocular hypertension model or 1-week optic nerve transaction model. The injection site was just below the limbus of the cornea to incur minimal injury to the retina. After the injection, 0.5% triclocamide sulfate ophthalmic ointment (Alcon-Kouvre) was smeared around the wound to prevent infection. The protein was administered once a week in the 4-week ocular hypertension group. The rats in the control groups received intravitreal injection of 2 μL PBS or 2 μg control protein (rat IgG1) in 2 μL PBS.

**Quantification of RGCs**

At predefined time, both eyes of each animal were enucleated and fixed in 4% paraformaldehyde (PFA) for 60 minutes after transcardial perfusion with 0.9% saline. Retinas were prepared and mounted on glass slides at the end of the experiments. The data were expressed as the density of cells (number of cells/mm²) and also analyzed in terms of relative percent RGC loss in the injured right eye compared with the contralateral left intact eye from the same animal.

**Immunohistochemistry for c-Fos and NgR1**

To determine whether sNgR-Fc can affect the synaptic input after the induction of ocular hypertension, the c-Fos protein which was transiently expressed in neurons after synaptic stimulation was used as the marker of neuronal connectivity.23–27 The expression of c-Fos protein in immunohistochemically stained RGC indicates the intactness of the neural synaptic pathways. A total of 42 animals were used for c-Fos staining (n = 6 for each group). FG labeling of RGCs was performed 7 days after euthanization. The animals treated with sNgR-Fc or PBS were exposed to 30 minutes of ambient light before euthanization and were euthanized with an overdose of anesthesia at 5 days, and 2 and 4 weeks after the first laser coagulation.28,29 The eyes were enucleated after transcardial perfusion with 0.9% saline and were fixed in 4% PFA for 1 hour. After removing the corneas and lens, the eyecups were fixed further in PFA for 4 hours and then transferred to 30% sucrose solution at 4°C for 16 hours. Ten-micron-thick frozen retinal consecutive sections were cut and placed on different slides. The sections containing the optic nerve stump were selected from a 1 in 5 series for immunostaining of c-Fos protein. After washing in 0.01 M PBS, the retinal sections were incubated in 0.01 M sodium citrate with 0.5% Tween-20 (pH 6.0) at 85°C–90°C for 30 minutes. After cooling to room temperature, the sections were washed and incubated in 0.5% Triton/PBS for 10 minutes, and then blocked with 10% normal goat serum for 1 hour. Incubation with rabbit anti-c-Fos antibody (1:100, CalBioChem, San Diego, CA) was performed at 4°C for 16 hours. Sections then were washed with PBS three times and incubated with AlexaFluor 568.
goat anti-rabbit second antibody (1:400, Molecular Probes, Eugene, OR) at room temperature for 2 hours. The sections were analyzed under fluorescent microscopes.

The eyes were enucleated at 0 and 4 weeks after injury for NgR1 immunohistochemistry. RGCs were retrogradely labeled with FG 7 days before euthanization. Retinal sections were prepared as above except that antigen retrieval was not performed. The sections were incubated with mouse anti-NgR1 (1:50; Biogen Idec, Inc.) and AlexaFluor 568 goat anti-mouse second antibody (1:400, Molecular Probes). After washing, the sections were mounted and analyzed under a confocal microscope (LSM 510 META; Carl Zeiss, Jena, Germany). The sections for NgR1 expression were also processed for immunoperoxidase staining. The sections were incubated with biotin-conjugated secondary antibody (goat anti-mouse; DakoCytonation, Glostrup, Denmark) and visualized with diaminobenzidine (DAB; 0.03% DAB, 0.003% H2O2, pH 7.2). The nuclei were counterstained by hematoxylin. The number of animals for each group is 5.

**Counting of Labeled RGCs and c-Fos Immunoreactivity in Retinal Sections**

The number of total FG-labeled RGCs and RGCs exhibiting colocalization of FG and c-Fos in each retinal section was counted. Three retinal sections from each eye were counted to generate the average value. The number of labeled cells in each retinal section was therefore used as an estimation of the labeled cell density in this study. FG-labeled RGCs were examined using an ultraviolet filter (excitation wavelength = 330–380 nm). Texas red-stained cells were examined using filters with excitation wavelengths of 510–560 nm (red).

**Western Blot Analysis for NgR1**

To measure NgR1 in the retina, the animals were euthanized at 0 and 4 weeks after laser coagulation and treatments with PBS or sNgR-Fc. The retinas were dissected and homogenized in lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1mM EGTA) supplemented
with 10% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail from Sigma (St. Louis, MO). After centrifugation at 13,000 rpm for 30 minutes to remove cell debris, the protein concentration of the supernatant was measured using a protein assay kit (Bio-Rad DC; Bio-Rad Laboratories, Hercules, CA). A 40 to 80 μg aliquot of proteins from each sample was subjected to 6% (for NgR1) or 10% (for synthesis model. ***P < 0.001 compared with PBS and control protein at the same time point. (B) Analyses for the density of RGCs between the right eyes and left eyes in each group (the number of RGCs/mm², mean ± SEM). ***P < 0.001 compared with the left eyes. Control protein: rat IgG1. (Δ)

FIGURE 2. sNgR-Fc rescues RGC loss in glaucoma. (A) Percentage RGC loss post laser coagulation. sNgR-Fc significantly promotes the survival of RGCs at 2 and 4 weeks in the ocular hypertension model. ***P < 0.001 compared with PBS and control protein at the same time point. (B) Analyses for the density of RGCs between the right eyes and left eyes in each group (the number of RGCs/mm², mean ± SEM). ***P < 0.001 compared with the left eyes. Control protein: rat IgG1. (n = 10.)

sNgR-Fc Promotes RGC Survival after the Induction of Ocular Hypertension

To explore the efficacy of sNgR-Fc on RGC survival, we induced an ocular hypertension induced glaucoma model in adult rats. The episcleral and limbic veins on the eye were sealed by two consecutive argon laser treatments (7 days apart) to elevate IOP in the eye. We previously noted that there is synaptic abnormality in the retina on imposing ocular hypertension. 7 We hypothesized that sNgR-Fc would prevent synaptic deafferentation while

RESULTS

NgR1 Expression in the Retina

Previous studies have demonstrated that normal RGCs express NgR1 mRNA in the adult mouse. 30 Here we further examined NgR1 protein expression in normal and injured rat retinas using immunohistochemistry. NgR1 positive staining was visible mainly in the ganglion cell layer (GCL) and the nerve fiber layer (NFL) in the normal retina (Figs. 1A, 1B). Normal RGCs expressed moderate staining for NgR1 but stronger immunoreactivity in the density of labeling for NgR1 occurred in RGCs 4 weeks after laser coagulation (Figs. 1A, 1B). This finding was confirmed by Western blot analysis (Fig. 1C). The increased IOP had a significant effect on NgR1 levels (one-way ANOVA, F(3,16) = 6.82; P < 0.001). NgR1 expression was low in normal retina and increased to 1.8-fold at 4 weeks after the injury (P < 0.05). The expression of NgR1 was similar for sNgR-Fc treatment versus control PBS group.

sNgR-Fc Prevents Synaptic Disconnection in Glaucoma

We previously noted that there is synaptic abnormality in the retina on imposing ocular hypertension. 7 We hypothesized that sNgR-Fc would prevent synaptic deafferentation while...
maintaining the efferent connectivity intact, thus preventing the injured neurons from dying. The c-Fos protein has been established as a neuronal connectivity marker in the retina and was employed in this study to indicate synaptic connectivity.27 The c-Fos was expressed in the nuclei of RGCs retrogradely labeled with FG in the normal retina (Fig. 4A). However, there were fewer c-Fos-positive RGCs after the induction of ocular hypertension (Fig. 4B). The ratio of c-Fos containing RGCs to the number of surviving RGCs was lower at 5 days ($P < 0.05$), 2 weeks ($P < 0.01$), and 4 weeks ($P < 0.01$) post laser-induced injury (PBS group, $n = 6$ for each group; Fig. 5A). The ratio was restored after sNgR-Fc treatment ($P < 0.05$ for 5 days and $P < 0.001$ for 2 and 4 weeks). Similar data were obtained by evaluating the number of c-Fos positive RGCs or total RGCs in the retina (Fig. 5B). Here we found that there was no significant RGC loss at 5 days after the first laser coagulation ($P > 0.05$; Fig. 5B). It suggests that synaptic disconnection before the loss of RGGs can be detected under the stress of ocular hypertension. Some surviving RGCs (FG-labeled) were not positive for c-Fos suggesting that there is continuous synaptic deafferentation under the elevated IOP. These results suggest a synaptotoxic mechanism, similar to that thought to occur in Alzheimer's disease,33 may operate in experimental glaucoma. RGC death may be a secondary event and inhibiting synaptic injury using sNgR-Fc may delay the progression of glaucoma.

**DISCUSSION**

Here we demonstrate that the NgR1 antagonist, soluble Nogo-66 receptor, attenuated synaptic degeneration as measured by c-Fos in the retina and in addition enhanced RGC survival in a rat chronic glaucoma model. The use of sNgR-Fc may be a novel approach for inhibiting synaptic injury and promoting RGC survival in glaucoma.

Glucoma is a common eye disease that can cause irreversible loss of vision if left undiagnosed and untreated. Recent studies showed that in a mouse model of glaucoma, RGCs manifest growing dysfunction via somatic shrinkage, retrograde labeling deficits, and downregulation of RGC-specific genetic programs before death.6 Our data also suggest that RGCs undergo synaptic degeneration before cell loss under ocular hypertension. The synaptic degeneration may play a crucial role in the subsequent RGC death.7 The visual field

![Figure 4](image-url) Expression of c-Fos in the retina after ocular hypertension. (A) c-Fos was expressed in the nuclei and nucleoli of FG-labeled RGCs in the normal retina (yellow arrows). Some c-Fos-positive cells in GCL were not FG-labeled RGCs (white arrows). The pictures in the boxes were enlarged. (B) c-Fos staining in RGCs at 2 weeks after ocular hypertension. Some FG-labeled RGCs did not express c-Fos (arrows). Scale bar, 25 μm.

![Figure 5](image-url) sNgR-Fc prevents synaptic disconnection in glaucoma. (A) Ratio of positive c-Fos RGCs in total FG-labeled RGCs. The ratio was much lower at 5 days ($#P < 0.05$), 2 weeks ($##P < 0.01$) and 4 weeks ($###P < 0.001$) post laser treatment (PBS group) compared with normal group, while the ratio was restored after the treatment of sNgR-Fc ($*P < 0.05$ for 5 day; $###P < 0.001$ for 2 and 4 weeks). (B) The number of total RGCs or c-Fos positive RGCs. (y-axis): the number of total Fluorogold-labeled RGCs or c-Fos positive RGCs in a section containing the optic nerve stump. There was more RGC loss at 2 and 4 weeks after ocular hypertension ($**P < 0.05$) compared with normal group. The data were expressed as mean ± SEM. ($n = 6$.)
changes in glaucoma may be caused by both synaptic degeneration and cell loss. Other than the primary degeneration, the self-perpetuation process of secondary degeneration may occur during which RGC bodies gradually succumb to the noxious environment in glaucoma.14 These results suggest inhibiting synaptic injury may provide a new insight for early intervention in delaying the progression of glaucoma.

NgR1 is associated with axon regeneration and neurite growth in the CNS. We found that there was NgR1 expression in the RGCs and upregulated after the induction of ocular hypertension. Soluble NgR1 protein can inhibit the activity of NgR1 and promotes axon regeneration after spinal cord injury,17,22 stroke,16 or dorsal root rhizotomy.18,22 Inhibiting the function of NgR by RNA interference19 or transfecting the dominant-negative form of NgR120 can stimulate optic nerve axon regrowth. Knockout of NgR1 is effective for enhancing axonal regeneration after optic nerve crush.21 However, there are no reports about the effect of sNgR-Fc on neuron survival and synaptic function. Using a model that mimics the etiology and progression of chronic glaucoma, we found that sNgR-Fc prevented RGC loss associated with an increase of intraocular pressure. These agents prevented RGC loss even over a long-term 4-week course of treatment. Because the RGC loss becomes stable after 4 weeks in this ocular hypertension model,32 the evident neuroprotection of sNgR-Fc for a 4-week course indicates a long-term prevention of RGC damage in this ocular hypertension model. More importantly, we found that there was no significant RGC loss but the number of RGCs expressing c-Fos was significantly lower in the experimental animals at 5 days. However, the treatment of sNgR-Fc promoted synaptic connection at 5 days even up to 4 weeks. It suggests that sNgR-Fc may promote RGC survival by prevention of the synaptic disconnection in the early stage of the RGCs, and inhibiting NgR1 activity can slow the progression of glaucoma by rescuing both the synapses and RGCs. We also tested the efficacy of sNgR-Fc in the extreme conditions of optic nerve transaction. Even in that stringent environment, NgR1 antagonist provided a significant level of RGC protection for up to a week.

A member of NgR1/p75 neurotrophin receptor and NgR1/TROY (an orphan receptor in the tumor necrosis factor TNF family) signaling complexes that prevent axonal regeneration in the presence of three myelin inhibitors is leucine-rich repeat (LRR) and Ig domain-containing Nogo receptor-interacting protein-1 (LINGO-1).35 We previously found that blocking LIN- GO-1 function with a soluble version of the extracellular domain of LINGO-1 or an antibody against LINGO-1 promotes survival of damaged RGCs in a chronic adult rat ocular hypertension model.28 Combination of brain-derived neurotrophic factor (BDNF) with LINGO-1 antagonist can provide long-term protection for RGGs. BDNF receptor, tyrosine kinase B (TrkB) may be the predominant mediator of this neuroprotection.29,31 As a glycosyl phosphatidyl inositol (GPI) anchored protein, NgR1 directly binds the myelin proteins, and then LINGO-1 and/or p75/TROY transduce the signal into the cells.36,37 Whether sNgR-Fc exerted neuroprotection in this study via the interaction with LINGO-1 and/or p75/TROY should be further investigated.

In this study, we found that there was RGC death after the induction of chronic ocular hypertension and that synaptic degeneration precedes RGC death. This would suggest that there may indeed be an opportunity for the rescue of RGCs that are undergoing synaptic alteration, but possibly not yet committed to die. NgR1 antagonism by sNgR-Fc prevents synaptic dysfunction and promotes RGC survival which may represent a novel therapy for treating glaucoma.

References


32. Li RS, Chen BY, Tay DK, Chan HH, Pu ML, So KF. Melanopsin-expressing retinal ganglion cells are more injury-resistant in a chronic ocular hypertension model. *Invest Ophthalmol Vis Sci.* 2006;47:2951–2958.


