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The expression patterns of Nogo-A, myelin associated glycoprotein and oligodendrocyte myelin glycoprotein in the retina after ocular hypertension

Running title: The expression of myelin proteins in the retina in glaucoma

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Abstract

Nogo-A, a major myelin inhibitory protein, inhibits axon growth and synaptic function in the central nervous system. Glaucoma is a progressive neuropathy as a result of retinal ganglion cell (RGC) death. Synaptic degeneration is thought to be an early pathology of neurodegeneration in glaucoma and precedes RGC loss. Here experimental ocular hypertension model was induced in adult rats with laser coagulation of the episcleral and limbal veins. The expression of Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) in the retina was investigated using immunohistochemistry and Western blotting. We found that Nogo-A was expressed in the RGCs and upregulated after the induction of ocular hypertension. OMgp was only expressed in the inner plexiform layer. There was no MAG expression in the retina. Our data provided, for the first time, the expression patterns of three myelin protein in the adult retina and suggested an important role of Nogo-A in the RGC death and synaptic degeneration in glaucoma.

Key words: Ocular hypertension, Nogo-A, Myelin-associated glycoprotein, Oligodendrocyte myelin glycoprotein, Retina
Introduction

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 synapses [1-3] and may underlie visual functional deficits in glaucoma. We previously identified that there is synaptic degeneration for RGCs in an ocular hypertension model of glaucoma and it may precede the RGC death [4]. These findings suggest that the synapse destruction 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 failure of axonal regeneration within the damaged adult central nervous system (CNS) has been ascribed in part to the presence of Nogo-A[5] myelin-associated glycoprotein (MAG) [6] and oligodendrocyte myelin glycoprotein (OMgp) [7,8]. All three bind the Nogo66 receptor (NgR1) [9] and the paired immunoglobulin-like receptor B (PirB) [10] to mediate their inhibitory influence. Although Nogo-A is thought to be the primary source of the Nogo related limitation of neuronal outgrowth after injury [5,11], much data demonstrates that Nogo-A is expressed by many populations of neurons in the CNS, and is not limited to oligodendrocytes [12-15]. Nogo-A expression was increased in the cortical neurons [16] and anti-Nogo-A antibody improves functional recovery in adult rats after stroke [17]. Nogo protein was detected in the retina of mouse embryos [18] and in the neonatal rat [19]. Neuronal Nogo-A may be involved in normal cell-functioning
separate from the post injury outgrowth-inhibitory role observed for oligodendroglial Nogo-A[15]. Nogo-A plays a role in the maintenance of inhibitory synapses in cerebellar Purkinje cells [20] and motor neuron in amyotrophic lateral sclerosis (ALS) [21]. Unlike Nogo-A, MAG is not expressed in neurons but in glia of CNS and peripheral nerves [22]. OMgp was first identified as a myelin protein but has subsequently been found to be expressed on some neurons in the CNS [23]. However, there were no reports for Nogo-A, MAG and OMgp in the retina of adult animals and in glaucoma. In this study, we provide evidence that Nogo-A, but not OMgp and MAG protein increased in the retina in an ocular hypertension model in rats. High levels of Nogo-A may be a possible mechanism of the RGC death and synapse degeneration in the glaucoma.

**Experimental Procedure**

**Animals**

Animal experiments were approved by the University of Hong Kong Faculty Committee on the Use of Live Animals in Teaching and Research. Twenty two adult female Sprague-Dawley (SD) rats (starting weight, 250-280 g) were used for the study. The animals were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) during the experiments and were euthanized at the end of the experiment with an overdose of pentobarbital sodium (150 mg/kg). Alcaine 0.5% (Alcon-Couvreur, Belgium) was applied to the eyes before all operations and antiseptic eye drops (Tobres [Tobramycin 0.3%], Alcon-Couvreur, Belgium) were
used to prevent infection after the surgery. Rimadyl (0.025mg/ml) in drinking water was used as analgesics for 7 days after the surgeries.

**Induction of experimental ocular hypertension**

Argon laser (Ultima 2000SE Argon Laser, Coherent, Palo Alto, CA) was used to coagulate the limbal and episcleral veins in the right eye of the rats. This technique was adopted from the method by WoldeMussie et al [24] and has been adopted in our laboratory for the retina degeneration and therapeutic effects on retina functions [4,25-27]. About 90 spots were applied on the three episcleral veins and 70 spots on the limbal vein (270° around the limbus, except on the nasal side) with the following settings: power of 1000 mW; spot size of 50 µm in diameter; and duration of 0.1s. A secondary laser surgery was performed to coagulate the reconnected blood vessel seven days later. The intraocular pressure (IOP) of the eyes were measured using a Tonopen XL Tonometer. Fluorogold (FG) labeling of RGCs was performed 7 days before sacrifice. Both superior colliculi (SC) were exposed after removing a small piece of skull and cortex, and a piece of Gelfoam (Pharmacia & Upjohn, Kalamozoo, MI, USA) soaked with FG (6% in distilled H₂O, Fluorochrome, Denver, CO, USA) was placed on the surface of SC. Rats were euthanized with an overdose of anesthesia 2 weeks after the first laser exposure for RGC labeling.

**Immunohistochemistry of Nogo-A, MAG, OMgp**

The animals were euthanized with an overdose of anesthesia at 0, 2 and 4 weeks after
the first laser coagulation. The eyes were enucleated following transcardial perfusion with 0.9% saline and were fixed in 4% PFA for 1 h. After removing the corneas and lens, the eye cups were fixed further in PFA for 4 h and then transferred to 30% sucrose solution at 4°C for 16 h. Ten-micron-thick frozen retinal sections were washed in 0.01M phosphate buffered saline (PBS) and were incubated in 0.5% Triton/PBS for 10 min. After being blocked with 10% normal goat serum for 1 h, the sections were incubated with rabbit anti-Nogo-A (1: 100, CalBioChem, CA, USA), mouse anti-MAG (1:100, Chemicon, Hofheim, Germany) and mouse anti-OMgp (1:100, Chemicon, Hofheim, Germany) antibodies at 4°C for 16 h. Sections then were washed with PBS three times and incubated with Alexa Fluor® 488 goat anti-rabbit second antibody (1:400, Molecular probes, Oregon, USA) at room temperature for 2 h. Negative control was performed on the retina sections at 2 weeks after the induction of ocular hypertension with only incubation of Alexa Fluor® 488 goat anti-rabbit second antibody. The positive control for Nogo-A was performed by staining Nogo-A in the optic nerve from normal retina in the adult rat. The sections were analyzed under Olympus IX71 fluorescent microscope with DP2-BSW software (Olympus, Japan). Six animals were used in each group.

**Western Blotting for Nogo-A**

To measure Nogo-A in the retina, the animals were euthanized at 0, 5 days, 2 and 4 weeks after laser coagulation. The retinas were dissected and homogenized in lysis buffer (10 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) supplemented...
with 10% protease inhibitor cocktail and 1% phosphatase inhibitor cocktails from Sigma. Following centrifugation at 13,000 rpm for 30 min to remove cell debris, the protein concentration of the supernatant was measured using a Bio-Rad DC protein Assay Kit (Bio-Rad Laboratories, CA, USA). A 40-80 µg aliquot of proteins from each sample was subjected to 6% (for Nogo-A) or 10% (for β-actin) SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membrane. The membranes were blocked with Odyssey blocking buffer (LI-COR, Inc., Lincoln, NE, USA) for 1 h in room temperature. Incubation with goat anti-Nogo-A (1:100, Santa Cruz Biotechnology, CA, USA) antibody was performed for 16 h at 4ºC. After washing, the membranes were incubated with IRDye 800CW goat anti-mouse secondary antibody (1:2000, LI-COR, Inc., Lincoln, NE, USA) in Odyssey blocking buffer for 1 h at room temperature. Protein loading was controlled using a mouse antibody against anti-actin (1:2000, Chemicon, Hofheim, Germany) and finally incubated with IRDye 800 goat anti-mouse secondary antibody (1:2000, LI-COR, Inc., Lincoln, NE, USA). The membranes were scanned and analyzed using Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE, USA). All experiments for Western blotting were performed with 5 animals in each group and repeated 3 times.

**Statistics**

All data are expressed as Mean ± SEM. Statistical analysis was evaluated by one-way analysis of variance (ANOVA) followed by post-hoc tests (Student-Neuman-Keuls) for comparisons between groups. The mean difference is significant at 0.05 level.
Data were analyzed statistically with the software SPSS 12.0 (SPSS Inc., Chicago, Illinois, USA).

**Results**

**Nogo-A expression in the normal rat retina**

The expression of Nogo-A in the retina was evaluated using immunohistochemistry. Negative control only with second antibody showed no positive staining in the retina at 2 weeks after ocular hypertension (Fig. 1a-b). Nogo-A was reported to express in the oligodendrocytes. Here we found strong immunostaining for Nogo-A in the optic nerve with myelin sheath of normal adult rat (Fig. 1c). Nogo-A positive staining was visible mainly in the ganglion cell layer (GCL) and the nerve fiber layer (NFL) in the normal retina (Fig. 1d-f). Less immunoreactive staining was observed in the other layers. Next we examined whether RGCs expressed Nogo-A. The retrograde FG staining for RGCs was mainly distributed in the cytoplasm. Here we identified that Nogo-A was expressed in the FG-labeled RGCs (Fig. 1j-l). Some Nogo-A positive cells in GCL were not FG-labeled RGCs. Since amacrine cells are the other major types of cells in the GCL, amacrine cells in GCL may also express Nogo-A protein.

**Increased Nogo-A expression after the induction of ocular hypertension**

We induced an ocular hypertension model in adult rats. Similar to our previous reports, the IOPs were found to be 23.45 ± 0.79 mmHg and 24.32 ± 0.82, respectively, in the glaucomatous eyes at 2 weeks and 4 weeks after laser treatments, and 13.24 ± 0.46 mmHg in the normal eyes ($P < 0.01$). In the glaucomatous retina, high levels of
Nogo-A was detected in the RGCs. Strong Nogo-A immunostaining was found in the inner plexiform layer (IPL) and outer nuclear layer (ONL) (Fig. 1g-i, m-o). This was confirmed using Western blotting. Nogo-A expression increased at 5 days after IOP elevation and there was up to 3 fold increase of the Nogo-A protein at 2 and 4 weeks (Fig. 2, $P < 0.05$ compared to normal retina). No difference was found for the Nogo-A levels between 2 weeks and 4 weeks ($P > 0.05$).

**MAG and OMgp expression after the induction of ocular hypertension**

We further examined the expression of the other two myelin proteins in the retina. MAG protein was not detected in the retinas of normal and glaucomatous animals (Fig. 3a). FG-labeled RGCs did not express MAG protein. No MAG expression was found in the beginning portion of the optic nerve without myelin sheath. MAG was only observed in the part of the optic nerve with myelin sheath (Fig. 3a). There was a clear ridge for MAG expression in the longitudinal section of the optic nerve with the optic disk. We further identified that there was low level of OMgp in the IPL in the normal retina using immunohistochemistry (Fig. 3b). There was no OMgp immunostaining in the FG-labeled RGCs (Fig. 3b). No difference was found for OMgp protein in the retina before and after the induction of ocular hypertension (Fig. 3b).

**Discussion**

Here we demonstrated that Nogo-A was expressed in the normal retina and was
increased in a rat glaucoma model. We further identified that RGCs expressed Nogo-A. There was no MAG expression in the retina and OMgp was only expressed in the IPL. It is the first report about the expression of three myelin proteins in the adult retina and in a rat model of ocular hypertension. The data suggest an important role of Nogo-A in the normal retina and after ocular hypertension.

Glaucoma is a common eye disease that can cause irreversible loss of vision if left undiagnosed and untreated. Compartmentalized degeneration in the synapses may underlie visual functional deficits in glaucoma [1-3]. Early changes in RGC dendrites have critical consequences on synaptic efficacy and may underlie functional deficits in glaucoma [28-30]. 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 [1]. We previously identified that synaptic degeneration for RGCs may precede the RGC death in an ocular hypertension model [4]. However, it is unclear for the mechanisms for the synaptic degeneration in glaucoma. The model used in this study mimics the slow, progressive neuropathy produced by elevated IOP in humans by partially obstructing aqueous humor outflow, similar to elevated episcleral venous pressure glaucoma. However, there are still some differences between this animal model and human glaucoma disease. Human chronic glaucoma is a progressive optic neuropathy characterized by slow visual field loss with a long-term—even several decades long-course, whereas there was significant RGC loss in several weeks in this rat ocular hypertension mode. Even so, the finding of increased Nogo-A expression partly indicates that the Nogo-A may play some role...
for vision loss in the human glaucoma.

Previous opinions for myelin proteins are that they are limited to oligodendrocytes. However, much current data demonstrate that Nogo-A is expressed in many populations of neurons in the CNS. Our findings of the expression of Nogo-A in the RGCs in rats is consistent with previous reports that Nogo-A is expressed in the embryo or neonatal animals [18,19]. Besides the inhibition of axonal regeneration or neurite outgrowth, Nogo-A plays an important role in synapse maintenance and neural survival. Nogo-A genetic deletion resulted in prolonged cell survival in addition to axonal regeneration in mice with amyotrophic lateral sclerosis [31]. Nogo-A could signal transsynaptically as observed e.g. for Ephrins to regulate synapse maintenance [32-34]. The reduction of Nogo-A expression in the cell-Deep Cerebellar Nuclei correlates with synapse maturation [20]. The upregulation of Nogo-A in our model is consistent with previous reports about the increased expression of Nogo-A in Alzheimer disease [35], in muscles and spinal cord motoneurons of amyotrophic lateral sclerosis [36]. The increased expression of Nogo-A may be a primary factor leading to the synaptic function deficits and further RGC death. It is interesting that we found increased Nogo-A expression at 5 days after IOP elevation. We previously identified that there was significant synaptic degeneration but no RGC loss at 5 days in this ocular hypertension model [4]. The early upregulation of Nogo-A provide further evidence that Nogo-A may firstly affect synaptic function and then lead to the secondary RGC death. Furthermore, our data showed that there was no difference for Nogo-A levels at 2 weeks and 4 weeks after IOP elevation. It suggests that Nogo-A
protein reaches a saturation under the balance of production and cleaning. In this model, RGC loss stabilizes after 4 weeks [37]. The stability of Nogo-A accumulation after 2 weeks may have some relationship with the stabilization of RGC loss.

Our study indicates that some amacrine cells may also express Nogo-A. Amacrine cells in the vertebrate retina are interneurons that interact at the second synaptic level in the pathways consisting of the photoreceptor-bipolar-ganglion cell chain. They are synaptically active and serve to integrate, modulate and interpose a temporal domain to the visual message presented to the ganglion cell. The expression of Nogo-A in amacrine cells may be one of stations of Nogo-A to affect the synaptic function.

LINGO-1 (LRR and Ig domain-containing, Nogo Receptor-interacting protein-1) [38] was a member of NgR1 signaling complexes that prevent axonal regeneration in the presence of three myelin inhibitors. Because NgR1 is a GPI anchored protein, LINGO-1 and the other co-receptors, p75/TROY transduce the myelin inhibitory signal into the cells. We found that blocking LINGO-1 function promotes survival of damaged RGCs in a chronic adult rat ocular hypertension model [25]. The upregulation expression of Nogo-A after IOP elevation provides a possibility that LINGO-1 antagonists disturb the function of Nogo-A and NgR1, and then exert the neuroprotection. It may be one of the mechanisms how LINGO-1 antagonist promotes RGC survival.

In this study, we found that Nogo-A, but not OMgp and MAG protein increased in the retina after ocular hypertension. It may provide a possible mechanism
underlying the synapse degeneration and RGC death in glaucoma.

Acknowledgements

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Reference


Figure Legends

**Figure 1** Immunohistochemistry of Nogo-A in the normal and ocular hypertensive retina. (a) Negative control for Nogo-A expression in the retina at 2 weeks after ocular hypertension. (b) The same visual field showed fluorogold labeled retinal ganglion cells in (a). (c) Nogo-A expression in the optic nerve in adult rat as positive control. Nogo-A was expressed in the ganglion cell layer in the normal retina (d, j). Retinal ganglion cells (RGCs) expressed Nogo-A (white arrow heads) (j-l). Some Nogo-A positive cells in the GCL were not FG-labeled RGCs (white arrows). Strong Nogo-A expression was found in RGCs (white arrow heads and red arrows), especially for RGCs in the GCL, in the IPL and INL at 2 weeks after ocular hypertension (m-o). The pictures in the boxes in i-l and m-o were enlarged below. Nogo-A staining (green); FG-labeled RGCs (blue); merge of Nogo-A and FG labeling (orange). We regarded red as the color of FG in the picture of merge. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; FG, fluorogold.

**Figure 2** Western blot of Nogo-A in the normal and ocular hypertension retinas. Nogo-A expression in the normal and injured eyes with Western blotting and densitometric analysis of Western blot analysis ($P < 0.05$, compared with normal retina). $n = 5$ animals per group.

**Figure 3** The expression of Myelin associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) in glaucoma. (a) MAG expression.
(a1) Normal retina; (a2) The retina after 2 weeks of ocular hypertension. (a3) optic
disc in the normal retina. There was no expression of MAG in the normal retinas (a1)
and after the induction of ocular hypertension (a2). MAG was expressed in the optic
nerve with myelin sheaths (a3, upper picture: light microscopy picture to show the
optic disc structure). (b) OMgp expression. (b1) Normal retina; (b2) The retina after 2
weeks of ocular hypertension. There was moderate expression of OMgp in the NFL,
GCL, IPL and INL in the normal retinas (b1). OMgp expression had no change at 2
weeks after the induction of ocular hypertension (b2). MAG or OMgp staining (green);
FG-labeled RGCs (blue); We regarded red as the color of FG in the merged picture.
NFL: nerve fiber layer; GCL, ganglion cell layer; INL, inner nuclear layer; ONL,
outer nuclear layer; FG, fluorogold. n = 5 animals per group. Bar = 25 µm.
Figure 1
Figure 2

The levels of Nogo-A (relative to normal) in ocular hypertension conditions.

*P < 0.05
Figure 3