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COMBINED EFFECT OF BRAIN-DERIVED NEUROTROPHIC FACTOR AND LINGO-1 FUSION PROTEIN ON LONG-TERM SURVIVAL OF RETINAL GANGLION CELLS IN CHRONIC GLAUCOMA

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Abstract—Glaucoma is a progressive neuropathy characterized by loss of vision as a result of retinal ganglion cell (RGC) death. There are no effective neuroprotectants to treat this disorder. Brain-derived neurotrophic factor (BDNF) is well known to transiently delay RGC death in ocular hypertensive eyes. The CNS-specific leucine-rich repeat protein LINGO-1 contributes to the negative regulation to some trophic pathways. We thereby examined whether BDNF combined with LINGO-1 antagonists can promote long-term RGC survival after ocular hypertension. In this study, intraocular pressure was elevated in adult rats using an argon laser to photocoagulate the episcleral and limbal veins. BDNF alone shows slight neuroprotection to RGCs after a long-term progress of 4 weeks following the induction of ocular hypertension. However, combination of BDNF and LINGO-1-Fc prevents RGC death in the same condition. We further identified that (1) LINGO-1 was co-expressed with BDNF receptor, TrkB in the RGCs, and (2) BDNF combined with LINGO-1-Fc activated more TrkB in the injured retina compared to BDNF alone. These results indicate that the combination of BDNF with LINGO-1 antagonist can provide long-term protection for RGCs in a chronic ocular hypertension model. TrkB may be the predominant mediator of this neuroprotection.

Key words: ocular hypertension, neural cells, neuronal survival, neurotrophic factors, TrkB.

Glaucoma represents a group of neurodegenerative diseases characterized by structural damage to the optic nerve and slow, progressive death of retinal ganglion cells (RGCs) (Quigley et al., 1995). Although the most important trigger for progression of glaucomatous damage is an elevation of intraocular pressure (IOP), the exact mechanism remains unknown. Current standard therapy for glaucoma is to lower the IOP by medication or surgery that may delay disease progression but does not alter RGC loss and axon degeneration. Therefore, treatment of glaucomatous neuropathy requires the preservation, protection, and rescue of RGCs. Although several approaches for neuroprotection have been described including the use of neurotrophins (Cui et al., 1998), no effective clinical neuroprotectants are available.

Neurotrophic agents have been implicated in survival- and growth-promoting activity in the CNS. Brain-derived neurotrophic factor (BDNF) is an important survival factor for RGCs. BDNF rescues RGCs from death after optic nerve axotomy (Yan et al., 1999; Zhang et al., 2005; Mansour-Robaey et al., 1994; Mey and Thanos, 1993) and in cell culture (Johnson et al., 1986). Exogenously applied BDNF can delay the death of RGCs in animal glaucoma models (Ko et al., 2001; Quigley et al., 2000). However, a series of studies showed that BDNF did not rescue all RGCs after optic nerve axotomy and only delayed RGC death (Mansour-Robaey et al., 1994; Mey and Thanos, 1993; Peinado-Ramon et al., 1996; Klocker et al., 1998). Even repeated intravitreal injections of BDNF, or persistent availability of bioactive BDNF did not promote long-term survival of RGCs after axotomy (Mansour-Robaey et al., 1994; Di et al., 1998; Isenmann et al., 1998). The protective effect of exogenous BDNF or overexpression of BDNF gene on RGCs was also limited in a chronic glaucoma model (Martin et al., 2003; Ko et al., 2001). A clearer mechanistic understanding of the limited neuroprotection of BDNF can be crucial for the development of treatments for glaucoma.

LRR and Ig domain-containing, Nogo Receptor-interacting protein 1 (LINGO-1) is a leucine-rich repeat Ig-containing protein first identified as a critical component of Nogo receptor/p75 or TROY signaling complexes that prevent axonal regeneration in the presence of myelin inhibitors in the CNS (Shao et al., 2005; Mi et al., 2004). LINGO-1 is specific to the CNS and functions as a negative regulator of axonal regeneration and neuronal survival. We previously found that blocking the function of LINGO-1

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Abbreviations: BDNF, brain-derived neurotrophic factor; FG, Fluoro-Gold; IOP, intraocular pressure; LINGO-1, LRR and Ig domain-containing, Nogo Receptor-interacting protein 1; PBS, phosphate-buffered saline; RGC, retinal ganglion cell.

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protected a major proportion of injured RGCs in rats with ocular hypertension (Fu et al., 2008a). LINGO-1 binds to the epidermal growth factor receptor to negatively regulates its activation (Inoue et al., 2007). We just identified that LINGO-1 binds with BDNF receptor, TrkB and inhibits TrkB activation *in vitro* and in the rat retinas (Fu et al., unpublished observations). These data suggest that the negative regulatory functions of LINGO-1 may be involved in the limited neuroprotective effect of BDNF and it could be reversed after blocking the LINGO-1 function.

In this study, we investigated the combined neuroprotective effect of BDNF and a LINGO-1 antagonist, soluble LINGO-1 protein in a rat ocular hypertension model. We also investigated the involvement of TrkB activation with neuroprotection in this model.

EXPERIMENTAL PROCEDURES

Generation of recombinant LINGO-1-Fc

LINGO-1-Fc protein (soluble LINGO-1) was prepared as described previously (Mi et al., 2004). Residues 1–532 of human LINGO-1 were fused to the hinge and Fc region of human IgG1, expressed in CHO cells and purified on Protein A Sepharose (Pharmacia, NJ, USA). The purified protein (>95% pure) ran on SDS-PAGE with M_r=90 kDa under reducing conditions and M_r=180 kDa under non-reducing conditions. Recombinant human BDNF was purchased from Regeneron Pharmaceutical (Tarrytown, NY, USA).

Animals

Adult female Sprague–Dawley rats weighing approximately 250–280 g were reared in a temperature-controlled room on a 12-h light/dark cycle in the Laboratory Animal Unit of The University of Hong Kong. A total of 32 rats were used for the study of RGC survival (n=8 for each group). The animal number is four to five for each group for the experiments on histochemistry and Western blotting. All the experimental and animal handling procedures complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were also reviewed and approved by the Faculty Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong. All efforts were made to minimize the number of animals used and their suffering. The animals were anesthetized with i.p. injection of ketamine (80 mg/kg, 10% alfasan, Woerden, the Netherlands) and xylazine (8 mg/kg, 2% alfasan, Woerden) during the experiments and were euthanized with an overdose of pentobarbital sodium (150 mg/kg, Alcon-Couvreur, Rijksweg, Puurs, Belgium). Alcaine 0.5% (Alcon-Couvreur) was applied to the eyes before all operations and antiseptic eye drops (Tobres [tobramycin 0.3%], Alcon-Couvreur, Rijksweg, Puurs, Belgium) were used to prevent infection after the treatment. Rimadyl (0.025 mg/ml, Pfizer, NY, USA) in drinking water was used to relieve the pain for 7 days after the surgeries.

Ocular hypertension model and treatments

To induce experimental ocular hypertension, the rats received argon laser (Ultima 2000SE argon Laser, Coherent, Palo Alto, CA, USA) photocoagulation of the episcleral and limbal veins two times, 7 days apart, in the right eye. This technique was adopted from the method by WoldeMussie et al. (2001) and has been used in our laboratory with a track record of publications (Fu et al., 2008a,b, 2009; Ji et al., 2004; Li et al., 2006a,b). 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 0.1 s. The contralateral (left) eyes were used as controls. Animals were allowed to survive for 4 weeks post–first laser exposure before they were sacrificed. The IOP of the eyes was measured using a Tonopen XL Tonometer (Mentor, Norwell, MA, USA) at different time points.

The eyes with elevated IOP received intravitreal injection of proteins once a week in the 4-week glaucoma animals on days 0, 7, 14 and 21. The animals were divided into four groups, 0.01 M phosphate-buffered saline (PBS) group (2 μl), combination of BDNF (1 μg in 1 μl) and PBS (2 μl) group, LINGO-1-Fc group (2 μg in 2 μl), combination of BDNF (1 μg in 1 μl) and LINGO-1-Fc group (2 μg in 2 μl). The protein solution was injected intravitreally into the right eye using a 10 μl Hamilton microsyringe fixed with a 26-s gauge needle (# 80300, Hamilton, Reno, NV, USA). The microsyringe was held inside the eyeball for more than 1 min before being pulled out. The site of injection was just below of the limbus of the cornea, which provides minimal possibility of injury to the retina. The injections for combined proteins were performed at 30 min intervals.

Retrograde labeling of RGCs and RGC counting

In order to retrogradely label RGCs, both superior colliculi were exposed and a piece of Gelfoam (Pharmacia & Upjohn, NJ, USA) soaked with Fluoro-Gold (FG, 6% in distilled H_2O, Fluorochrome, Denver, CO, USA) was placed on the surface of superior colliculi seven days before sacrifice. At 4 weeks after laser coagulation, the rats were transcardially perfused with 0.9% saline for 30 min. Both eyes of each animal were enucleated and fixed in 4% paraformaldehyde for 60 min. Retinas were prepared as flat-mounts and the FG-labeled RGCs were counted under fluorescence microscope using an ultraviolet filter (excitation wavelength=330–380 nm). The RGCs were quantified under an eyepiece grid of 200×200 μm^2 5 along the midline of each quadrant, from the optic disc to the border at 500 μm intervals (Ji et al., 2004). Eight microscopic fields for each quadrant and a total of 32 per retina for four quadrants were counted. The data were expressed as the density of cells (number of cells/mm^2) and also analyzed in terms of relative percent RGC loss in the injured right eye to the contralateral left intact eye from the same animal.

Immunohistochemistry for TrkB and LINGO-1

RGCs were retrogradely labeled with FG 7 days before sacrifice. The eyes were enucleated at 4 weeks after laser treatment following transcardial perfusion with 0.9% saline and were fixed in 4% paraformaldehyde for 1 h. After removing the cornea and lens, the eye cups were fixed further in paraformaldehyde for 4–6 h and then transferred to 30% sucrose solution at 4 °C for 16 h. The eye cups were embedded in OCT compound and cryosections (10 μm thick) were prepared at −20 °C. The retinal sections were washed with 0.01 M PBS (PH 7.4), incubated in 0.5% Triton/PBS for 10 min, and blocked with 10% normal goat serum and 0.1% Triton/PBS for 1 h. Incubation with mouse antibody for LINGO-1 (1:100, Biogen Idec, Inc., MA, USA) and chicken IgY TrkB antibody (1:100, Promega, Mannheim, Germany) was performed at 4 °C for 16 h. After washing with PBS, the sections were treated with Qdot@655 goat IgG anti-chicken IgY (1:400, Molecular Probes, OR, USA) or Alexa-labeled goat anti-mouse 488 antibodies (1:400, Molecular Probes, OR, USA) at room temperature for 2 h. After washing, the sections were mounted with fluorescent mounting medium (Dako, Cytomation, Denmark) and analyzed under a Carl Zeiss LSM