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Expression of Slit2 and Robo Receptors in a Sprague-Dawley Rat Glaucoma Model

Yoon-Jung Choy[1], Jae-Ho Shin[2], Jee-Hyun Choi[3]

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ABSTRACT

Background: This study investigated immunoreactive changes in Slit2 and Robo receptors in the retinal ganglion cell layer of a rodent model of acute glaucoma.

Methods: Glaucoma model using Sprague-Dawley rats was made via weekly intracameral injections of hyaluronic acid. Intraocular pressure (IOP) was measured twice weekly for 4 weeks using a rebound tonometer in an experimental group of 10 rats and a control group of five rats for 4 weeks. The trimmed retinas were processed for anti-glial fibrillary acidic protein (GFAP), anti-Slit2 and anti-Robo1, anti-Robo2, anti-Robo3 and anti-Robo4 immunochemical analysis.

Result: The IOPs in the experimental group were approximately four times higher than IOPs in the control group. The GFAP, Slit2 and Robo4 immunoreactivity in the experimental group was higher than the corresponding values in the control group. Our results indicate that Slit2 and Robo4 potentially contribute to the progression of high tension glaucoma, especially in inducing ischemic injury.

Key words: Glaucoma, Immunochemistry, Robo receptors, Slit2.

INTRODUCTION

Elevated intraocular pressure (IOP) is a major risk factor for glaucoma and is characterized by an irreversible decrease in retinal ganglion cells (RGCs). Although the hypothesis that glaucoma is a consequence of axonal damage ending with the death of RGCs is generally accepted, recent studies have revealed the important role played by neuroglia in the pathogenesis of the disease (Gallego *et al*., 2012).

Slit2 is a secreted glycoprotein that was originally identified as an axonal repellent in central nervous system development and has also been documented to play a role in neuronal migration during development (Li *et al*., 2015). Slit2 has four cognate roundabout receptors (Huminiecki *et al*., 2002). In the developing rodent visual system, Slit2 is expressed in the eye, optic stalk and ventral diencephalon (Niclou *et al*., 2000). Projection of retinal axons to the brain is controlled by Slit proteins, which contribute to the guidance of RGC axons from the retina through the optic nerve to the mammalian optic tract (Plachez *et al*., 2008). Robo1 and Robo2 are also expressed in the inner retinal layer and in the optic tract (Plachez *et al*., 2008). Robo2 is expressed by RGCs whereas slit2 are expressed around the pathway of the RGC axons (Niclou *et al*., 2000). Through inhibitory signaling, these molecules are critical for formation of the optic chiasm and function to restrict the RGC axons to the optic pathway (Plump *et al*., 2002). These facts suggest that Slit2 and Robo receptors may play significant roles in the development of glaucoma.

Previously, experimental glaucoma models in rats resulting from episcleral vein injection with hypertonic saline,(Guo *et al*., 2011) and cauterizing of episcleral veins (Bai *et al*., 2014). Our study involved intracameral injections of cohesive hyaluronic acid (Moreno *et al*., 2005). When high IOP develops over a short period (within 24 hours) due to the presence of a viscoelastic material, the direct flow of aqueous humor from the ciliary process to the pupil is blocked.

This study investigated the changes in Slit2 and Robo receptors when high IOP was induced in an experimental rat model with a similar visual system and aqueous humor dynamics to humans (Kim *et al*., 2014). Analyzing immunohistochemistry results in regards to the type of neuroglia that expressed Slit2 and Robo receptors using glial fibrillary acidic protein (GFAP).

MATERIALS AND METHODS

Five-week-old male Sprague-Dawley (SD) rats weighing approximately 200 g and reared by ORIENTBIO were used in this study. All experimental procedures were approved by the Animal Review Board of Eulji University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23,

[1]Department of Optometry, Eulji University, Seongnam, Korea.
[2]Department of Biomedical Laboratory Science, Eulji University, Seongnam, Korea.
[3]Laboratory Animal Center, Osong Medical Innovation Foundation, Cheongju, Korea.

Corresponding Author: Yoon-Jung Choy, Department of Optometry, Eulji University, Seongnam, Korea.
Email: yjchoy7@eulji.ac.kr

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revised 1996). The study was carried out during the period of September 2019 to June 2020, at the Department of Biomedical Laboratory Science at Eulji University in Seongnam.

They were divided into between an experimental group (n=10) and a control group (n=5). The laboratory environment was maintained at a temperature 20-25°C, humidity 40-60% and a 12-hour day/night cycle (7:00 am to 7:00 pm).

Intracameral hyaluronic acid injection

Ten six-week-old male SD rats weighing 250-300 g were anesthetized using an intraperitoneal injection of tiletamine and Zolazepam (Zoletil 50 inj, Virvac Korea, St. Ogeum, Seoul, Korea) and xylazine hydrochloride (Rompun inj, Bayer Korea, St. Boramae, Seoul, Korea) in a 2:1 ratio (0.1mg/100g) weekly for 4 weeks. The rats were immobilized on the sample plate of a stereoscopic microscope (AKS-IIILF, OMAX, St. Maesil, Sejong, Korea) and 25µl sodium hydronate [Hyaluronic acid eye drop (18mg/1.2ml), Kukje Pharma, St. Yatap, Sungnam, Korea] was injected into the right eye using a Hamilton syringe (Gastight #1705, Hamilton Co., Reno, Nevada, USA) and a 30-gauge needle directed through the corneal limbus (Moreno *et al*., 2005).

IOP assessment

After injecting hyaluronic acid intracamerally, IOP was measured using a rebound tonometer (Tonolab, Tiolat, OY, Helsinki, Finland). IOP was measured in both the experimental and control groups at 3:00pm twice weekly. The measurements were obtained by a skilled researcher who fixed the neck and body of the rat with one hand, not allowing the rat to move. (Kim *et al*., 2014) On days that hyaluronic acid was injected the rats were anesthetized via intraperitoneal injection, while on the days that only IOP was measured, inhalational anesthesia was performed using isoflurane 2.5-3% in oxygen. Eye drops containing 0.5% proparacaine hydrochloride (Alcaine®, Alcon Inc., Fort Worth, TX, USA) were used as topical anesthesia. The probe was positioned perpendicular to the cornea and horizontal to the ground such that there was 1-4mm between the tip of the probe and the cornea.(Yu, Kim *et al*., 2007) After six measurements had been obtained, the mean IOP was shown in the display window. If an error message appeared at any time, the measurement was repeated.

Tissue preparation

After injecting hyaluronic acid for four consecutive weeks, enucleation was performed on postoperative week 4. The eyeballs were soaked in 4% paraformaldehyde (pH 7.4) and sectioned along the posterior margin of the ora serrata. The retina was peeled off with a number 0 brush and the retinal tissue was divided into four quadrants. The mid-papillary region of the retinas were then cut into small rectangular sections. The retinal tissues were fixed in 4% paraformaldehyde for 2 hours at 4°C. After fixation, they were transferred to a 30% sucrose solution (pH 7.4) and refrigerated overnight. The retinal tissues were flash frozen in liquid nitrogen and stored at -70°C. Finally, they were rinsed in phosphate buffered saline (PBS) (pH 7.4), dehydrated, embedded in wax and cut into 5- µm sections.

Immunohistochemistry

The retinal tissue that was selected for immunohisto chemistry displayed optic disc hemorrhage, as this is a sign of glaucoma and showed gradual thinning of the retinal vessels. For blocking, the retinal tissue was treated with 5% normal donkey serum (pH 7.4) (Jackson Immuno Research, West Grove, PA, USA) for 1 hour, then soaked overnight at room temperature in a solution containing 1% Triton X-100. The retinal tissue was then incubated in primary antibodies [goat polyclonal anti-Slit2 (1:50; Santa Cruz, Santa Cruz, CA, USA), rabbit polyclonal anti-Robo1 (1:200; Santa Cruz), goat anti-Robo2 (1:50; Santa Cruz), goat anti-Robo3 (1:50; Santa Cruz), goat anti-Robo4 (1:50; Santa Cruz), mouse monoclonal anti-GFAP (1:500; Milipore)] for 3 days in a refrigerator and rinsed with PBS three times for 15 minutes. After rinsing, they were incubated with secondary antibodies [anti-goat-horseradish peroxidase (HRP) (1:100; Milipore) or anti-rabbit-HRP antibody] for 2 days in a refrigerator and rinsed with PBS for 45 minutes. All retinal preparations were evaluated by microscope (Nikon ECLIPSE 80i, Nikon Corporation, Japan) and were captured with I-Solution™ (IMT I-Solution Inc., Vancouver, BC, Canada). Subcellular components were segmented using a hematoxylin counterstain.

RESULTS AND DISCUSSION

IOP values

All eyes with induced glaucoma (n=10) developed elevated IOP within 1 hour of intracameral injection. Induced local corneal oedema at the injection site lasted less than 24 hours. Two of the 10 experimental rats developed hyphema, two developed cataracts and one developed corneal inflammation; all were then excluded from the study. The IOP values of the experimental group consistently measured over 30mmHg (first and second IOP measurements after intracameral hyaluronic acid injection on week 0: 39.20±9.36 and 49.0±19.49mmHg, respectively; week 1: 35.20±9.09 and 36.40±11.0mmHg, respectively; week 2: 63.80±12.50 and 41.60±11.0mmHg, respectively; week 3: 41.20±11.0 and 42.20±9.50mmHg, respectively) whereas IOP of the control group measured 10mmHg. All the IOP values for the experimental group were significantly different from those of the control group (Fig 1)($P < .05$, Mann-Whitney U test).

Immunohistochemistry

GFAP and Slit2 immunoreactivity

Morphological observation revealed a reduction in the thickness of the entire retina due to elevated IOP. GFAP immunoreactivity in the control group appeared mainly in the astrocytes and the end feet of the Müller glial cells distributed in the nerve fiber layer (Fig 2A). In the

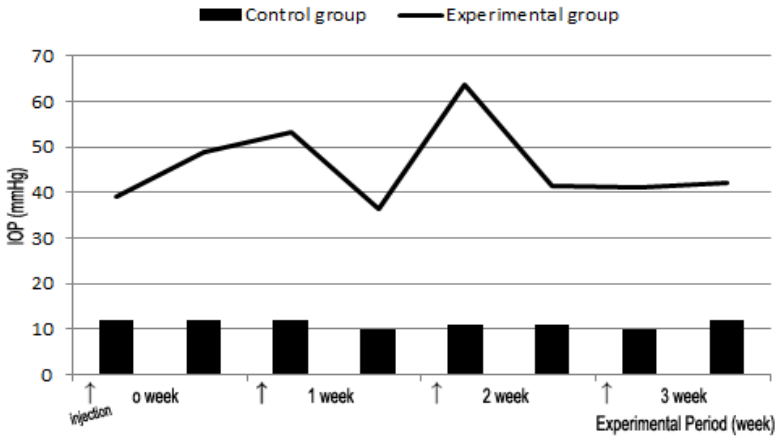

Fig 1: Changes in intraocular pressure (IOP) after intracameral hyaluronic acid injections. IOPs were measured in both experimental and the control groups at 3:00pm twice weekly from postoperative week 1 to week 4. The solid line shows IOP changes in the experimental group. The bar shows changes in the control group.

glaucomatous retinal sections, GFAP immunoreactivity extended into the proximal radial processes of the Müller cells in the inner plexiform layer and into the distal radial processes of the Müller cells in the outer plexiform layer and the photoreceptor layer (Fig 2B). GFAP immunoreactivity in the experimental group was increased compared to the control group.

Slit2 immunoreactivity was localized to astrocytes, which are distributed between RGCs and retinal vessels in the ganglion cell layer (GCL) (Fig 2C, D). Slit2 immuno reactivity was increased in the experimental group than in the control group.

Robo receptors immunoreactivity

Immunoreactivity of the Robo receptors was localized to the astrocytes. Robo1 immunoreactivity was decreased in the experimental group (Fig 3A, B). Robo2 and Robo3 immunoreactivity appeared weak in both groups. Interestingly, Robo2 immunoreactivity was localized to the cell bodies of the ganglion cells in the experimental group. However, the differences in immunoreactivity were not significant for Robo1, Robo2, or Robo3 (Fig 3C, D, E and F). In contrast, Robo4 immunoreactivity was significantly increased in the experimental group compared to that in the control group (Fig 3G, H).

Glaucoma is recognized as one of the major ocular neurodegenerative diseases leading to blindness. Excess aqueous humor caused by overproduction or blockage of the drainage channels results in elevated IOP and subsequent ischemic or mechanical injury to the optic nerve axons.

Our results revealed that Slit2 and Robo4 immunoreactivity was increased which resulted in an abrupt increase in IOP. It is well known that Slit2 and Robo4 contribute to the dynamic morphologic changes that occur in astrocytes in response to ischemic injury. Slit2 is involved in the pathophysiology of ischemic injury, including neuronal damage and the glial response (Park *et al*., 2016). Our rat

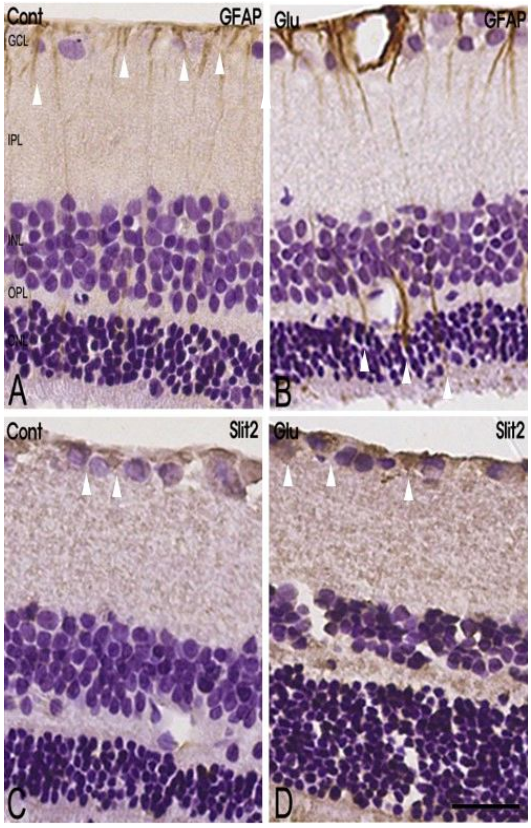

Fig 2: Micrographs of retinal sections processed for glial fibrillary acidic protein (GFAP) and Slit2 immunohistochemistries. Scale bar represents 35µm. Cont = Control group; Glu = Glaucoma group; GCL = ganglion cell layer; IPL = Inner plexiform layer; INL = Inner nuclear layer; OPL = Outer plexiform layer; ONL = Outer nuclear layer.

glaucoma model was successful in inducing ischemic injury to the retina and optic nerve axons. Pressure-induced ischemic injuries, such as optic disc hemorrhage, are known to result in a neuroglial reaction and neuronal degenerative responses,

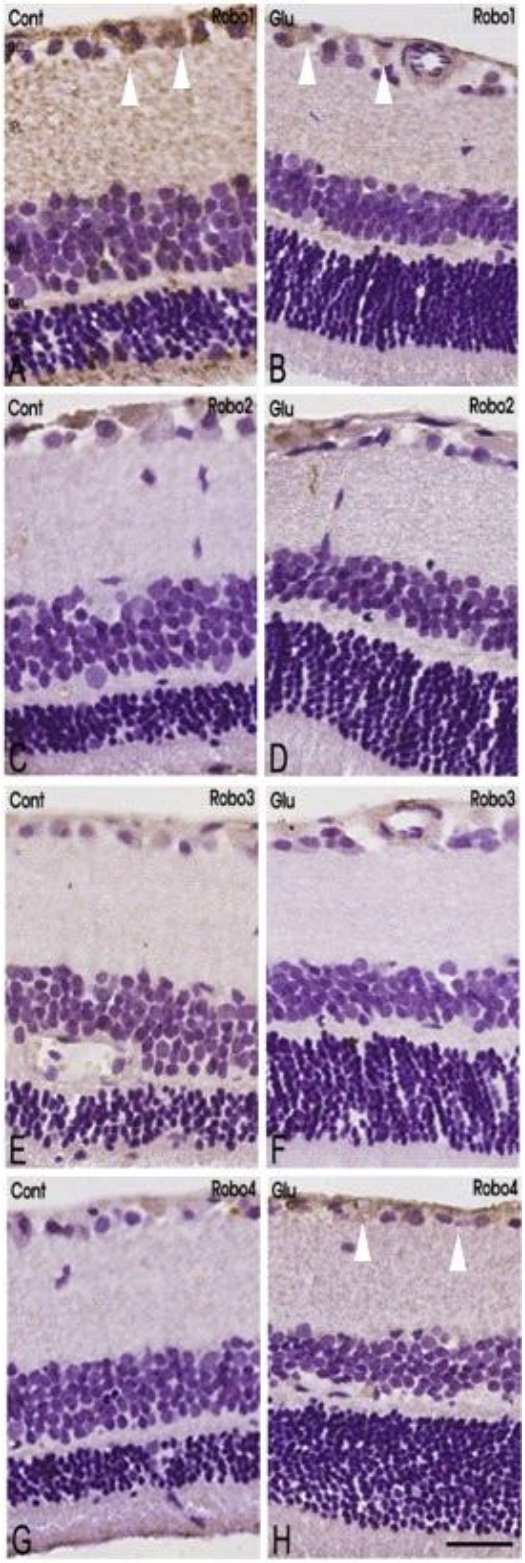

Fig 3: Representative immunohistochemical images of Robo 1, 2, 3 and 4. Scale bar represents 35µm. Cont = control group; Glu = Glaucoma group; GCL = Ganglion cell layer; IPL = Inner plexiform layer; INL = Inner nuclear layer; OPL = Outer plexiform layer; ONL = Outer nuclear layer.

including cell death (Ahmed *et al*., 2001).

The retinal neuroglia are organized into three layers of neuronal somata namely the outer nuclear layer, inner nuclear layer and GCL. Astrocytes are located in the RGC close to the retinal blood vessels and microglia are normally restricted to the retinal nerve fiber layer and the GCL (Lee *et al*., 2014). Glaucoma causes retinal neuroglia to display abnormal features, which results in the expression of GFAP and the appearance of reactive astrocytes (Ringstedt *et al*. 2000). GFAP is generally used as an astrocyte identification marker protein to detect neuroglial reaction to injury (Lee *et al*., 2011). The cellular marker for a neuroglial reaction is the upregulation of GFAP (Bringmann and Reichenbach 2001). In our study, GFAP immunoreactivity increased in the experimental group.

The neuroglial expression profile is altered during activation, presumably exerting neuroprotective or damaging influences at different phases of disease progression (Erskine, Williams *et al*. 2000). It has been suggested that reactive neuroglia are potentially neuroprotective of RGCs (Di Polo *et al*. 1998). Conversely, Kawasaki (Kawasaki *et al*., 2000) described reactive neuroglia that exacerbated neuronal damage and may have contributed to the etiology of glaucoma. This could negatively influence ganglion cells, which would lose their normal functional support. Thus, our results suggest that Slit2 and Robo4 blockade could potentially be used therapeutically to inhibit optic disc damage due to glaucoma. Robo1 and Robo2 regulate the targeting of RGC axons along the entire visual projection (Plachez *et al*. 2008). Slit2 activation is abolished by the inactivation of Robo1 and Robo2, indicating that decreased activity of these signal proteins may contribute to defective sprouting (Rama *et al*. 2015).

Our study had several limitations. The main limitation was that immunoreactivity was not quantified. Second, we only performed a short term follow-up on Slit 2 and Robo receptors. A more detailed study is necessary. However, the results of this study demonstrated that Slit2 and Robo receptors are specifically expressed at GCL, which is the origin of afferent axons leading to the optic disc. In addition, Slit2 and Robo4 have the potential to promote the survival of retinal ganglion cells and the optic disc.

CONCLUSION

In conclusion, Slit 2 and Robo4 are likely to be associated with the development of acute high tension glaucoma when ischemic injury is induced in the retina and optic disc.

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