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Original Article

Axonal regeneration of optic nerve after crush after PirBsiRNA transfection

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Abstract: Axon can't regenerate after central nervous system injury because of myelin-associated protein exerting its effects through the paired immunoglobulin like receptor (PirB). In our study, axon regeneration of retinal ganglion cells (RGCs) after optic nerve (ON) crush was investigated both in vivo and in vitro in rat after PirBsiRNA transfection. The eyes transfected with AAV-PirBsiRNA were as experimental group. Partial ON injury was induced by using ON clip to crush the ON 1 mm behind the eyeball for 9 s. PirB protein was analyzed by western blot. Growth Associated Protein 43 (GAP-43) was analyzed by immunofluorescence staining on the frozen sections. Image analyzer was used to the axonal growth of RGCs. PirB expression in retina and optic nerve of experimental group is less than control group. The regenerated axon of experimental group is longer compared to control group. Our result indicate that PirB genes play an important role in the axonal regeneration after ON injury, wheresas knokdown of PirB is effective to improve axonal regeneration.

Keywords: Paired immunoglobulin like receptor, knockdown, axonal injury, regeneration, GAP-43

Introduction

Due to the C-terminal of NogoA (Nogo66) [1, 2], myelin-associated glycoprotein (MAG) [3, 4], and Omgp [5] exerting their effects via the Nogo receptor (NgR) [6] axons of the adult mammalian central nervous system (CNS) can't regenerate after injury. However, NgR knockout does not improve regeneration of corticospinal tract (CST) axons after injury, although raphespinal tracts regeneration after spinal cord injury has been reported [7]. Our previous result confirmed that knockout of Nogo-A/B/C or NgR can raise axonal regeneration after optic nerve injury to some extent [8, 9].

Jasvinder K. Atwal [10] found that paired immunoglobulin like receptor-B (PirB) can be a therapy target and discovered that the knockout of PirB enhance axonal regeneration. PirB has been confirmed as a necessary receptor for neurite inhibition by the myelin-associated inhibitors Nogo66, MAG, and OMgp. We will be investigating whether AAV-PirBsiRNA transfection can heighten the axonal regeneration of RGCs in the present study.

Materials and methods

Animal grouping

The investigation adhered to the principles concerning the care and use of animals according to the ARVO Statement for the use of animals in ophthalmic and vision research. The left eyes of 30 female (8 or 12-week-old) rats transfected with PirBsiRNA were used as the experimental group, and the right eyes as the control group.

Isolation and culture of retinal ganglion cells

The retinal tissue was dealed with DMEM (Invitrogen, California, USA) containing 15 U/ml papain (Worthington, Lakewood, USA). The cell suspension was incubated in a polypropylene tube coated with anti-rat ED1 antibody (Sigma, St. louis, USA), and then in another one coated with anti-rat Thy 1.1 IgG (Sigma, St. louis, USA). RGCs were collected by centrifugation for 5 min after the tube was washed three times with PBS. Then cultured at a concentration of 6×10^6 cells/ml in DMEM containing 100 U/ml penicillin, 100 μg/ml gentamicin, and B-27 medium

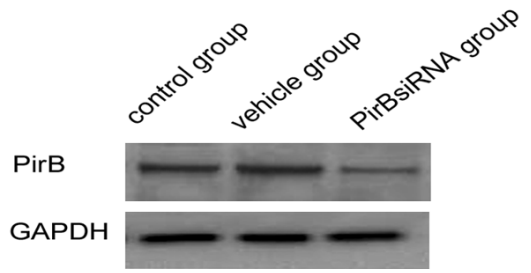

Figure 1. There is little expression of NgR protein after transfection of NgR shRNA (group A, lane 1). Expression of NgR protein can be seen in negative control group (group B, lane 2). Expression of NgR protein can be seen in blank control group (group C, lane 3).

(Invitrogen, California, USA). 2.5×10^5 cells were then cultured into poly-lysine coated 24-well plates and incubated at 37°C with 5% CO_2 ventilation [11].

Culture retinal ganglion cells and transfected with AAV-PirBsiRNA

The RGCs suspension was incubated in a polypropylene tube coated with a monoclonal anti-rat macrophage IgG with the purpose of removing the macrophages, and then in another one coated with a monoclonal anti-rat Thy 1.1 IgG. RGCs were cultured in DMEM medium containing B-27 supplement and incubated at 37°C with 5% CO_2 ventilation. Cells were examined under phase-contrast microscope every day. AAV-PirBsiRNA was added into the medium, whereas the medium without AAV-PirBsiRNA was presented as the control group.

Immunocytochemistry

4% paraformaldehyde was used to fix RGCs for 15 min and then blocked them with 5% goat serum. RGCs were incubated with the primary antibody anti-Thy 1.1 (1:500) at 4°C for 24 h and secondary antibody FITC-IgG (1:1000) at 37°C for 1 h to identify the RGC. The slides were investigated under immunofluorescence microscope. RGCs were incubated with anti-GAP-43 (1:1000) at 4°C for 24 h and anti-IgG (1:1000) at 37°C for 1 h. After stained with DAB and treated with dehydration, dimethyl benzene, slides were examined under phase-contrast microscopy.

The number of axons extend ≥1 mm from the injury site of six longitudinal sections per case, were counted under 400 magnification. Then these numbers by the cross-sectional width of the nerve at the point were divided where axons were counted. The number of axons per unit width of optic nerve was averaged across the six sections and used to calculate the total number of axons regenerating in the nerve.

Western blot

Optic nerve were prepared in extraction buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton-100, 0.1% SDS, 1 mM EDTA, 1 mM AEBSF, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. 10% SDS-PAGE was used to separate total protein (10 μg) and then transferred it to Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After blocking with 5% nonfat dry milk in PBS with 0.1% Tween-20, membranes were probed with rabbit anti-PirB antibody (1:500, Abcam company, Cambridge, USA), followed by subsequent incubation with goat anti-rabbit secondary antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The enhanced chemiluminescence kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to visualize the protein bands. Band intensity was quantified using Quantity One 4.4.1 software (Bio-Rad, USA).

Surgery procedure

Prior to ON crush, general anesthesia was induced in each animal with an intraperitoneal injection of 1% sodium pentobarbital solution (50 mg/kg bodyweight). After the meningas was cut, the ON was crushed by an ON forceps with 40 g pressure for 9 s. The left ON was crushed, but the right ON was intact in the control group.

Preparation for pathological and immunohistochemistry examinations

Seven days after ON crush, perfusion through aorta was performed. Frozen sections (15 μm) of eyes with ON segments were cut longitudinally on a cryostat, thaw mounted onto coated glass slides, and stored at -80°C. Frozen sections were stained in GAP-43 monoclonal antibody (1:500 dilution), followed by fluorescent secondary antibody (1:500).

Statistical analysis

All computations were carried out using the software of SPSS version 18.0 for Windows. All data are presented as the mean ± SD and have been evaluated for normality of distribution.

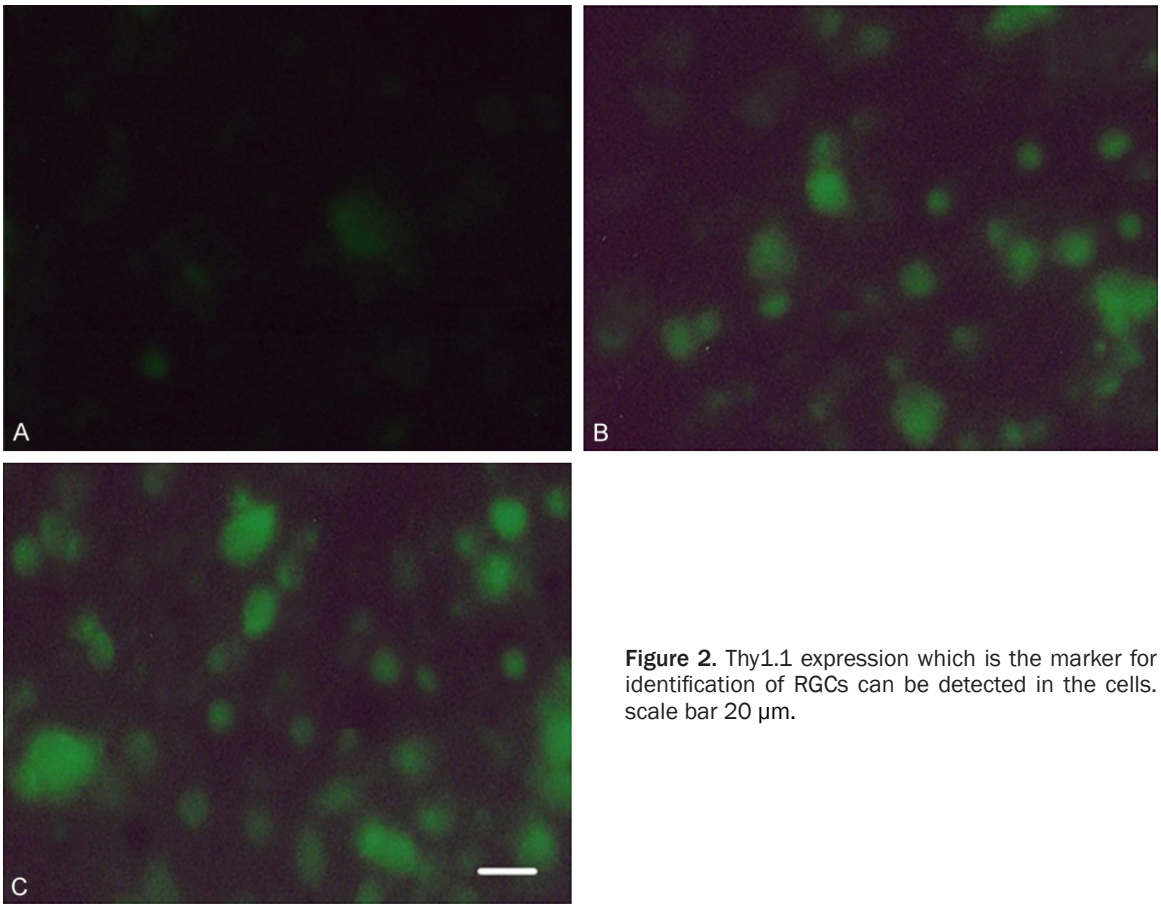

Figure 2. Thy1.1 expression which is the marker for identification of RGCs can be detected in the cells. scale bar 20 μm.

The data were analyzed by the two-tailed Student's t-test Difference between mean values was tested for statistical significance with student t test, with a *P* value <0.05 was considered significant statistically significant and less than 0.01 statistically borderline significant.

Results

PirB expression after transfected with AAV-PirBsiRNA

There is little expression of PirB protein after PirB knockout in retina and optic nerve (**Figure 1**, lane 2, lane 4) compared with the control group (**Figure 1**, lane 1, lane 3).

Expression of Thy1.1 by immunofluorescence

Immunochemistry showed positive Thy1.1 staining in cultured RGCs with red fluorescence (**Figure 2A-C**).

AAV-PirBsiRNA on axon length of RGC in vitro

Adherence of RGCs began at 12 h after seeded into poly-lysine-coated 24-well plates then incubated at 37°C 5% CO_2 ventilation, and finished in 24 h. After this, a single layer of round and oval RGCs was seen on the plates.

The length of the axon was defined as the distance from the soma to the tip of the process. An average of 50 neurons from each group was selected from a number of chamber slides. Axonal length of the experimental group was longer in the third day than that of the control group. Borderline significant difference was observed in axonal length between the experimental and the control group (*P=0.05*) on the first postoperative day. While significant difference was observed in axonal length by the third and seventh days between the experimental and control groups. (**Figure 3A-C**; **Table 1**, *P= 0.003*).

Transfection with AAV-PirBsiRNA on axon regeneration of optic nerve in vivo

The number of axonal length ≥1 mm from the crush position in experimental group is 955.26±1.15 after optic nerve crush for seven days. The number of axonal length ≥1 mm from

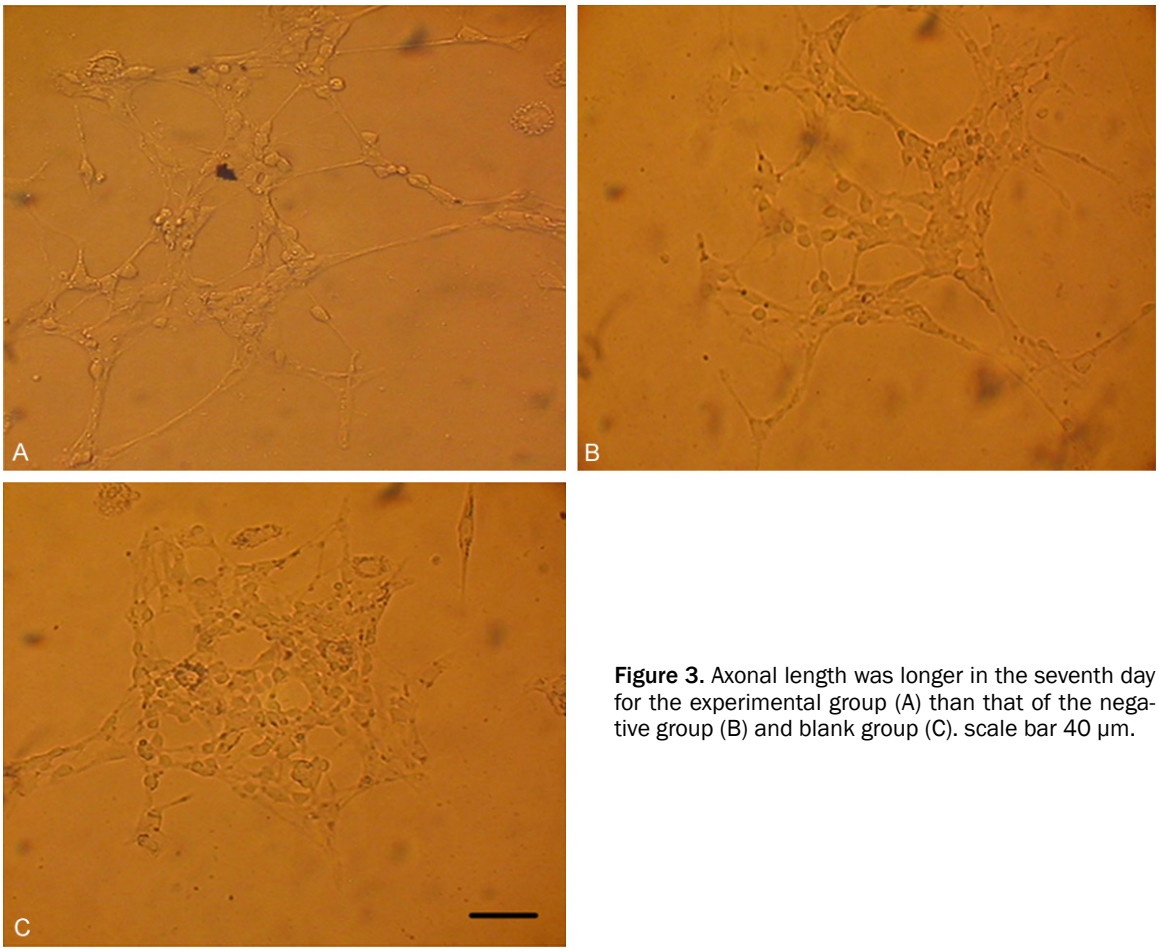

Figure 3. Axonal length was longer in the seventh day for the experimental group (A) than that of the negative group (B) and blank group (C). scale bar 40 μm.

Table 1. Numbers of axonal length ≥1 mm of RGCs of different time ($\bar{x}\pm s$)

Survival time (d)	Experimental group	Negative control group	Blank control group	*F*	*P*
7	715.12±0.95	11.21±0.75	11.09±0.35	25.15	<0.01

Table 2. Axonal length of RGCs of different time in vitro (μm, $\bar{x}\pm s$)

Culture time (d)	Experimental group	Negative control group	Blank control group	*F*	*P*
1	2.13±1.05	2.09±0.85	2.15±0.72	25.15	>0.05
3	35.15±1.12	7.12±0.53	7.25±0.62	29.12	<0.01
7	13.25±1.15	5.12±0.95	5.13±0.82	25.15	<0.01
F	35.12	32.25	32.13		
P	<0.01	<0.01	<0.01		

the crush position in control group is 15.32± 1.16. There was a significant difference in axonal length ≥1 mm from the crush position that stained positively for GAP-43 on the ON sections between experimental and control group (**Table 2**, *P=0.003*). The crush position of ON of mice can be investigated under normal light microscopy.

Discussion

Kim et al. [12] and Simonen et al. [13] have showed that there is significant corticospinal tract fiber regeneration in Nogo-A-/- mice. However, Zheng et al. [14] confirmed that there is no significant regeneration in either Nogo-A/B-/- line or Nogo-A/B/C-/- line mice. Our previous results showed that knockdown of Nogo-A/B/C and NgR can effectively improve axonal regeneration after ON crush both in vitro and in vivo.

Kim et al. [15] also reported that NgR-deficiency improves the raphe spinal and rubrospinal axon

regeneration but not corticospinal neurons. Zheng et al. [16] also failed to observe any improvement of NgR knockout mice in terms of corticospinal regeneration. The animal model of different background which different research groups used may lead to different results.

The PirA and PirB have been identified recently whereas these two genes share sequence similarity with a gene family [17]. It was confirmed that inactivation of either PirB or NgR alone could inhibit the growth-cone collapse although PirB is more important than NgR [18]. PirB can act through Shp-1 and Shp-2 phosphatases and regulate cytoskeletal dynamics, cell motility. PirB could also confine the response of neurons and limit synaptic plasticity [19, 20].

Our result showed that there is PirB expression in RGCs of retina by ISH and western blot previously. We also found that there is little expression of PirB protein both in retina and optic nerve after knockdown of PirB gene, while the expression of PirB was detected in the control group. Our results indicate that it is possible to knockout PirB in retina. We confirmed that NgR can be successfully knockdown. Knockdown of PirB makes it possible to study the effect of PirB on axonal regeneration of RGCs in vivo and vitro.

GAP-43 expression in growing cone is a symbol of regenerated axon. We did detect the expression of GAP-43 in RGC after culturing for seven days in both experimental and control group. Our data indicates that knockout of PirB can effectively enhance axonal regeneration of RGCs in vivo. RGCs have instinctive competence of axonal regeneration in vivo. Axonal length of experimental group increased from the first day to the seventh day, but the axonal length of the RGCs in control group grows slowly. The difference in axonal lengths of RGCs between the experimental group and control group was significant either at the third day or the seventh day. Extinguishing of PirB enhances axonal regeneration of RGCs in vitro.

Taken together, our data above suggest that PirB play important roles in inhibiting axonal regeneration of RGCs. These result showed that PirB is a necessary receptor for Nogo66, MAG and OMgp. Our observation will help to promote going efforts to further elucidate the role of molecular mediators of optic nerve outgrowth inhibition.

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Disclosure of conflict of interest

None.

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