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Original Article

Transfection with AAV-NgR1siRNA on axonal regeneration of optic nerve after injury

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Abstract: Nogo receptor (NgR) play important role of inhibition axonal regeneration after central nervous system (CNS) injury. Rat retinal ganglion cells were transfected with AAV-NgR1siRNA in vivo and vitro to investigate its effect on axonal regrowth. NgR protein expression was assayed by western blot. The sections of cultured RGCs and optic nerve (ON) were stained with GAP-43 antibody. There is little expression of NgR after transfection of AAV-NgR1siRNA compared with control group. There was a significant difference of axonal length between experimental and control group. The present finding indicates that NgR genes play an inhibitive role in the axonal regeneration of RGCs, while transfection of AAV-NgR1siRNA is an effective way to enhance axonal regeneration of ON after injury.

Keywords: Nogo, receptor, RNA interference, axonal injury, regeneration

Introduction

CNS neurons can't regenerate after injury due to the proteins associated with myelin. The C-terminal of NogoA (Nogo66) [1, 2], myelin-associated glycoprotein (MAG) [3, 4], and Omgp [5] play important role to inhibit axonal regeneration via the Nogo receptor (NgR) [6, 7]. The Nogo gene has three isoforms (Nogo-A, -B and -C) and Nogo-A was confirmed to exert its role through NgR.

The NgR family has three CNS-linked proteins (named NgR1, NgR2 and NgR3) [8-10]. NgR1 has been considered served as the ligand binding to p75NTR [11]. It is reported that NgR1 knockdowned by antibodies [12, 13] or short haipin RNA [14] indicating NgR be essential for myelin inhibition.

In present study, we will use siRNA-mediated deletion of NgR to clarify the possible NgR contribution in inhibiting the axonal regeneration of retinal ganglion cells (RGCs).

Materials and methods

Animal grouping

Sprague Dawley rats (160-180 g m), fifteen to nineteen weeks old, were used in our experiment. Transfection with AAV-NgRsiRNA was used as experimental group (group A). Group B was transfected with vehicle only, and blank control was group C. There were seven rats in each group. The investigation complied with the ARVO Statement for the Use of Animals and Ophthalmic and Vision Research.

Surgical procedure

1% sodium pentobarbital solution (50 mg/kg body weight) was injected into abdomen cavity before the ON was crushed. The ON was crushed at 1 mm distal to the eyeball by an ON forceps with 40 g pressure (Martins Instruments, Tullingen, Germany, donated by Professor Gu Zhao-bin, Gifu University of Japan), for 9 s after the dural sheath of the ON was opened longitudinally. The vascular integrity of the retina was examined by funduscope after dilating the pupil with atropine. After withdrawing 2 μl of vitreous body from the eye of both eyes, 1 μl (20 μM) of AAV-NgRsiRNA (experimental group), negative control (negative control group) or PBS (blank control group) was injected into the vitreous body using a micropipette avoiding the lens of injury.

Preparation for immunohistochemistry examination

The animals were perfused through the aorta artery with heparin saline, 4% paraformalde-

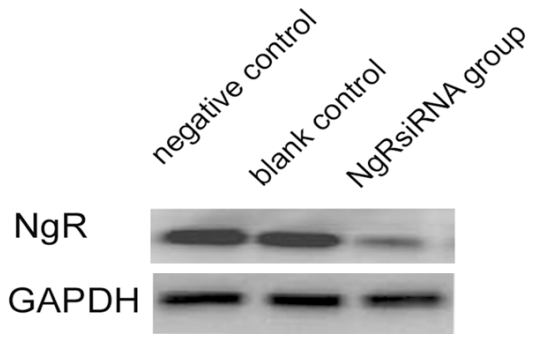

Figure 1. There is little expression of NgR protein after transfection of NgR shRNA (lane 1). Expression of NgR protein can be seen in negative control group (lane 2). Expression of NgR protein can be seen in blank control group (lane 3).

hyde successively seven days after ON crush. Eyes with ON segments up to the optic chiasm were transferred to a 30% sucrose solution overnight after separated and postfixed overnight (4°C). Frozen sections (15 μm) were cut longitudinally and stored at -80°C.

Immunofluorescence staining

GAP-43 monoclonal antibody (1:500 dilution) (Serotec, Raleigh, USA) was used in all cases. Then secondary antibodies (1:500 fluorescein-conjugated anti-sheep IgG) were used, and coverslipped with Vectashield mounting medium (Vector laboratories, Burlington, Canada).

Axon regeneration quantitation

The amounts of GAP-43-positive axons from the crush position were counted and divided by the cross-sectional width of the nerve in six longitudinal sections per case, under 400 magnification. The number of axons per unit width of optic nerve was averaged across the six sections to calculate the total number of regenerated axons [15, 16].

Isolation and culture of retinal ganglion cells

The retinas were washed three times in Hank's balanced salt solution after they were dissected under an anatomic microscope (SZ-PT, Olympus, Tokyo, Japan).

The retinal tissue was dissociated into single cells in 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) (Leon S et al., 2000; Yin Y et al., 2003). 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 (Invitrogen, California, USA). 2.5×10^5 cells were then cultured into polylysine coated 24-well plates and incubated at 37°C with 5% CO_2 ventilation.

Transfection and NgR knockdown in vitro

3 μl hiperfect transfection reagent, 50 nmol/L AAV-NgRsiRNA and 100 μl DMEM without supplements were added into per well of a 24-well plate. The complexing reaction continued for 20 min at room temperature. One hundred microlitres of the siRNA-hiperfect transfection reagent complex (Qiagen inc, Valencia, CA, USA) were added to each well. RGCs were cultured for seven days. Then washed twice with phosphate-buffered saline (PBS) and lysed.

Western blot

Cell lysates were prepared in extraction buffer. Equal amounts of total protein (10 μg) were transferred to Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) after separated by 10% SDS-PAGE. After blocking with 5% nonfat dry milk in PBS with 0.1% Tween-20, membranes were probed with rabbit anti-NgR66 antibody (1:500, Zymed laboratory Inc, USA), followed by incubation with goat anti-rabbit secondary antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein bands was visulized by the enhanced chemiluminescence kit (Santa Cruz Biotechnology).

Immunocytochemistry

Cells were incubated with anti-GAP43 antibody (1:500) at 4°C overnight after fixed with 4% paraformaldehyde for 10 min, followed by secondary antibody goat-anti-rabbit IgG (1:500) for 1 hr at room temperature. After that, SABC was used at 37°C for 30 min and colored with DAB. After coloration with DAB, dehydration, and dimethyl benzene treatment, slides were mounted. Controls were stained by omitting the primary antibody.

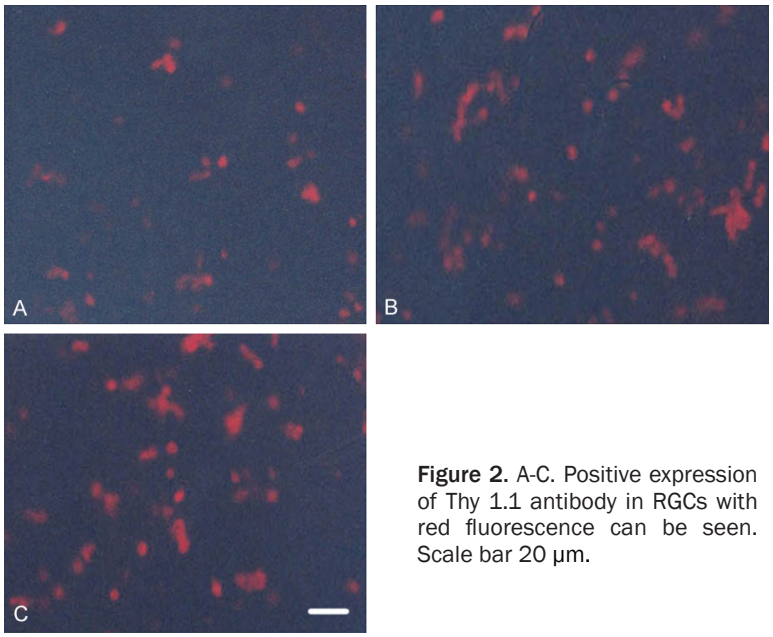

Figure 2. A-C. Positive expression of Thy 1.1 antibody in RGCs with red fluorescence can be seen. Scale bar 20 μm.

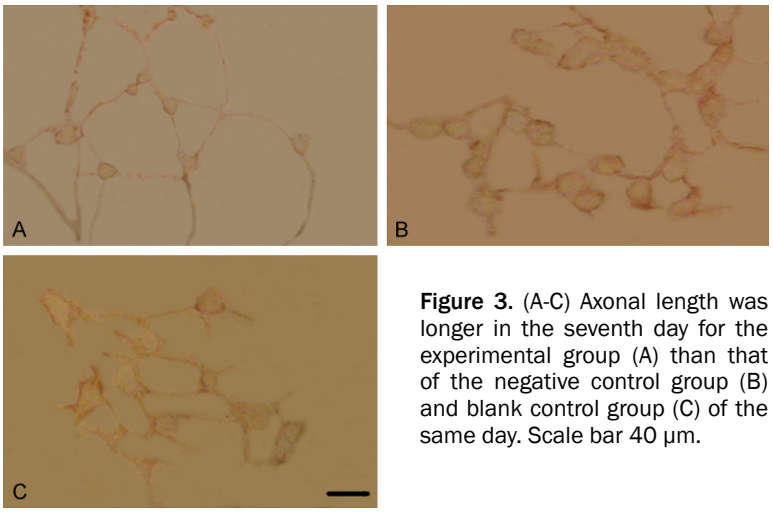

Figure 3. (A-C) Axonal length was longer in the seventh day for the experimental group (A) than that of the negative control group (B) and blank control group (C) of the same day. Scale bar 40 μm.

Statistical analysis

The computations were done using the software of SPSS version 18.0 for Windows. Difference between mean values was calculated for statistical significance with student t test, with a *P* value <0.05 indicative of statistical significance.

Results

Expression of NgR protein by western blot

Little expression of NgR protein after transfection of AAV-NgRsiRNA was detected (**Figure 1**, lane 3), however, expression of NgR protein can be found in negative control group (**Figure 1**, lane 1) and blank control group (**Figure 1**, lane 2). The result showed that transfection with AAV-NgRsiRNA achieved significant decline of NgR expression.

Expression of Thy 1.1 by immunofluorescence

Adherence of RGCs began at 12 h after they were seeded into poly-lysine-coated 24-well plates and incubated at 37°C with 5% CO_2 ventilation. Then a single layer of round and oval RGCs was seen on the plates. Immunochemistry showed positive Thy 1.1 staining in cultured RGCs with red fluorescence (**Figure 2A-C**).

In vitro axonal growth of the RGCs in experimental and control groups

Axonal length of the experimental group (**Figure 3A**) was longer in the seventh day than that in negative control group (**Figure 3B**) and blank control group (**Figure 3C**) on the same day.

For neurite length determinations, the neurites were traced and the morphometry analysis was performed using Image-Pro Plus version 4.0 (Media Cybernetics, Silver Spring, MD). An average of 50 neurons from each group was selected from a number of chamber slides. The difference in axonal length of group A (AAV-NgRsiRNA) increased from the first day to the seventh day. The axonal length of groups B and C increased slowly. There is significant difference of axonal length among experimental group, negative control and blank control group on the seventh day (**Table 1**).

Expression of GAP-43 in vivo

Axon of positive GAP-43 expression distributes along the longitudinal axis of optic nerve. We

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	825.15±1.25	13.35±1.59	11.35±1.25	32.25	<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	3.15±1.26	3.13±1.15	3.12±1.13	26.23	>0.05
3	25.12±2.25	8.16±2.12	8.16±2.15	32.15	<0.01
7	12.13±2.21	6.18±2.13	6.23±2.12	28.26	<0.01
F	41.35	33.26	35.15		
P	<0.01	<0.01	<0.01		

found that GAP-43 expression of optic nerve increased significantly with the survival time. There was a significant difference in axonal length ≥ 1 mm from the crush position between experimental and negative control or blank control group (**Table 2**).

Discussion

It was confirmed that there is significant axonal regeneration after corticospinal tract injury in Nogo-A/B-/- mice [17-19]. However, there is no significant regeneration found in either Nogo-A/B-/- or Nogo-A/B/C-/- mice [20, 21]. It is believed that the various capacity after Nogo knockdown because of the different backgrounds of the mice in these experiments. Our previous research found that elimination of either Nogo-A or Nogo-A/B/C can effectively enhance axonal regeration of optic nerve after injury [22, 23].

It was showed that NgR knockout enhance axon regeneration after spinal injury [24]. But Zheng et al. [25] can't observe corticospinal regeneration in NgR knockout mice.

RNA interference was showed to be effective way to knockdown gene expression [26-28]. We detected the expression level of NgR by western blot and found that there is little expression of NgR protein after transfection in experimental group. However, expression of NgR can be detected in negative control and blank control groups. There is significant difference between experimental group and negative control or blank control group. The above results confirm that it is possible to knockout of NgR by transfection with AAV-NgRsiRNA in retina. We concluded that NgR can be successfully knockdowned by transfection with AAV-NgRsiRNA. Knockdown of NgR make it possible for us to research on effect of NgR on axonal regrowth of RGCs in vivo and vitro.

Expression of GAP-43 in RGC cultured was also found for seven days in both experimental and control group. Our data indicate that transfection with AAV-NgRsiRNA can effectively enhance axonal regrowth of RGCs in vivo and vitro. As discussed above, RGCs have instinctive competence of axonal regeneration in vivo and vitro in experimental group. Our result also showed that axonal length of experimental group increased from the first day to the seventh day. The axonal length of the RGCs in group B control group grows slowly. There is significant difference in axonal lengths of RGCs between experimental group and control group on the seventh day. Extinguishing of NgR enhances axonal regeneration of RGCs in vitro. Our data indicated that NgR play an important role for inhibition of axonal regeneration of RGCs.

Taken together, in view of the growing interest in NgR as a target for treatment after optic nerve injury, it is our hope that the data presented here 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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