Nogo-A antibody treatment enhances neuron recovery after sciatic nerve transection in rats


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Abstract. – OBJECTIVE: The use of an antibody to block the neurite outgrowth inhibitor Nogo-A has been of great interest for promoting axonal recovery as a treatment for peripheral nerve injuries. The present study aimed to investigate the signaling pathway of p75 neurotrophin receptor (NTR) and Nogo receptor (NgR) in a sciatic nerve transection (SNT) rat model and evaluate the underlying mechanisms.

MATERIALS AND METHODS: Seventy-five Sprague-Dawley (SD) rats were randomly divided into 3 groups (n=25), namely the sham group, sciatic nerve transection (model) group and Nogo-A-pAb group. Following euthanasia, spinal cord and sciatic nerve of the operation site were harvested, fixed in formalin. Hematoxylin and eosin (HE) staining was used to evaluate the sciatic nerve pathology. The mRNA and protein expression levels of Nogo-A, NTR were assessed by Real-time polymerase chain reaction (RT-PCR) and Western blotting, respectively.

RESULTS: Histology showed enhanced regeneration of spinal axon in the anti-Nogo-A antibody group. At 48 hours after operation, mRNA of Nogo-A and NTR were higher in model group compared with control group. mRNAs were at their highest levels at 1 week, while these were at normal levels after 4 weeks in Nogo-A-pAb group. The protein levels of Nogo-A and NTR were higher in model group compared with sham-operation group at 1-week after operation; Nogo-A-pAb could reduce these proteins levels.

CONCLUSIONS: The results suggested Nogo-A antibody might represent a promising repair strategy to promote recovery following SNT.

Key Words:
Sciatic nerve transection, Nogo-A, NTR.

Introduction

Peripheral nerve injury is an under-appreciated clinical problem, even though it is more common than injury to the central nervous system. Repair and regeneration of peripherally injured nerves is an extremely complex process. Several myelin components, including the extracellular domain of Nogo-A (Nogo-66), oligodendrocyte myelin glycoprotein (OMgp) and myelin-associated glycoprotein (MAG), exert their inhibiting neurite outgrowth effects through the same Nogo receptor (NgR). Also NgR requires additional transmembrane protein to transduce the inhibitory signals into the interior of responding neurons. The p75 neurotrophin receptor (NTR), a member of the tumor necrosis factor receptor superfamily, specifically interacts with NgR. Blocking the p75-NgR interaction also reduces the activities of neurite outgrowth inhibitors. Another study demonstrated that a truncated p75 NTR protein lacking the intracellular domain attenuated the same set of inhibitory activities. Thus, elucidation of the mechanisms that modulate p75 NTR-Nogo-A mediated signaling may increase our understanding of neural development and nerve injury.

Materials and Methods

Animals

Adult Sprague-Dawley rats, weighting 180-220 g, obtained from the Experimental Animal
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Center of Suzhou Aiermaite Technology Co. Ltd. (Suzhou, Jiangsu, China), which were housed in a 12-h light-dark cycle with free access to food and water. Before experiments, the animals were allowed to habituate to the housing facilities for a week. The present study was conducted with approval from the Animal Ethics Committee of Yantaishan Hospital. Rats were randomly divided into 3 groups (n = 25), namely the sham group, sciatic nerve transection (model) group and Nogo-A-pAb group.

**Surgical Procedure**

Animals were anesthetized with 10% chloroform hydrate [350 mg/kg, intraperitoneal injection (i.p.)], the left thigh was shaved, sterilely prepped, and draped. A 5-cm incision was carefully made to the buttock through the gluteal muscles exposing the sciatic nerve. The sciatic nerve at 0.8 cm distal end of piriformis was sharply transected with a scalpel followed by ligation of the two severed ends with sutures. The sciatic nerves of rats in sham-operated control group were exposed, but no deliberate injury was performed. The rats of Nogo-A-pAb group were administrated 0.2 mL Nogo-A-pAb i.p. after surgery. The wound was then closed and secured using suture clips. The wound was treated with antibiotics.

**Tissue Collection**

Animals of each group were subdivided into five subgroups. The rats were euthanized at 24-h, 48-h, 1-week, 2-week and 4-week. Meticulous microsurgical dissection was then performed to carefully harvest the sciatic nerve and spinal cord. The proximal and distal ends were identified and stabilized on cork squares where they were fixed in 10% formalin solution for 24 hours, then rinsed and placed in a phosphate buffer (pH 7.4) solution for storage at 4°C.

**Histopathological Analysis**

The nerves were dehydrated in increasing concentrations of ethanol, rinsed with Histoclear and embedded in paraffin and cut on a microtome into 5 µm thick slices from proximal end to distal end according to previously described. After dewaxing, slides were boiled for 48 hours at 45°C, and then for 1 hour at 60°C. Sections were stained with hematoxylin and eosin. Briefly, they were placed in hematoxylin for 5 min, then washed with tap water and left for 5 min, followed by rinsing in 1% acid alcohol and washing in tap water. After that, sections were added in eosin solution (1%) for 5 min, followed by washing in tap water, dehydrated. All stained sections assessed under light microscope.

**Nogo-A and NTR mRNA Assay**

The relative levels of Nogo-A and NTR transcription were determined by RT-PCR (n=5, each group). Total RNA was extracted with Trizol reagent (Gibco, Grand Island, NY, USA), and 1 µg of each isolated RNA was subjected to cDNA synthesis. RT cDNA synthesis was conducted in a 14 µl reaction buffer, containing 1 µl reverse transcriptase (50 U) and 1 µl ologe (dT) primer, according to manufacturer’s instructions (Takara, Otsu, Shiga, Japan). With the obtained for cDNA as a template, the relative expression levels of Nogo-A and NTR from rats receiving experimental treatment were determined by polymerase chain reaction (PCR). The sequence of the primers for RT-PCR are as follows: Nogo-A, Forward, 5'-CGCTGGTGCTTCTGTAGTGC-3'; Reverse, 5'-CTTTGTGCTGGGCTACTG-3' (30 cycles); NTR, Forward, 5'-TCTGACATTAGGCGTCTC-3', and Reverse, 5'-CCAGCCCACACTTCTCTTC-3' (30 cycles) β-actin, Forward, 5'-CTTTCTGGGATGGAGTCCTG-3' and Reverse, 5'-GGAGCAATGATCCTTCCTC-3' (30 cycles). Each 20 μl reaction system comprised 2 µl of cDNA, 10 µl SYBR Premix Ex Taq II, 10 µmol/l of both sense and antisense primers. For normalization, β-actin was used to normalize mRNA. The final PCR products were analyzed on an agarose gel, and the relative intensity was determined using semi-quantitative densitometry.

**Nogo-A and NTR Western Blot Analysis**

The protein samples from each group were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The spinal cords were homogenized in lysis buffer (20 mM Tris, 1% TritonX-100, 0.05% SDS, 5 mg of sodium deoxycholate, 150 mM NaCl and 1 mM PMSF) containing protease and phosphatase inhibitor cocktail. The protein concentrations were determined using a BCA Protein Assay reagent kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein (40 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, the non-specific binding of antibodies were blocked with 5% non-fat dried milk in PBS and then incubated with the primary antibodies rabbit monoclonal anti-Nogo-A, 1:500 (Santa Cruz Biotechnology,
Santa Cruz, CA, USA), rabbit monoclonal anti-NTR, 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse polyclonal anti-β-actin, 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used followed by the application of the secondary antibodies consisting of, HRP-conjugated goat anti-rabbit IgG, 1:5000 (Wuhan Boster Biological Technology, Ltd, Wuhan, Hubei, China). The protein band images were collected and the relative optical density (ROD) was analyzed with molecular image, ChemiDocXRS+Image System (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analysis
Data statistic analysis was performed by SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and all data were expressed as mean values ± standard deviation. Changes between samples were compared by Student’s t-test, and difference between groups were compared by the method of one-way ANOVA. To validate the ANOVA, the LSD test was used. p<0.05 was considered statistically significant.

Results

Histopathological Analysis
In the sham group, the myelinated nerve fibers and sheaths were uniformly distributed and there was no inflammatory cell infiltration. By contrast, in the model group, the myelinated nerve fibers were sparsely distributed, the thickness of sheaths was uneven, and the axons were bulky (Figure 1).

Also there were a large number of infiltrated lymphocytic cells (including macrophage, neutrophil and T lymphocyte) in model group at 48 hours after operation, while the degree of degeneration was ameliorated in Nogo-A-pAb group. Animals that received anti-Nogo-A Ab treatment began to exhibit improvement at 2 weeks after treatment and myelinated nerve fibers and sheaths demonstrated no difference compared to sham group. However, the vacuolization of nerve fiber with decreased myelin was even worse in model group at 4 weeks (Figure 2).

Nogo-A and NTR mRNA Assay
This experiment conducted in the current study sought to examine alterations in the expression of Nogo-A and NTR in the sciatic nerve after transection. There was no difference between groups of Nogo-A and NTR mRNA expression levels at 24 hours after operation. Compared with the sham group, the Nogo-A and NTR mRNA expression levels were found to rise at 48 hours in model group and this difference was significant (p<0.05). The upregulation of Nogo-A and NTR expression reached a maximum at 1 week after transection (p<0.01) and lasted for 4 weeks (p<0.05). When comparing the response of the Nogo-A antibody group and model group, the anti-inhibitory effect of the Nogo-A antibody was observed at 48 hours and 2 weeks after injury (p<0.05, Figure 3). As shown in Figure 3, at the end of the experiment, animals treated with anti-Nogo-A antibody demonstrated a significant improvement of mRNA levels of Nogo-A and NTR, when compared to the
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model group ($p<0.05$), with no difference between sham group and Nogo-A antibody group. These results demonstrated that neutralization of Nogo-A with a specific function-blocking antibody started at 48 hours after sciatic nerve injury can result in remarkable improvement on the mRNA levels of Nogo-A and NTR.

**Nogo-A and NTR Western Blot Analysis**

At 24 hours after operation, there was no difference between groups of Nogo-A and NTR protein expression levels ($p>0.05$, Figure 4). After sciatic nerve transection, the mean protein levels of Nogo-A and NTR were increased significantly in model group compared to sham-operation group from 48 hours to the end of the study ($p<0.05$, Figure 3). At 48 hours after injury, the significant improvements in protein levels of Nogo-A and NTR were found in the Nogo-A-pAb group when compared to the model group ($p<0.05$), with no difference between sham group and Nogo-A antibody group. At 4 weeks after surgery, the protein levels decreased significantly in Nogo-A-pAb group compared to the model group ($p<0.05$), with similar levels of sham group. These results again suggested that anti-Nogo-A antibody treatment could reduce the protein levels of Nogo-A and NTR, which was reduced by sciatic nerve injury.

**Discussion**

The results of this study demonstrated that the production of Nogo-A and NTR mRNA and protein is stimulated 48 hours after sciatic nerve transection, and such nerve injury induced upregulation of Nogo-A and NTR could be suppressed by treatment with anti-Nogo-A antibody. When peripheral nerve is damaged, nerve fibrosis is thought to interfere with growth, create a mechanical barrier to the sprouting axons and impair normal function. Studies have shown that anti-Nogo-A immunotherapy improves functional recovery, neuro-regeneration, and compensatory fiber growth after nerve injury. Nogo protein is one of the myelin-associated growth inhibitory proteins and is thought to play an important role in spinal cord regeneration. Nogo-A, a high-molecular-weight membrane protein (1,163 amino acids), is a myelin-related azonal growth inhibitory factor secreted by oligodendrocytes and stored in the
white matter of the central nervous system, which is rich in myelinated membranes. Intracellular Nogo-A is released by oligodendrocytes and myelin into the extracellular matrix after spinal cord injury. At the injury sites, Nogo-A binds Nogo receptor (NgR) on the nerve and inhibits its regeneration. The NgR needs to form a complex with the p75 neurotrophin receptor (NTR) to mediate this inhibitory signal. NTR mediates nerve growth inhibition by myelin-associated inhibitor via functioning in part as a co-receptor of NgR.

In the present study, the results demonstrated that the sciatic nerve transection model had low protein and mRNA expression of Nogo-A and NTR at 24 hours after injury, and rapidly rose to a peak at 1 week and gradually declined at 4 weeks in model group. Compared with sham-operated group, the expression of Nogo-A and NTR pro-

Figure 3. Comparison of mRNA levels of Nogo-A, NTR in all experimental groups by RT-PCR (mean ± SD). *p<0.05, **p<0.01, compared with Sham group; ’p<0.05, ’’p<0.01, compared with model group at the corresponding time points.

Figure 4. Comparison of mRNA levels of Nogo-A, NTR in all experimental groups by RT-PCR (mean ± SD). *p<0.05, **p<0.01, compared with Sham group; ’p<0.05, ’’p<0.01, compared with model group at the corresponding time points.
tein and mRNA were significantly increased after 48 hours ($p<0.05$). This confirmed that sciatic nerve injury was responsible for the high expression of Nogo-A and NTR. Soon after the growth inhibitory properties of myelin were discovered, the antibody was produced against Nogo-A. The antibody had a strong neutralizing activity for neurite growth inhibition exerted by Nogo-A. In vivo study of the antibody in the spinal cord of rats demonstrated that it enhanced compensatory fiber growth from unlesioned tract system. These anatomical findings were paralleled by improved functional recovery after spinal cord injury and brainstem lesions. Those results suggested that the anti-Nogo-A antibody could penetrate into the lesion site and block the Nogo-A molecules, which were likely expressed on the lesioned sites. As a result, axons at the site of injury no longer recognize the environment as inhibitory and regeneration of these axons occurs. In this study, the anti-inhibitory effect of the Nogo-A antibody was observed at 48 hours and 2 weeks after injury and also at the end of the experiment, animals treated with anti-Nogo-A antibody demonstrated a significant declined of mRNA and protein levels of Nogo-A and NTR with no difference between sham group and Nogo-A antibody group. Although it remains to be determined whether other proteins are present in the Nogo-A and NTR signaling pathway that mediates the inhibitory activities of Nogo-A, our data showed that NTR is a required factor that participates in propagating the inhibitory signals into responding neurons.

**Conclusions**

These results indicated that Nogo-A plays an important role in sciatic nerve transection model. Transected sciatic nerve injury induced an upregulation of Nogo-A and NTR expression in injured neurons. The anti-Nogo-A antibody could suppress the upregulation and intervene with NTR and Nogo-A signaling after sciatic nerve transection.

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**Conflict of interest**

The authors declare no conflicts of interest.

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