Nanoparticles carrying neurotrophin-3-modified Schwann cells promote repair of sciatic nerve defects

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Abstract

Schwann cells and neurotrophin-3 play an important role in neural regeneration, but the secretion of neurotrophin-3 from Schwann cells is limited, and exogenous neurotrophin-3 is inactivated easily in vivo. In this study, we have transfected neurotrophin-3 into Schwann cells cultured in vitro using nanoparticle liposomes. Results showed that neurotrophin-3 was successfully transfected into Schwann cells, where it was expressed effectively and steadily. A composite of Schwann cells transfected with neurotrophin-3 and poly(lactic-co-glycolic acid) biodegradable conduits was transplanted into rats to repair 10-mm sciatic nerve defects. Transplantation of the composite scaffold could restore the myoelectricity and wave amplitude of the sciatic nerve by electrophysiological examination, promote nerve axonal and myelin regeneration, and delay apoptosis of spinal motor neurons. Experimental findings indicate that neurotrophin-3 transfected Schwann cells combined with bridge grafting can promote neural regeneration and functional recovery after nerve injury.

Key Words
neural regeneration; peripheral nerve injury; neurotrophin-3; nanoparticle liposome; Schwann cells; sciatic nerve; neuroelectrophysiology; gene transfection; grants-supported paper; neuroregeneration

Research Highlights
(1) Neurotrophin-3 can be successfully transfected into Schwann cells via a nanoparticle carrier, where it is effectively expressed at steady levels.
(2) A composite of neurotrophin-3 transfected Schwann cells and poly(lactic-co-glycolic acid) copolymer biological conduit for nerve defects can effectively promote sciatic nerve regeneration and reduce motor neuronal apoptosis.

INTRODUCTION

Schwann cells are the most important factor in the microenvironment of peripheral nerve regeneration, and can secrete neurotrophic factors that repair damaged nerve, but their presence can be limited at times[1-3]. Neurotrophin-3 is a crucial factor in the regenerative milieu and exerts a variety of physiological effects on nervous system development. It is known to improve motor neuron survival by either a paracrine or autocrine mechanism. In addition, they promote the formation of neuromuscular junctions and axonal outgrowth[4-6].
However, exogenous neurotrophin-3 is very easy deactivated in the body, and has a low absorption rate, so its clinical effectiveness is poor\[7\]. Furthermore, there is no available therapy for its effective transfer.

Gene therapy is described as the delivery of nucleic acids to patients via a vector\[8\]. There are two kinds of vectors used for gene delivery, viral and non-viral. Viral vectors have the potential to induce an immune response and low safety\[9-10\]. Synthetic non-viral vector systems have lower toxicity and immunogenicity; however, they have low efficiencies\[11-12\]. Nanoparticle carriers could be potentially applied for cell and drug targeted sustained delivery of various therapeutic agents as they have simpler quality control and substantially easier pharmaceutical and regulatory requirements\[13-15\]. Diksha and Roy\[16\] have recently used silicon nanoparticles as gene carriers in the transfection of pcDNA.

Development of highly efficient non-viral gene delivery vectors still remains a great challenge. In this study, we reported a new gene delivery vector based on nanoparticles with significantly higher gene transfection efficiency. This study represents a new approach to repair nerve defects by applying ex vivo nanoparticle carrier plasmid-transfected Schwann cells for axonal regeneration. We examined the mechanisms of neurotrophin-3-transfected Schwann cells on functional recovery and axonal regeneration after nerve injury.

RESULTS

Schwann cell purity increased after neurotrophin-3 gene transfection

Boundaries of Schwann cells were clear, with oval or spindle shape. Both ends had prominences, the cytoplasm was deeply stained and the cell nuclei were lightly stained by inverted phase contrast microscopy. Flat fibroblasts were negative after 1 week of neurotrophin-3 gene transfection, and Schwann cells rapidly grew forming an interconnected network, with a high purity (Figure 1). The number of cells was increased after neurotrophin-3 transfection of Schwann cells, compared with primary cultured Schwann cells. This indicates that the purity of Schwann cells was increased following transfection ($P < 0.01$; Figure 2).

Quantitative analysis of experimental animals

Ninety-six adult rats were randomly divided into four groups ($n = 24$): model group, Schwann cells group, neurotrophin-3 group, and neurotrophin-3 + Schwann cells group. One rat in the model group died of wound dehiscence at 28 days and one rat in the Schwann cells group died of wound infection at 41 days. The remaining 94 rats were involved in the final analysis.

Neurotrophin-3 mRNA expression increased in the neurotrophin-3 gene transfected Schwann cells

At day 7 after transfection, neurotrophin-3 transgene expression in Schwann cells was examined by real-time PCR. The sciatic nerve injury tissue from rats in the pcDNA3.1(+)–neurotrophin-3-transfected Schwann cells expressed higher levels of neurotrophin-3 mRNA than that in the control group (Figure 3).

Neurotrophin-3 gene-modified Schwann cell transplantation increased electromyographic activity in sciatic nerve injured rats

At 12 weeks after sciatic nerve injury, the latency was prolonged, amplitude was shortened, and motor nerve conduction velocity was extended in the Schwann cells group and neurotrophin-3 group compared with the model group ($P < 0.01$). There was no significant difference between the Schwann cells group and neurotrophin-3 group. The neurotrophin-3 + Schwann
cells group was better than Schwann cells group and neurotrophin-3 group regarding the above indicators ($P < 0.05$; Table 1).

The Schwann cells group and neurotrophin-3 group showed greater myelin thickness, nerve fiber diameter, axonal number, percentage of nerve tissue area, and survival rate of motor neurons than the model group ($P < 0.05$), but there were no significant differences between the Schwann cells group and the neurotrophin-3 group. The above properties in the neurotrophin-3 + Schwann cells group were increased compared with the Schwann cells group and neurotrophin-3 group ($P < 0.05$; Table 2).

Transplantation of neurotrophin-3 gene modified Schwann cells improved the morphology of injured sciatic nerve
Under the light microscope and electron microscope, regenerating nerve fibers in rats of the model group sparse and irregular, the myelin sheath was thin, and the nutrient vessels were sparse. Compared with model group, regenerating nerve fibers in rats of the Schwann cells group and neurotrophin-3 group were more dense and orderly arranged, the myelin sheath was thicker, and the nutrient vessels were increased, but the fibers were rarely seen around the scar. There was no significant difference between the Schwann cells group and neurotrophin-3 group. In contrast, the morphology of the sciatic nerve in the neurotrophin-3 + Schwann cells group was improved significantly (Figure 5).

The Schwann cells group and neurotrophin-3 group showed greater myelin thickness, nerve fiber diameter, axonal number, percentage of nerve tissue area, and survival rate of motor neurons than the model group ($P < 0.05$), but there were no significant differences between the Schwann cells group and the neurotrophin-3 group. The above properties in the neurotrophin-3 + Schwann cells group were increased compared with the Schwann cells group and neurotrophin-3 group ($P < 0.05$; Table 2).

Transplantation of neurotrophin-3 gene-modified Schwann cells reduced the apoptosis of spinal cord anterior horn motor neurons in injured sciatic nerve
The apoptosis rate of spinal cord anterior horn motor neurons in the Schwann cells group and neurotrophin-3 group was significantly reduced compared with the model group ($25.02 \pm 0.93 \%$ and $20.95 \pm 0.92 \%$ vs. $65.01 \pm 1.64 \%$, $P < 0.05$), and no significant difference was found between the Schwann cells group and neurotrophin-3

Table 1  Electromyograph recorder of regenerating nerve fibers in sciatic nerve of rats at 12 weeks after transfection

| Group          | Latency (ms) | Amplitude (mV) | Motor nerve conduction velocity (m/s) |
|----------------|-------------|----------------|--------------------------------------|
| Model          | 1.12±0.11   | 18.01±1.25     | 17.6±0.9                             |
| SCs            | 1.62±0.10   | 13.55±1.32     | 21.4±0.9                             |
| NT-3           | 1.64±0.12   | 14.32±1.35     | 22.6±0.8                             |
| NT-3+SCs       | 2.11±0.12   | 12.13±1.39     | 24.8±1.0                             |

Data are expressed as mean ± SD of 12 rats in each group. $^{a}P < 0.01$, vs. model group; $^{b}P < 0.05$, vs. neurotrophin-3 (NT-3) + Schwann cells (SCs) group (two sample t-test).

Table 2  Diameter and cross-section area of regenerating nerve fibers in rats at 12 weeks after transfection

| Group          | Thickness of myelin (μm) | Fiber diameter (μm) | Axonal number (/mm) | Percentage of nerve tissue area (%) |
|----------------|--------------------------|---------------------|---------------------|------------------------------------|
| Model          | 0.56±0.18                | 4.58±1.19           | 9 846±629            | 25.7±1.9                           |
| SCs            | 0.86±0.23$^{a}$          | 7.15±1.98           | 10 372±781$^{a}$    | 30.8±2.2$^{a}$                     |
| NT-3           | 0.91±0.25$^{ab}$         | 7.49±2.06           | 10 659±837$^{ab}$   | 32.1±2.4$^{ab}$                    |
| NT-3+SCs       | 1.05±0.29$^{a}$          | 8.12±2.13$^{a}$     | 13 163±935$^{a}$    | 40.1±2.8$^{a}$                     |

Data are expressed as mean ± SD of 12 rats in each group. $^{a}P < 0.01$, vs. model group; $^{b}P < 0.05$, vs. neurotrophin-3 (NT-3) + Schwann cells (SCs) group (two sample t-test).
group. The apoptosis rate in the neurotrophin-3 + Schwann cells group was lower than that in the Schwann cells group and neurotrophin-3 group (P < 0.05), accounting for 14.03 ± 0.82%.

DISCUSSION

Schwann cells have a great potential to repair damaged nerve. It is very important for facilitating nerve regeneration and protecting neuronal survival, mainly in the early active stages[1,17-18]. Furthermore, Schwann cells are a perfect target for gene therapy of nerve injury. A great many of apoptotic cells appear following nerve injury. Armstrong et al[19] showed that regeneration at the distal nerve following 8 weeks of denervation was significantly impaired compared with earlier time points. However, Kou et al[22] showed that there were no differences in the number of axonal sprouts in the distal nerve stump between denervation periods of 2 and 6 months. Neurotrophin-3 can maintain the survival of sensory, motor and sympathetic neurons, prevent neuronal apoptosis after spinal cord injury, and induce axonal regeneration[4]. We investigated the combined use of Schwann cells and neurotrophin-3, which not only increased the number and thickness of axons, the diameter of nerve fibers, the percentage of nerve tissue area in the distal stump, but also promoted the survival of ventral horn motor neurons after spinal cord injury.

Nanoparticle carriers have shown a great potential as a platform for delivering drugs into biological systems due to their biocompatibility, high loading capacity, and ability to cross cellular membranes[21-22]. These particles have imparted sustained-release and hydrophilic characteristics to water-insoluble drugs, and have opened up the possibilities for systemic administration of highly effective, hydrophobic chemotherapeutic molecules[23-25]. In the present study, the purity of Schwann cells increased significantly after neurotrophin-3 transfection. This shows that the nanoparticle carrier could deliver neurotrophin-3 cDNA into Schwann cells effectively. Therefore, the nanoparticle carrier can be utilized as a gene delivery vehicle for cells and biological factors.

The detected indicators in neurotrophin-3 + Schwann cells group were superior to those in the Schwann cells group and neurotrophin-3 group. The present study provides a new approach to repair injured nerve by applying ex vivo gene-transfected implants in an attempt to create more conducive axonal regeneration[26]. This gene transfer technique provides a new strategy for the application of neurotrophin-3 and allows for the insertion of the neurotrophin-3 gene into Schwann cells, which produced neurotrophin-3 continuously and exerted physiological effects.

Our experimental findings indicate that compared with the model group, the Schwann cells group and neurotrophin-3 group had higher levels of motor nerve conduction velocity, number and thickness of axons, nerve fiber diameter, percentage of nerve tissue area, and survival of motor neurons in the ventral horn of the spinal cord. However, there was no significant difference between the Schwann cells group and neurotrophin-3 group. The apoptosis rate in the neurotrophin-3 + Schwann cells group was lower than that in the Schwann cells group and neurotrophin-3 group. Our experimental findings indicate that: (1) Schwann cells can be transfected efficiently with a nanoparticle carrier. (2) Neurotrophin-3 gene-transfected Schwann cells can improve the functional recovery of injured nerve by promoting nerve and axonal regeneration, compensating for the shortage of the low content of Schwann cells and neurotrophic factors, and reduce the apoptosis of ventral horn motor neurons after sciatic nerve injury.

MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
Experiments were performed from February 2010 to December 2010 in the Central Laboratory (Postdoctoral Training Base) of the First Affiliated Hospital of Xinxiang Medical College, China.

Materials
Animals
(1) Source of Schwann cells: 2-day-old newborn male Wistar rats of clean grade were used in this study.
(2) Grouping: A total of 96 adult male Wistar rats, including 24 males and 72 females, were provided by the Animal Laboratory of Xinxiang Medical College, in China with license No. SCXK (Yu) 2008-0001. All rats were raised under standard conditions with a 12-hour light/dark cycle, 35% humidity and accessed to water and food ad libitum. Experimental disposals were in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China[27].
Biomaterials
Extracellular matrix gel and neurotrophin-3 gene were provided by SBS Genetech Co., Ltd., Beijing, China. Poly(lactic-co-glycolic acid) biological conduit was provided by Sigma (Santa Clara, CA, USA).

Methods
Isolation and purification of Schwann cells
Two newborn Wistar rats were killed by intraperitoneal injection of 1% pentobarbital (30 mg/kg) and decapitated. The sciatic nerves were dissected, rinsed twice, minced and incubation at 37°C for 15 minutes with continual shaking. Dulbecco's modified Eagle's medium (Sigma) and Ham's F-12 (Sigma; 1:1 mixture) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) was added to stop the trypsinization and the specimen was briefly centrifuged. The cells were diluted to 1 × 10^6 cells/mL, following which they were selected with G-418.

pcDNA neurotrophin-3 transfection into Schwann cells with a nanoparticle carrier
Neurotrophin-3 was amplified by PCR and cloned into pcDNA neurotrophin-3 eukaryotic expression vector. Analysis by restriction enzyme digestion and DNA sequencing were performed to confirm the sequence of the plasmid, as described previously. We used a nanoparticle carrier liposome (Sbsbio; 10 mmol/L) in 50 μL of media for pcDNA neurotrophin-3 plasmid transfection into Schwann cells (1 × 10^6 cells/μL), following the manufacturer's instructions. The plasmids were diluted in 50 μL of Opti-MEM I Reduced Serum Medium without serum (Sigma) and mixed gently with the nanoparticle liposome before use, for 5 minutes. The diluted DNA was combined with the diluted nanoparticle carrier, and incubated for 20 minutes at room temperature, until they were ready to assay for transgene expression.

Schwann cell purity before and after neurotrophin-3 gene transfection as detected by immunohistochemical staining
The purity of Schwann cells was determined by S-100 immunohistochemistry after neurotrophin-3 gene transfection. The results were observed by inverted phase contrast microscopy (Nikon, Tokyo, Japan).

Establishment of sciatic nerve injury model and transplantation of Schwann cells
Adult Wistar rats were anesthetized by intraperitoneal injection of pentobarbital. The left sciatic nerve was exposed and 10 mm of nerve was excised, 2 mm from the sciatic macroporous outlet under a microscope at 16-fold magnification. A 15 mm poly(lactic-co-glycolic acid) biological conduit was used to connect the defected nerve ends. The sciatic nerve was bridged by 15 mm-long grafts. After mixing 10 μL Schwann cells, 10 μL pcDNA neurotrophin-3 plasmids, 10 μL neurotrophin-3-transfected Schwann cells (1 × 10^5/μL) and 10 μL extracellular matrix gel, 20 μL was collected using a microinjector and injected into the bridged biological conduit.

Neurotrophin-3 expression in Schwann cells at sciatic nerve injury site detected by real-time PCR
Real-time PCR was performed at day 7 after transfection to evaluate transgene expression. The total cellular mRNA was isolated from the injury area using Test Kit (Applied Biosystems, Forst, CA, USA), following the manufacturer's instructions. The real-time PCR reaction was performed in 25 μL volume containing 5 μL diluted cDNA, 7 μL of Power SYBR green (Applied Biosystems, Forst, CA, USA) and 2 μL primer dilution (1.75 μmol/L each), and mixed in a 96-well MicroAmp reaction plate (Applied Biosystems). Real-time PCR was performed using the StepOnePlus-thermocycler (Applied Biosystems). Reaction conditions were: 94°C for 10 minutes, followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

Electromyogram survey of sciatic nerve muscle electrical changes
The musculus gastrocnemius of each animal was examined at 12 weeks after transfection for motor nerve conduction velocity via electromyogram. Surveys from the bilateral sides were averaged and a single score was submitted for each animal at each testing session. Nerve electrophysiology was detected and analyzed by the POWERLAB polygraph (ADI Company, Australian).

Immunohistochemical detection of neurofilament expression in the sciatic nerve
Rats were sacrificed at 12 weeks after transfection, and sciatic nerves were harvested for immunohistochemical staining for neurofilament protein expression. In brief, the defected distal sciatic nerve was cut and fixed, embedded in paraffin, and then cut in 4-μm thick sections. Mouse anti-rat neurofilament SM131 antibody (1:1 000; Sternberger Monoclonals Inc., Baltimore, MD, USA) was diluted in 1% normal serum, applied to the tissue sections at 37°C for 1 hour and then at 4°C overnight. Sections were incubated with an appropriate biotinylated goat anti-mouse IgG (1:200; Vector Laboratories,
Apoptosis of spinal cord ventral horn motor neurons detected by terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) assay

Spinal cord from the contused site was cut and fixed in a 10% formalin solution for 2 hours, embedded in paraffin, and consecutively sliced into sections at 30-μm thickness. The apoptosis was detected by TUNEL assay. The TUNEL reaction mixture (Boster, Wuhan, Hubei Province, China) was dropped onto the spinal cord sections and incubated for 1 hour at 37°C under parafilm. TUNEL-positive cells (apoptotic spinal cord anterior horn motor neurons) were analyzed under a light microscope (Olympus, Tokyo, Japan). Five damage areas were randomly selected under non-overlapping 400-fold field of view at high magnification, and the number of apoptotic (positive staining was brownish-yellow) and total spinal cord anterior horn motor neurons were calculated. Spinal cord anterior horn motor neuron apoptotic rate (%) = number of apoptotic cells/number of total cells × 100%.

Statistical analysis

Statistical data were analyzed using SPSS 15.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Differences between groups were compared using two sample t-test, and a P < 0.05 value was considered statistically significant.

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Conflicts of interest: None declared.

Ethical approval: Animal experiments were approved by the Animal Ethics Committee of Xinxiang Medical College, China.

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