Original Article Implantation of neurotrophin gene modified bone derived mesenchymal stem cells to repair spinal cord complete transection injury in adult rats

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Abstract: Recovery from spinal cord injury (SCI) after mesenchymal stem cells (MSCs) implantation is minimal due to the limited capacity for the reduction in the cystic cavitation, the axonal regeneration, and neural plasticity in the spinal cord. We combined MSC implantation with neurotrophin gene therapy in an attempt to enhance regeneration and functional recovery after thoracic spinal cord complete injury in adult rats. Primary MSCs were transfected with reconstructed vectors encoding rat brain-derived neurotrophic factor (BDNF) or human neurotrophin-3 (NT-3). MSCs expressing *BDNF* or *NT*-3 or both were implanted into the transected spinal cord of rats. Enhanced regenerative sprouting of the fibers and recovery of hind limb function was observed in rats implanted with MSCs expressing BDNF and NT-3. Efficiency of the treatment was improved in dual combination of BDNF and NT-3 in MSCs. This combined neurotrophin genetic engineering of MSCs not only resulted in cells that were more effective in promoting axonal outgrowth but could also lead to enhanced recovery after SCI. Cell transplantation combined with two gene therapy provides better effect in the restoration of SCI.

Keywords: Spinal cord injury, gene therapy, cell transplantation, mesenchymal stem cells (MSCs), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3)

Introduction

Repairing of the spinal cord injury (SCI) has been one of the most challenging issues in the field of medical science at present, though series of progress have been made in the treatment during the last 20 years. New treatment methods tested and studied in the animal models has shown certain prospect in the clinical application, and the cell transplantation seems to be more promising in the treatment of SCI. Till date, various cell types were used for therapeutic transplantation for spinal cord injury. With the increase in the development of ce-Il therapeutic transplantation, some treatment strategies have been applied at the clinical level [1, 2]. But the overall effect of the above intervention is still limited, even the results are quite different obtained by the same method from different laboratories. In addition, few literatures report the achievement of promoting functional recovery and the recent issues around the olfactory ensheathing cell transplantation for SCI are challenging but still promising [3, 4]. In view of pathophysiological change and spinal regeneration failing after SCI, various researchers realized that rely on single means to achieve the good repair and functional improvement after SCI is impossible. In recent years, besides the search for new factors that promote repair and regeneration after SCI, an attention towards the combination of the existing methods, in which combination of gene therapy and cell transplantation is an actively under study and reported [5, 6].

Neurotrophic substances are of the most promising candidates for therapy, which can protect the nerve and promote neurite growth. Neurotrophic factors can reduce pathological damage and induce regeneration reaction in fresh or obsolete SCI. In the repair of SCI, most researches focuses on neurotrophic factors like BDNF and NT-3. Earlier studied have reported that BDNF and NT-3 play important roles in promoting neuronal survival and axonal regeneration after SCI. Many researchers have reported that direct local application or transplanted cells or viral vector coding the above genes can effectively promote the repair of the injured spinal cord and improve the motor function [7, 8]. In addition, the two genes have selective neuron types, the combined application of these two genes are expected to have a synergistic effect.

Due to easy isolation, high in vitro proliferation activity and strong differentiation plasticity, MSCs is very promising in the cell transplantation and gene therapy. Earlier studies have shown that in vitro proliferated mesenchymal stem cells (MSCs) can settled down in the tissue and differentiated to relative cells, repair tissue damage caused by trauma, and partially restore relative tissue function [1, 2, 9]. They can revive to produce mesenchyme originated tissue and also can differentiate into other mesoderm derived cells. These studies confirmed the differentiation plasticity and the possibility of using MSCs in various tissue repairs. Recently, a number of studies reported the application of rat or human MSCs in the treatment of brain and spinal cord disorders [10]. Localized transplantation of rat MSCs after SCI showed that the transplanted cells can survive long term, and well integrated into the spinal cord tissue to differentiate into neural cells, and the animal experiments also show a certain degree of functional recovery [7, 11-15]. MSCs are also ideal for gene therapy and will not cause immune rejection after being local or systematic transplantation [16]. Some reports have also shown that the genetic transfection of MSCs can obtain high transfection rate, with the foreign gene being stably expressed for many generations in vitro, and when these cells are transplanted in vivo, these genes can also be stably expressed at a certain level in the process of differentiation [17].

In our research, we constructed the green fluorescent protein (GFP) and red fluorescent protein (RFP) carrying BDNF and NT-3 eukaryotic expression plasmid, respectively. The constructed vectors were stably transfected into rat MSCs by cationic liposome mediation. MSCs with BDNF or NT-3 were transplanted to completely injured spinal cord in rats. We compared the differences in nerve regeneration and improvement in motor function in the application of MSCs having none, single, and dual genes on the injury site. We expect to explore a beneficial effect of combining multiple methods to repair spinal cord injury and also investigate the feasibility of blood transplant therapy in old spinal cord injury.

Materials and methods

MSCs isolation and culture

All animal experiments were conducted in the accordance with the Institutional Animal Ethics Committee and Animal Care Guidelines of First Affiliated Hospital of PLA General Hospital. The MSCs were harvested from three-month-old male Wistar rats as followings: rats were anesthetized with 1% pentobarbital sodium and MSCs were harvested from hind leg by washing the bone marrow cavity with 4 mL sterile anatomy liquid (3 g glucose, 7.5 g sucrose, 2.34 g Hepes, 8 g NaCl, 0.4 g KCl, 0.24 g Na₂HPO₄. 12H₂O, 0.03 g, KH₂PO₄, 0.001 g phenol red, 1×10⁵ U penicillin in 950 mL ddH₂O). The suspension was gradient centrifuged using 1.073 g/mL Percoll at 2500 g for 5 min, then the MSCs were collected from the middle layer and passaged in culture solution (a-MEM with 10% fetal calf serum and 100 U/mL penicillin). Cells from passage 5 were used for further experimental studies.

Construction of transfection vectors of GFP-BDNF and RFP-NT3

Specific primers BDNF-ORF-5' (5'-GCAGAATTC-ATGACCATCCTTTTCCTTACT-3) and BDNF-ORF-3'(5'-GGTGGATCCCGTCTTCCCCTTTTAATGGT-3') for BDNF as well as hNT3-ORF-5' (5'-GCAG-AATTCTGATGTCCATCTTGTTTTATGT-3') and hN-T3-ORF-3' (5'-GGTGGATCCCGTGTTCTTCCGATT-TT-3') for NT3 were designed using Primer V5.0. pcDNA3.1-rBDNF and pcDNA3.1-hNT3 were used as templates for BDNF and NT3, respectively. The final PCR mixture in 50 µL containing 1 µL each of the primers, 1× Pyrobest Buffer, 2 µL dNTP Mix (10 mM each), 1 µL Pyrobest DNA polymerase, 1 μ L template, and 39 μ L ddH₂O. The thermal cycling parameters consisted of a denaturation step of 94°C for 2 min followed by 30 cycles of 94°C for 20 sec and 60°C for BDNF (57.5°C for NT3) for 2 min. The targeted DNA sequences were separated by electrophoresis in a 1% agarose gel and recovered using the QIAquick Gel Extraction Kit. All the purified PCR products were digested with EcoR I and BamH I. Products of BDNF were ligated to pEG-FP-N1 and products of NT3 were ligated to pDsRed2.



Figure 1. MSC injection Site Selection: A: Injection depth in the middle area, 1.75 mm, 1.25 mm, 0.75 mm, 0.25 mm and injection depth in the side area, 0.75 mm, 0.25 mm; B: Processing of injection.

Preparation of implanted cells

Transfection was performed using Lipofectamine 2000 according to the manufacturers protocol (10 µL lipidosome, 100 µL SFM (serum-free medium), and 1 µg recombinant plasmid were mixed in sterile condition). The mixed solution was placed for 40 min in room temperature and added with 800 µL SFM. One mL medium with plasmids was mixed gently with MSCs and cultured for 6 h in 37°C, 5% CO₂ condition before 1 mL serum culture was added. After 48 h incubation, protein expression in the transfected cells was observed with an inverted fluorescence microscope and transfection solution was replenished with 2 mL 0.5 mg/mL G418 culture. Clones were picked and culture in 0.5 mg/mL G481 medium for following experiments.

Spinal cord injury model preparation and MSCs implantation

Fifty three-month-old male Wistar rats were classified into 5 groups, ten for each group: Group 1: Control group (no implantation); Group 2: Animals implanted with MSC labeled with Hoechst (MSC was incubated in 1 μ g/ml Hoechst33258 for 20 min at room temperature); Group 3: Animals implanted with MSC transfected with *BDNF*; Group 4: Animals implanted with MSC transfected with MSC transfected with MSC transfected with *BDNF* and *NT-3* together. Rats were anesthetized with 1% pentobarbital sodium and a T8

laminectomy was performed, and a complete transection of the spinal cord was made with a scalpel, which was further verified histologically. After hemostasis, $0.5 \times 10^5/\mu$ L MSCs suspended in culture medium was injected into the transection site with 4-µm-diameter micro glass injection pipette, with 0.2 µL for each site as shown in **Figure 1**.

Behavioral assessment

The Basso-Beattie-Bresnahan (BBB) locomotor rating scale was used to assess hind limb locomotor function in the MSC-implanted groups and control group. BBB scores were obtained every week until 16 weeks after SCI by an investigator who was blinded to the experimental groups.

Fluro-gold retrograde tracing, tissue processing, and neurofilament immunohistochemistry

Eight weeks since the treatment, two individuals randomly selected from each group were anesthetized using 1% pentobarbital sodium and quickly perfused with 500 mL 4% paraformaldehyde. Endorhachis was removed immediately after perfusion and placed in 20% w/v sucrose solution overnight. Precipitated samples in the solution were sectioned using a freezing microtome. The sections were mounted on sliders and stored at -20°C in dark. Neurofilament immunohistochemistry was carried out on the basis of the protocol developed by Shi et al. [18]. 14 weeks since the treatment,

BDNF and/or NT3 engineered MSCS for spinal cord injury



Figure 2. Migration of implanted MSCs with or without foreign gene expression in transected spinal cord. A: Group 2 implanted with MSCs labeled with Hoechst33258 (25×); B: Group 3 implanted with MSCs expressing *BDNF* (50×); C: Group 4 implanted with MSCs expressing *NT*-3 (25×); D: Group 5 implanted with MSCs expressing *BDNF* and *NT*-3 (25×).

another two individuals were randomly selected from each group. A T10 laminectomy was performed and 2% fluro-gold solution was injected into cross section to assess the regeneration of the neurons. Sites selection and volume were determined as above shown in **Figure 1**. The execution and material collection of the animals were performed as described above at the 16th week of the treatment.

Statistical analysis

All the data were expressed in the form of mean \pm SD. ANOVA and LSD tests were performed

with significant level of 0.05. All the statistical analysis were conducted using SPSS version 16.0 (IBM, Armonk, NY, USA).

Results

Migration of implanted MSCs and foreign gene expression in transected spinal cord

Before implantation, MSCs in group 2 were labeled with Hoechst33258 in order to trace these cells. After 16 weeks since SCI, the implanted MSCs were not only limited in the injected sites but also migrated into the center



Figure 3. Basso, Beattie, and Bresnahan (BBB) scores obtained during the course of recovery.

of injury sites along the vertical axis of spinal cord (**Figure 2A**). For the MSCs transfected with *BDNF*, *NT-3*, *BDNF* and *NT-3*, in group 3, group 4, and group 5, respectively, showed good expression, and the distribution pattern of MSCs in these three groups were in accordance with those in group 2 (**Figure 2B-D**).

Effect of MSCs implantation on functional recovery of hindlimb locomotion in transected rats

After transection, all animals immediately showed flaccid paralysis in both hindlimbs and the BBB scores were zero. Within three weeks after implantation, the hindlimb locomotion improved gradually in the MSC-implanted groups (Figure 3). The recovery was accelerated in the following 4-8 weeks and sustained for 12 weeks before leveled off. The recovery initially started with hip joint followed with the locomotor activities in all the joints in the hindlimbs. Animals in group 5 having MSCs with BDNF and NT-3 exhibited the best functional recovery of the hindlimbs compared to other groups, and BBB score was higher in group 3 compared to group 4, but was statistically insignificant (Figure 3).

Implantation of BDNF-MSCs and NT3-MSCs promoted axonal regeneration in SCI rats

Fluro-Gold retrograde tracing analysis revealed that fluro-gold labeled neurons were detected

in MSC implanted groups whereas no labeled neurons were found in control group. Transplantation of MSCs seemed to increase the number of fluro-gold labeled neurons in upper side and dorsal horn of spinal cord and reticular formation of brain stem. Labeled area in group 3 and group 4 expanded to red nucleus and vestibular nuclei, respectively. Moreover, the densities of labeled neurons in these two groups were significantly larger than that in group 2. The most extensive distribution of labeled neurons was detected in group 5 (Figures 4 and 5), including all the areas mentioned above. However, no

fluro-gold labeled neuron was found in telencephalon in any group.

Implantation of BDNF-MSCs and NT3-MSCs increase the survival of neurons

In control group without MSCs implantation, no staining neurofilament was detected after observation (Figure 6B); and for group 2 in which animals were implanted with MSCs only, dotted and short dendroid staining neurofilaments were sparsely distributed in lesion sites was observed (Figure 6C). More ordered and intensive positive neurofilaments were observed in group 3 and group 4 (Figure 6D and 6E). However, compared with the other four groups, neurofilaments in spinal cord injury area in group 5 exhibited the higher recovery rate, with the most orderly distributed neurofilaments being stained (Figure 6F).

Discussions

Implantation of cultured MSCs is a potential means of repairing neural loss due to neurodegenerative disease or traumatic damage because they can be used as auto grafts and easily genetically modified to overexpress neurophic factors. It is reported that implantation of MSCs into transected SCI promote functional recovery [7, 19]. However, results focusing on the implantation of MSCs are inconsistent [20, 21].



Figure 4. Fluro-gold retrograde tracing of neuron in group 5 implanted with MSCs expressing *BDNF and NT*-3. A: 50×; B: 200×.

In the present study, we have proposed an effective therapeutic strategy based on MSC implantation expressing BDNF and NT-3 genes. In the rats with a transected spinal cord, we found the improvement of their hindlimb locomotion was relatively little in the simple MSCimplanted group or single-gene MSC-implanted groups. Although the benefits produced by these approaches in SCI recovery were high, the structure repair was not enough to produce behavioral improvement. Higher functional repair was seen in the SCI rats that received MSCs genetically expressing both BDNF and *NT-3*. This functional recovery was further supported by fluro-gold retrograde tracing and neurofilament immunohistochemistry showing a dramatic increase in the regeneration of axon in SCI animals compared with the groups implanted with MSCs only or implanted with MSCs modified with single gene and also more robust positive neurofilaments in the transected site of the spinal cord was observed compared to other groups.

A key characteristic of SCI is the presence of cystic cavitations at the injury sites [22]. Previous reports indicated that there was some correlation between the formation of cystic cavitation and hindlimb functional loss [19, 23]. In our study, although MSCs implantation alone or with single-gene expressed could improve the functional improvement of hindlimb was found in the double-gene expressed group. MSCs can secrete various trophic factors, which may help to repair the injured spinal cord, but there is no direct evidence that MSCs can produce BDNF and NT-3, which are important neurotropin fac-

tors. These trophic substances enhance nerve fiber outgrowth, neural differentiation and have the ability of neuroprotection. They can rescue axotimozed neurons from retrograde atrophy and death [24, 25], which would help to repair the tissue, including the reduction of cavity formation in the injured spinal cord. However, either BNDF or NT-3 alone seemed not to account for all the benefits of MSCs implantation, since better functional repair and axonal regeneration were seen in double-gene expressed group. In group 5, SCI animals achieved the highest BBB scores with more neurofilament regeneration after the implantation of genetically modified MSCs. In addition, the origin area of regeneration was much wider in the upper brain regions in group 5.

Even with an improvement in the functional recovery of completely transected spinal cords, the therapeutic effect was still limited with double-gene modified MSCs. The cell level improvement didn't result in the complete locomotor functional recovery. Most SCI rats didn't regain the ability of coordinating to walk with hindlimbs. This situation will be much worse when the similar therapy is applied to human's as human body is highly complicated and might have lower recovery ability compared with animals. However, our results are still inspirable in that the concatenated use of two genes in genetic modification highlights a novel methodology in treatment of SCI. Moreover, gene therapy is far from perfection, most research lack the knowledge of the biological factors and detail pathway involving in the treatment. Thus, additional studies are required to accurately control the expression of genetically modified products



Figure 5. Detail distribution of fluro-gold retrograde tracing of neuron in group 5 implanted with MSCs expressing *BDNF and NT*-3. A-C: Brain maps of rats; D-F: Brain sections of rats (25×); G-I: Morphology of neuron labeled with fluro-gold (200×).

and to determine whether multiple MSCs injection at different pathological stages will be necessary to promote walking in the SCI models. Based on MSCs implantation, genetically modified MSCs with *BDNF* and *NT-3* genes in our study have greater potential as therapeutic



Figure 6. Regeneration of neurofilaments in immunohistochemistry. A: Sagittal sections of SCI area; B: Control group; C: Group implanted with MSCs alone; D: Group implanted with MSCs expressing *BDNF*; E: Group implanted with MSCs expressing *NT*-3; F: Group implanted with MSCs expressing *BDNF* and *NT*-3.

agents against SCI. These cells could be readily obtained through established clinical procedures, and were easy to isolate and expanded for auto transplantation with limited risk of rejection. The results from our study suggested that the dually expressed *BDNF* and *NT-3* genes in MSCs could provide a functional recovery of hindlimb movement in rats with completely transected spinal cords, compared implantation of normal or single gene expressed MSCs. However, further study is required to ensure the long-term safety and efficacy of manipulated MSCs to improve locomotor function and other qualities of life issues after SCI.

Disclosure of conflict of interest

None.

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