Therapeutic effect of co-transplantation of neuregulin 1-transfected-Schwann cells and bone marrow stromal cells on spinal cord hemisection syndrome

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Abstract

The aim of the present study is to evaluate the therapeutic effect of co-transplantation of neuregulin-1-transfected-Schwann cells (SCs) and bone marrow stromal cells (BMSCs) on a rat model of spinal cord hemi-section injuries (Brown–Séquard syndrome), which is relevant to human clinical spinal cord injury. Both in vivo and in vitro data we received demonstrated that co-transplantation BMSCs with NRG1-transfected SCs reduced the size of cystic cavities, promoted axonal regeneration and hindlimb functional recovery in comparison with SCs or BMSCs transplantation alone or together, and this treatment could provide important insights into potential therapies of spinal cord hemi-section injuries.

Keywords:
Neuregulin-1
Schwann cells
Bone marrow stromal cells
Co-transplantation
Spinal injury

Injury to the adult mammalian spinal cord results in a non-permissive environment for the regeneration of axons in lesion [3], leading to only partial recovery of axons. One potential strategy for promoting axonal regeneration after injury is the implantation of autologous Schwann cells to support and guide axonal growth. Neurotrophic factors alone, as well as combined with Schwann cell grafts, could further amplify axonal growth and extension after injury [5]. Neuregulin 1 (NRG1), a neuronal growth factor for myelination, is essential for glial or neuronal survival, the proliferation of SCs and their terminal differentiation [5,10]. Disruption of NRG1 signaling leads to an almost complete loss of SCs and of the neurons that they support [13]. ErbB proteins, including ErbB1, ErbB2, ErbB3, ErbB4, are neuregulin receptors, which are expressed on the surface of SCs. NRG1, which binds on ErbB2, could elevate levels of ErbB2 receptor and responsiveness to NRG1 [8,9]. Researches lately have reported that the transplantation of SCs derived from bone marrow stromal cells in vitro effectively promoted the regeneration of lesioned sciatric nerves and damaged axons and hindlimb functional recovery in completely transected adult rat spinal cord [12]. The aim of this study is to evaluate the efficacy of NRG1-transfected-SCs and bone marrow stromal cell co-transplantation in therapy for spinal cord hemi-section injuries. And here we show that transplantation of NRG1–SCs–BMSCs exert much more effective axonal regeneration and functional recovery in rat spinal cord hemi-section injuries compared with SCs–BMSCs transplantation and other control groups, implying a promising strategy for injured spinal cord therapy.

WISTAR rats (4–6 weeks old, 50 males and 50 females) were divided randomly and used for model of spinal cord hemi-section injury. Ten Wister rats, 1–2 and 2–4 weeks old were used for BSMCs and SCs preparation, respectively. Animals were provided by the animal center of the Harbin Medical University of China and maintained under specific-pathogen-free conditions at 21 ± 2 °C and 45 ± 5% humidity. All animal handling and experimental procedures were performed in accordance with the guidelines of the Care and Use of Laboratory Animals published by the China National Institute of Health.

SCs cultures were established as previous description [6,4] with some modifications. The dissected nerves were obtained from Wistar rats and treated with an enzyme mixture consisting of 0.125%
Fig. 1. Transfection with NRG1 significantly increased NRG1, ErbB2 expression level in cultured SCs. Culture supernatant or cell lysate were obtained from different cells at various time points. NRG1 expression in supernatant was shown in (A) and (B). NRG1 in lysate was depicted in (C) and (D). While ErbB2 in cell lysate was shown in (E) and (F). *P < 0.05, #P < 0.01, compared with the NRG1-SCs group. All data were reported as the mean ± SD of triplicates of independent experiments.

Bone marrow stromal cells were cultured as previously described [7]. Briefly, total bone marrow cells were flushed out of the femurs and tibias of 1–2 weeks old Wister rats. After 2 washings by PBS, cells were planted onto plastic culture dishes at a final concentration of 1 × 10^6 nucleated cells per mL in BMSCs culture medium (Cyagen Biosciences Inc.). Adherent cells after 4–6 passages were used for next procedure.

A mixture consisting of 94 μL DMEM, 6 μL FuGen6 reagent (Roche, Inc. USA) and 3 μL plNCX2-NRG1 or plNCX2-GFP (1 μg/μL) was gently mixed, and placed at RT for 20 min. 1 × 10^6 SCs cultured on 90 mm petri dish a day before were incubated with this mixture for 6 h, then refreshed the culture medium by BS medium containing 10% FBS. After 48 h, G418 was added in culture medium and SCs were cultured at 5% CO₂, 37 °C. At the same time, GFP transfected cells were checked to determine the transfected efficiency. After 1 week, stable transfected SCs were used for in vivo or in vitro experiments.

Supernatants of NRG1-transfected SCs and protein of cell lysates of different time point were collected and the concentration of NRG1 as well as ErbB2 was determined by ELISA (USCNLIFE).

Spinal cord hemi-section injuries were performed in rats deeply anesthetized and a laminectomy was performed at the T8 level. Confirmation of a complete hemi-section was determined by spasmodic swinging of the rat tail and the retraction and flutter of the ipsilateral leg. Rats only undergoing Lamina Removal, but without spinal cord hemi-section were used for control.
The BBB [1] scale is a valid and predictive measure of locomotor recovery. The scale used for measuring hindlimb function from a score of 0, indicating no spontaneous movement, to a maximum score of 21, with an increasing score indicating the use of individual joints, coordinated joint movement, coordinated limb movement, weight-bearing, and other functions. Rats were evaluated according to BBB locomotive rating scale every day by four blinded observers, until 8 weeks after cell transplantation.

Seven days after injury, rats scoring less than 4 BBB score evaluation were randomly divided into six groups and accepted cell transplantation. Cells were injected at three sites using a glass micropipette (1 mm deep into the medial and 0.5 mm into both sides of the injury site, 5/µL each site). The total number of cells each rats received was 3 × 10⁵ (equal ratio for two types of cells). Each rat was administered immunosuppressant cyclosporine (2.5 mg/kg, Novartis) i.p. after transplantation.

Rats were anaesthetized and cortical somatosensory evoked potentials (SEP) were recorded using keypoint electromyogram evoked potential diagnostic apparatus (Medtronic, USA). Dipole surface stimulating electrode was placed on the surface of the tibial nerves in the posterior limb of the surgical side. The collecting electrode was placed on subcutaneous tissue in the rear edge of the ear beside the sagittal line. Reference electrode was placed on the corresponding area in the front edge of the ears. The optimal stimulus intensity was defined as the current intensity that evoked a slight trembling of the hind toe. The data obtained included a wave curve recording of the latency and amplitude of somatosensory evoked potentials. The latency and amplitude response was converted into velocity as a measure of spinal cord dysfunction.

Four weeks following cell transplantation, four rats in each group were randomly selected and sacrificed. About 2 cm of the spinal cords covering the injured segment were collected, paraffin-wax embedded, sliced (6 µm) and stained with hematoxylin and eosin (HE). Anti-NF-200 (1:100, Sigma) was also stained on duplicated section and visualized using HRP labeled second antibody and DAB.

Four weeks after cell transplantation, four rats in each group were randomly selected and anaesthetized. MRI scanning was performed using Siemens Magnetom Avanto1.5 T MRI imaging system. The surface coils and ventricumbent position were employed and the scanning center was focused on the injured areas of the spinal cord. FSE sequential sagittal scanning was performed for imaging (TR/TE = 400 ms/13 ms; 512 × 512).

BBB scale scores were analyzed using one-way ANOVA test and LSD pairwise comparison. The results of the ELISA experiments were analyzed by Dunnett test. Data were analyzed using SPSS.

At 48 h after transfection, NRG1 expression was slightly higher in supernatant of NRG1-transfected SCs, and statistical significance was observed at the end of 72 h incubation (P < 0.05, Fig. 1A). NRG1 in cell lysate of NRG1-transfected SCs had a similar increasing trend as that in supernatants (Fig. 1C). Besides, a slight increase of ErbB2 level in cell lysate of NRG1-transfected SCs was observed at 24 h after transfection, and significant elevation of ErbB2 expression also appeared at the end of 72 h incubation (Fig. 1E, P < 0.05, compared with other groups). High levels of NRG1 and ErbB2 expression continued for weeks (Fig. 1B, D and F).

Seven days after indirect contact co-culture of BMSCs and NRG1-SCs in transwell system, there was a greater percentage of
Fig. 3. Percentages of differentiated BMSCs in indirect co-culture system with NRG1-SCs or SCs. Differentiated BMSCs expressed NSE, GFAP or S100 were counted on each section. Statistical data were obtained from 3 independent experiments, and results were expressed as the mean ± SD. *P < 0.05, compared with the SCs group.

differentiated BMSCs with the positive expression of NSE (neuron marker), GFAP (astrocyte marker) or S-100 (Schwann cell marker) (Fig. 2A–C), as compared with the BMSCs and SCs co-culture group (Fig. 2D–F). Natural differentiation of BMSCs (Fig. 2G–I) were used as control groups. Positive cells were counted on each section and data were shown in Fig. 3. All these data indicated that BMSCs indirect co-culture with NRG1-SCs was associated with a significant increase in the differentiation of neuron-like cells.

HE staining was performed 4 weeks after transplantation for evaluating efficacy of cell transplantation for tissue sparing after injury. Horizontal sections of the injured animals treated with DMEM lost normal tissue construction showing encephalomalacia foci and large cystic cavities (Fig. 4B*). In groups treated with SCs (Fig. 4C*), BMSCs (Fig. 4D*), NRG1-SCs (Fig. 4E*), and SCs–BMSCs (Fig. 4F*), the area of cystic cavity were significantly fewer and smaller than that of DMEM treated group. Nearly no cystic cavities were seen in NRG1–SCs–BMSCs-treated group (Fig. 4G*). Normal spinal cord was prepared as control (Fig. 4A*).

Four weeks after cell transplantation, thoracic injured spinal cords were imaged using MRI. A low T1 signal intensity (Fig. 4B, arrows indicated) was similar to that of cerebrospinal fluid on MRI in the DMEM-transplanted group, suggesting a formation of malacia lesions and a continuous interruption of the epithelium and muscle tissue in the injured spinal cord. The spinal cord morphology of the SC-, BMSC-, NRG1–SC- and SC–BMSC-treated groups was normal, although a long and uniform T1 signal with clear boundaries were detected (Fig. 4C–F), indicating that the injured spinal was not fully restored. In the NRG1–SC–BMSC-treated group, no abnormal signals within the spinal cord were detected (Fig. 4G). There were heterogeneous signals in all mid-thoracic spinal cords, where the surgery took place.

Before injury, hindlimbs of all rats were scored at 21 points (normal). After injury, the ipsilateral hind limb was paralyzed, with a BBB score of 0–1 points. The scores gradually increased after cell transplantation. There were significant differences among NRG1–SCs–BMSCs group and other groups 6 weeks after cell transplantation (Fig. 5H). This phenomenon indicated apparently therapeutic effects of NRG1–SCs–BMSCs transplantation.

Somatosensory evoked potential is a test showing the electrical signals of sensation going from the body to the brain. All injured rats had the same low amplitude and long latency. Varied degree of elevated amplitude and shortened latency were recorded in different groups 4 weeks after different cell transplantation and maintained at the end of 8 weeks and the most obvious changes were received in BMSCs–NRG1–SCs transplantation group, indicating a best therapeutic effect of BMSCs–NRG1–SCs (Table 1).

Given the fact that neurofilament 200 (NF-200) plays important roles in neuronal axons and recovery after injury [11], we examined the number and distribution of NF-200 after cells transplantation in all groups. Eight weeks after cell transplantation, highly significant increase in NF-200 immunoreactivity appeared in the NRG1–SCs–BMSCs group in comparison with other groups (Fig. 5A–G).
was observed by group blinded observers and given scores according to the BBB scale once a week from 1 week before cell transplantation until 8 weeks after cell transplantation. NF-200 expression was detected by immunohistochemistry. Pictures were captured at 200× magnification. Arrows indicated the NF-200 positive objects. Positive objects are shown in brown. We counted on each section by group blinded observers and results were expressed as positive objects on the whole area of each section. Each rat indifferent groups was observed in BBB scores. In vitro experiments, we co-cultured BMSCs and SCs in indirect contact system and found that NRG1 transfected SCs could secrete high levels of NRG1 and expressed great amount of ErbB2 receptor. NRG1:ErbB2 signaling contributes a powerful autocrine or paracrine production of trophic factors, such as insulin-like growth factor (IGF), platelet-derived growth factor PDGF, transforming growth factor-beta (TGF-beta), leukemia inhibitory factor (LIF) and so on [2]. These trophic factors might form a microenvironment to facilitate the differentiation of BMSCs into neuronal-committed lineages in vitro experiments. When NRG1-SCs and BMSCs were co-transplanted into spinal cord hemi-section injury rats, on one hand, NRG1-SCs could secrete trophic factors to improve the recovery of injured neurons and Schwann cells, on the other hand, BMSCs might differentiate associated cells to replace dead cells. In addition, NRG1-SCs might facilitate the differentiation of BMSCs. Interestingly we observed that NRG1-SCs and BMSCs combination exerted an obviously better improvement of limb function than SCs or BMSCs alone or together. The mechanism of this improvement and which kind of cells played a prominent role need to be further studied. However, our results provide a new approach to treat spinal injury or other relative diseases. This new approach, i.e. the combination of BMSCs and NRG1-SCs, is more efficient than transplanted BMSCs or SCs alone or together. Because BMSCs were accompanied by more powerful SCs, NRG1-SCs, which could secrete many trophic factors, it might be easier for BMSCs to be conducted to exert suitable contribution.

In summary, all these findings indicated that co-transplantation of NRG1 transfected SCs and BMSCs exerted significant neuroprotective effect on promoting survival of neurons and regeneration of nerve fibers, reduced the size of the cystic cavity, promoted axonal regeneration and sparing and resulted in a better hindlimb function.

Table 1
The SEP latency and amplitude of different groups before operation and after cells transplantation (ms, μV, n = 6× ±1).

<table>
<thead>
<tr>
<th></th>
<th>Pre-operation</th>
<th>4 weeks after cells-trans</th>
<th>8 weeks after cells-trans</th>
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<tr>
<td>Latency</td>
<td></td>
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<tr>
<td>DMEM</td>
<td>2.61 ± 0.10</td>
<td>10.47 ± 2.07</td>
<td>9.83 ± 1.40</td>
</tr>
<tr>
<td>SCs</td>
<td>2.44 ± 0.72</td>
<td>7.29 ± 1.36</td>
<td>7.02 ± 1.54</td>
</tr>
<tr>
<td>BMSCs</td>
<td>2.50 ± 0.73</td>
<td>7.38 ± 1.22</td>
<td>7.13 ± 1.47</td>
</tr>
<tr>
<td>NRG1-SCs</td>
<td>2.54 ± 0.48</td>
<td>6.29 ± 1.12</td>
<td>6.02 ± 1.48</td>
</tr>
<tr>
<td>BMSCs + SCs</td>
<td>2.51 ± 0.43</td>
<td>6.13 ± 1.09</td>
<td>5.77 ± 1.16</td>
</tr>
<tr>
<td>BMSCs + NRG1-SCs</td>
<td>2.74 ± 0.28</td>
<td>4.20 ± 1.46</td>
<td>2.91 ± 0.86</td>
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<tr>
<td>Amplitude</td>
<td></td>
<td></td>
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<tr>
<td>DMEM</td>
<td>4.60 ± 0.78</td>
<td>0.49 ± 0.20</td>
<td>0.87 ± 0.05</td>
</tr>
<tr>
<td>SCs</td>
<td>4.58 ± 0.50</td>
<td>2.16 ± 0.54</td>
<td>2.50 ± 0.09</td>
</tr>
<tr>
<td>BMSCs</td>
<td>4.38 ± 0.61</td>
<td>2.39 ± 0.63</td>
<td>2.52 ± 0.17</td>
</tr>
<tr>
<td>NRG1-SCs</td>
<td>4.84 ± 0.79</td>
<td>3.07 ± 0.71</td>
<td>3.10 ± 0.20</td>
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<tr>
<td>BMSCs + SCs</td>
<td>4.56 ± 0.63</td>
<td>3.02 ± 0.55</td>
<td>3.12 ± 0.26</td>
</tr>
<tr>
<td>BMSCs + NRG1-SCs</td>
<td>4.77 ± 0.95</td>
<td>3.97 ± 1.04</td>
<td>4.46 ± 0.12</td>
</tr>
</tbody>
</table>

* P < 0.05, compared with DMEM group.
# P < 0.05, compared with NRG1-SCs + BMSCs.
tional recovery. This method could be an integrated approach to the treatment of spinal cord injuries or other similar diseases.

References