Stem cells modified by brain-derived neurotrophic factor to promote stem cells differentiation into neurons and enhance neuromotor function after brain injury

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Objective: To promote stem cells differentiation into neurons and enhance neuromotor function after brain injury through brain-derived neurotrophic factor (BDNF) induction.

Methods: Recombinant adenovirus vector was applied to the transfection of BDNF into human-derived umbilical cord mesenchymal stem cells (UCMSCs). Enzyme linked immunosorbent assay (ELISA) was used to determine the secretion phase of BDNF. The brain injury model of athymic mice induced by hydraulic pressure percussion was established for transplantation of stem cells into the edge of injury site. Nerve function scores were obtained, and the expression level of transfected and non-transfected BDNF, proportion of neuron specific enolase (NSE) and glial fibrillary acidic protein (GFAP), and the number of apoptosis cells were compared respectively.

Results: The BDNF expression achieved its stabilization at a high level 72 hours after gene transfection. The mouse obtained a better score of nerve function, and the proportion of the NSE-positive cells increased significantly (P<0.05), but GFAP-positive cells decreased in BDNF-UCMSCs group compared with the other two groups (P<0.05). At the site of high expression of BDNF, the number of apoptosis cells decreased markedly.

Conclusion: BDNF gene can promote the differentiation of the stem cells into neurons rather than glial cells, and enhance neuromotor function after brain injury.

Key words: Brain-derived neurotrophic factor; Stem cells; Cell differentiation; Brain injuries

METHODS

Material and reagents
Fetal bovine serum (FBS, Hangzhou, China), trypsin (Gibson, USA), pAdeno-BDNF adenovirus (Zhongshan University, China), FITC tag streaming antibody CD29, CD31, CD44, CD45 and CD105 (Erotic, UK), rabbit anti-human CD34 antibody (Santa Cruz, USA), goat anti-mouse glial fibrillary acidic protein (GFAP) and rhodamine fluorescent antibody (Beijing, China), rabbit anti-mouse neuron specific enolase (NSE) antibody and goat anti-mouse BDNF antibody (Boshide, Wuhan, China) were used in this study. A total of 36 male thy-
mus-free mice, weighing 50 g each, purchased from the Chinese Academy of Military Medical Sciences, were raised in the animal laboratory of Tianjin Medical University.

Culture and identification of UCMSCs

Umbilical cord was immersed into serum-free DMEM under aseptic condition, and the residual blood was washed off with PBS. Then cut the umbilical cord tissue into 1 mm³ sections, which were thereafter digested by collagenase IV and 0.1% trypsin, filtered with cell sieve, and then centrifuged. We inoculated the cell suspension into 10 cm plastic dishes with the density of $1.0 \times 10^6$ each, and cultivated them under the condition of 37°C, 5% CO₂ and saturated humidity in cell incubator. About 10-14 days later, when single-adherent cells grew into 90% fusion, use 0.25% trypsin for digestion, with 1:2 passage. Select the 4th passage cells ($1 \times 10^6$) into PBS to suspension, respectively add CD29, CD31, CD44, CD45 and CD105 antibody that marked with their matching fluorescein isothiocyanate (FITC) and stained in darkness about 30 minutes. The CD34 was detected by indirect fluorescent label and tested by the flow cytometer.

BDNF gene transfection and secretion phase detection

Well-grown UCMSCs were selected and inoculated into 24-well culture dishes. When the cellular density grew into 60% to 70% confluent, we proceeded recombinant adenovirus transfection of BDNF gene. At the time points of 24 h, 48 h, 72 h, 96 h, 120 h after transfection, the expression level of BDNF was detected by ELISA, and the non-transfected UCMSCs served as control. The procedures of ELISA were carried out according to specification.

Establishment of brain injury model

The thymus-free mice were anesthetized by intraperitoneal injection of 10% chloral hydrate and fixed on a stereotaxic apparatus and exposed the skull under sterile conditions. At the point of 2.0 mm rearward from the anterior fontanelle and 2.0 mm right to the midline, open a 4 mm diameter bone window and maintain the integrity of the dura mater. Then fix a percussion-tube on the edge of bone window, connect it to the hydraulic craniocerebral trauma instrument, keep the tube closed, and implement an impact injury at a peak impacting stress of 0.20 MPa and mean time of 20 ms.

Experimental group and UCMSCs transplantation

All the animals were divided into three groups, i.e. control group (n=12, without stem cell transplantation), simple UCMSCs transplantation group (n=12, simple UCMSCs transplantation) and BDNF-UCMSCs transplantation group (n=12, UCMSCs transplantation modified by BDNF gene). The well-grown UCMSCs of transfected and non-transfected BDNF genes were selected to accept conventional trypsin digestion and sufficient suspension, so as to make single cell suspension ready for use. Twenty-four hours after injury, at a speed of $1 \times 10^6/10 \mu l$, UCMSCs were evenly injected via the edge of bone window into the traumatic brain cortex.

Measurement of parameters

(1) Neural function scores: Neural function score was carried out in 8 mice in each group on the 7th day, 14th day, 21th day and 28th day before treatment. The scoring method we used was from the literature.

(2) The expression BDNF, NSE and GFAP at the edge of the traumatic site: Four mice in each group were executed on the 7th day, and their brain tissues were made into paraffin sections which afterwards were detected by immunofluorescence test. The dilution ratios of BDNF antibody, GFAP and NSE were 1:1000, 1:500, and 1:1000, respectively. (3) Detection of apoptosis in situ was conducted according to instructions of in situ apoptosis detection kit.

Statistical analysis

SPSS 13.0 statistical software was used for data analysis. The comparison between two groups was made by Student’s t test, and multi-group comparisons by ANOVA test. $P<0.05$ was considered to be statistically significant.

RESULTS

Identification of UCMSCs

The results of the surface markers identified by flow cytometry were: CD29 (97.69%), CD44 (98.17%), CD105 (98.38%) were positively expressed, and CD31 (2.67%), CD34 (3.09%), CD45 (3.38%) negatively expressed. Among them, CD29, CD44, CD105 were the specific markers of UCMSCs expression. CD31 was the marker of endothelial cells, CD34 and CD45 were hematopoietic stem cell markers. Multi-indicator detection confirmed that UCMSCs with a high purity of variety could meet the experimental requirements (Fig. 1).
BDNF secretion phase

The constantly low expression of BDNF was detected in the UCMSCs medium of non-transfected BDNF gene (Fig.2), but continuously increased expression of BDNF was seen in the UCMSCs medium of transfected BDNF, and maintained a relatively high level 72 hours later.

Expression of BDNF, GFAP and NSE at the wound edge and apoptosis detection in situ

The selected regions were just on the edge of brain traumatic site, i.e. the site of stem cell transplantation (Fig.3). The green fluorescence in the cytoplasm suggested FITC-positive cells, while red ones suggested TRATIC-positive cells. The expression of BDNF was low both in the control group and simple UCMSCs transplant group. There was no significant difference between them (P>0.05). A large number of BDNF-positive cells can be seen at the edge of traumatic focus in BDNF-UCMSCs transplant group. Compared with the control group and simple UCMSCs transplant group, there were significant statistical differences (P<0.01). Compared with the control group, the number of NSE and GFAP-positive cells increased significantly in UCMSCs transplant group, but the proportion of GFAP-positive cells (91.3%±7.8%) was much more than that of NSE-positive cells (8.7%±3.1%). At the same time, the proportion of GFAP-positive cells (57.3%±8.9%) decreased significantly in BDNF-UCMSCs transplant group, while the proportion of NSE-positive cells (41.7%±7.4%) increased. It indicated that UCMSCs modified by BDNF gene had strong differentiation potential into neurons rather than into glial cells.

Cell apoptosis in situ appeared near the traumatic focus. It can be seen that the number of apoptosis cells under microscopic vision in UCMSCs transplant group (84.5±21.3) was less than that in the control group (191.5±27.1). The BDNF-UCMSCs transplant group had a smaller number of apoptotic cells than the other two groups, and few apoptotic cells (21.5±7.3) were seen locally. There was significant difference when compared with simple UCMSCs transplant group (P<0.01).

Neural function scores

At all the 5 time points, the neural function of UCMSCs transplant group was better than that without stem cell transplantation (Table 1). Meanwhile, the BDNF-UCMSCs transplant group had better neural motor function than the simple UCMSCs transplant group (P<0.05).

Table 1. Comparison of neurologic impairment scores in different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Before treatment</th>
<th>7d</th>
<th>14d</th>
<th>21d</th>
<th>28d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>37.85 ± 4.29</td>
<td>24.12 ± 3.29</td>
<td>19.01 ± 4.41</td>
<td>15.03 ± 3.31</td>
<td>14.19 ± 1.78</td>
</tr>
<tr>
<td>UCMSCs</td>
<td>8</td>
<td>36.98 ± 3.72</td>
<td>19.74 ± 2.73</td>
<td>16.35 ± 2.44</td>
<td>14.15 ± 2.31</td>
<td>11.37 ± 2.11</td>
</tr>
<tr>
<td>BDNFUCMSCs</td>
<td>8</td>
<td>37.91 ± 2.02</td>
<td>15.23 ± 3.06</td>
<td>12.31 ± 2.73</td>
<td>9.11 ± 1.37</td>
<td>7.36 ± 1.04</td>
</tr>
</tbody>
</table>

| t¹ | 0.433 | 2.898 | 1.493 | 0.617 | 2.889 |
| P¹ | >0.05 | <0.05 | <0.05 | <0.05 | <0.05 |
| t² | 0.036 | 5.598 | 3.653 | 4.676 | 3.312 |
| P² | >0.05 | <0.05 | <0.05 | <0.05 | <0.05 |

t¹: compared with the control group, t²: compared with the UCMSCs group.
DISCUSSION

There are two major pathophysiological events for traumatic brain injury patients, i.e. nerve tissue restoration and motor function recovery. The structural basis of nerve tissue reparation is glial proliferation and regeneration, while motor function commutation is based on the functional compensation of undamaged neurons and the differentiation into neurons from neural stem cells that are stored sub-ependymal and the back side of the hippocampus region. For the majority of adults and adolescents, the function compensation is very limited, which is also an important reason why brain injury patients cannot effectively restore to normal motor function after long-term motor dysfunction. In addition, the proliferation of a large number of glial cells have also become a potential factor for seizures.

In recent years, stem cell transplantation makes it possible to fundamentally improve motor function of brain trauma patients. However, many studies have confirmed that most of the transplanted stem cells, under the induction of brain micro-environment, differentiate into glial cells rather than neurons, which to some extent limits the application of stem cells transplantation. As a result, whether we can solve this problem will lead a profound impact on stem cell transplantation. Studies have shown that BDNF can promote stem cell differentiation into neurons to certain degrees. In light of previous researches on UCMSCs, our study used adenovirus vector to transfet BDNF into UCMSCs, observed the differentiation results of BDNF-UCMSCs into neurons or glial cells in vivo, and tested the nerve function restoration in rats subjected to brain injury.

The reasons why we transplanted stem cells to the edge of traumatic focus are mainly considered two points: (1) There are a large amount of toxic substance accumulation in traumatic brain area because of severe hypoxia and ischemia, which is not conducive to the survival, growth and differentiation of local stem cells. (2) There are a few normal nerve cells near the traumatic sites, to whom timely nutrition and effective treatment are essential to the nerve function recovery after
traumatic brain injury.

In addition, in order to reduce post-transplant immunologic rejection when injecting human-derived UCMSCs into animals brain tissue, we chose thymus-free mice as transplantation objects. By establishing a good brain injury model, as well as a stable environment for stem cell transplantation, we detected the gene expression of BDNF, NSE (characteristic protein marker for neurons), and GFAP (characteristic protein marker for glial cells) near the brain trauma sites. It was found that there were a large number of GFAP-positive cells, but very few NSE-positive cells in the UCMSCs transplant group, suggesting that these grafted UCMSCs, under the influence of brain microenvironment, tend to differentiate into glial cells rather than neurons. This result is consistent with other previous reports.

Meanwhile, in BDNF-UCMSCs transplant group we found high expression of BDNF and numerous GFAP-positive cells and some NSE-positive cells at the edge of the traumatic brain area. Compared with simple UCMSCs transplant group, the proportion of GFAP-positive cells were significantly reduced (from 91.3% ± 7.8% down to 57.3% ± 8.9%), while the proportion of NSE-positive cells increased (from 8.7% ± 3.1% up to 41.7% ± 7.4%). These results indicate that the effective transfection and expression of BDNF gene can promote the proportion of UCMSCs differentiation into neurons and at the same time retard the differentiation into glial cells. In addition, we observed that apoptosis cells were greatly diminished at the site of the high expression of BDNF, and motor function scores were superior to that of low expression of BDNF groups (control group and simple UCMSCs transplant groups). In general, BDNF, by promoting the proportion of stem cells differentiation into neurons, plus its role in nerve nutrition, can improve the motor function of experimental animals after traumatic brain injury.

REFERENCES


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