Implantation of bone mesenchymal stem cells overexpressing miRNA-705 mitigated ischemic brain injury

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Abstract. Ischemic brain damage remains the major cause of death and disability worldwide. Bone mesenchymal stem cell (BMSC) transplantation has been identified to serve important roles in cerebral infarction due to its multi-directional differentiation and proliferative ability. However, the function of miR-705 combined with BMSCs on ischemic brain injury remains to be fully elucidated. In the present study, an ischemic brain injury mouse model was constructed, and the mice were injected with BMSCs infected with lentiviral particles expressing miR-705 (BMSCs-Ad-miR-705) to explore the mechanism by which BMSCs-Ad-miR-705 mitigates neurological deficits in ischemic brain damage. In the sham group, no significant neurological injury evaluated via neurological deficit scores was identified, the morphological structure of brain stained with HE was almost normal, and few apoptotic cells were detected by TUNEL assay. However, the PBS group exhibited significant brain damage (P<0.05). BMSCs-Ad (BMSCs infected with control lentiviral particles) and BMSCs-Ad-miR-705 markedly mitigated neurological injury, suppressed morphological damage and inhibited neuronal apoptosis, however promoted the mRNA levels of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) examined by reverse transcription-quantitative polymerase chain reaction and western blotting. Notably, BMSCs-Ad-miR-705 improved the outcome of BMSCs-Ad transplantation. These data indicated that BMSCs-Ad-miR-705 promoted the secretion of VEGF and BDNF, suppressed neuronal apoptosis, and stimulated neuronal regeneration, in turn mitigating the impairment of ischemic brain damage.

Introduction

Despite the development of medical technology, cerebrovascular disease, approximately 80% of which is ischemic brain damage, remains as the major cause of death and disability worldwide (1). Based on its multi-directional differentiation and proliferation ability, bone mesenchymal stem cell (BMSC) transplantation has attracted increasing attention (2). When BMSCs were injected into mice with a cerebral infarction, the cells were observed to survive in the ischemic brain, migrate to the cerebral infarct and promote neurological recovery (3). In addition, BMSCs have been observed to secrete various cytokines and neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) (4). BDNF has been demonstrated to promote functional recovery of the ischemia brain (5), and VEGF induces neuroprotection and neurogenesis following cerebral ischemia (6).

MicroRNAs (miRNAs) are a class of single-strand non-coding RNAs consisting of 18-24 nucleotides in length (7), which are involved in multiple physiological and pathological processes (8) by negatively regulating gene expression at the post-transcriptional level by either target mRNA degradation or translational repression (9). Increasing evidence has demonstrated that miRNAs are crucial for BMSC differentiation (10). miRNA (miR)-705 was first reported to be associated with steatohepatitis (11). Then miR-705 was identified to be a novel negative regulator of cell lineage commitment of BMSCs by directly targeting the homeobox A10 (HOXA10) mRNA 3' untranslated region (12). Furthermore, the transfection of miR-705 mimics or inhibitors in BMSCs affected the expression of forhead box O1 (FoxO1) (13), suggesting miR-705-transfected BMSCs might be a good model for study of function. The present study indicated that the expression of miR-705 was associated with ischemic brain damage of mice. Although previous studies have observed that BMSCs could rescue ischemic brain damage (2-4), the role of miR-705 combined with BMSCs in ischemic brain injury remained to be elucidated.

In the present study, a lentiviral expression vector was designed for miR-705 and BMSCs were infected with lentiviral
particles. Subsequently, the BMSCs overexpressing miR-705 were injected into mice with ischemic brain injuries induced by middle cerebral artery occlusion (MCAO). The effect of BMSCs infected with miR-705 on neurological deficit scores, neuronal morphology and cell apoptosis was investigated in order to confirm the function of miR-705 in ischemic brain damage therapy.

Materials and methods

Experimental animals. A total of 50 male C57BL/6J mice in a SPF grade, aged 8 weeks, weight 200±10 g, were purchased from Beijing Huafukang Biotechnology Company (Beijing, China). Mice were housed in a colony room under controlled temperature (22±3°C), and a 12:12 light-dark cycle, with food and water available. All animal care and use was approved by the ethics committee of Beijing Chao-yang Hospital.

Preparation of BMSCs. The C57BL/6J male mice in SPF grade were anesthetized with chloral hydrate (30 mg/100 g; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) via peritoneal injection. The diaphyses of the bones were cut in sterile conditions in order to open the marrow cavity. Bone marrow was pushed out of the bone with a syringe with a no. 9 needle containing Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). A total of 5 ml bone marrow was aspirated and then mixed with phosphate-buffered saline (PBS) at a volume ratio of 1:1. The mixture was added to the lymphocyte separation solution at a volume ratio of 2:1, and centrifuged at 3,500 x g for 20 min at 4°C. The cloudy layer (lymphocytes) was harvested and washed in serum-free DMEM twice after centrifugation at 1,500 x g for 10 min at 4°C. The cells were incubated in complete medium in a humidified environment with 5% CO₂ at 37°C. The medium was first changed after 24 h, and changed again after 48 h to remove non-adherent cells. Cells were diluted with 9 ml Hanks buffer and mixed with 1 ml 0.4% trypan blue for detection of the cell viability. The BMSC passage was completed at a ratio of 1:2. The cells of passage 3 were used in the following experiments.

Vector construction of a lentiviral expression vector for miR-705. The target sequence of miR-705 was amplified by polymerase chain reaction (PCR) and was inserted into the pCDH-CMV-MCS-EF1-cop-green fluorescent protein (GFP) plasmid (System Biosciences, Mountain View, CA). The recombinant plasmid was verified by restriction endonuclease analysis and DNA sequencing. 293T cells were purchased from American Type Culture Collection and cultured in 25 cm² flask containing 4 ml DMEM with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). At a density of 70-90%, 293T cells were cotransfected with lentiviral vector pCDH-CMV-MCS-EF1-copGFP-miR-705 (System Biosciences) and lentivirus package plasmid mixture. After 8 h, the old medium was replaced with fresh medium for 48 h incubation at 37°C. The supernatant was collected and filtered with a 0.45 μm membrane. The control cells were transfected with the control lentiviral vector. Virus titer was measured according to the expression of GFP. Furthermore, the expression level of miR-705 was measured by reverse transcription-quantitative PCR (RT-qPCR). Lentiviral particles were collected and stored at -80°C for further experiments.

BMSCs were seeded into the 10 cm plate. At a density of 80%, cells were infected with lentiviral particles of miR-705 [BMSCs-adenovirus (Ad)-miR-705], and the control was incubated with control lentiviral particles (BMSCs-Ad). The cell density was adjusted to 1x10⁶ cells/ml prior to transplantation.

Animal model and grouping. Mice were fixed on the table, anesthetized with an intraperitoneal injection of chloral hydrate (30 mg/100 g), and subjected to MCAO for ischemic brain damage. In brief, the skin of the middle of the neck was opened, and the left common carotid artery, external carotid artery (ECA) and internal carotid artery (ICA) were exposed. The origin of MCA was occluded with a monofilament suture with a distal cylinder from ECA to ICA. The sham-operative mice received the same surgery, except that the filament was inserted only 10 mm and withdrawn a minute later.

A total of 40 mice underwent 24 h reperfusion after 2 h ischemic brain injury and were divided into four groups as follows: i) Sham group, underwent sham operation and received PBS (n=10); ii) PBS group, subjected to MCAO and received PBS (n=10); iii) BMSCs-Ad group, subjected to MCAO and injected with BMSCs-Ad (200 µl, 1x10⁶ cells/ml) through ECA (n=10); iv) BMSCs-Ad-miR-705 group, subjected to MCAO and injected with BMSCs infected with Ad-miR-705 (200 µl, 1x10⁶ cells/ml) via ECA (n=10). After 7 days, brain tissues were isolated for further experiments.

RT-qPCR. Total RNA was extracted from the brain tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The purity of RNA was determined by absorbance at 260 nm and 280 nm, and RNA integrity was verified by agarose gel electrophoresis. A total of 5 mg of RNA was converted to cDNA using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). RT-qPCR was performed using Ultra SYBR mixture (CW Biotech, Beijing, China) containing SYBR Green I staining on a CFX96™ Real-time system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The temperature protocol for PCR was as follows: 95°C for 5 min, 95°C for 5 sec, 60°C for 45 sec and 72°C 45 sec, repeated for 40 cycles. The primers for RT-qPCR were as follows: BDNF, forward primer 5'-AGGTCTGACGACAGACACT-3' and reverse primer 5'-CTTGTTGGGCGCAACTT-3'; VEGF, forward primer 5'-GGCAATAGAGAATGACCTTT-3' and reverse primer 5'-CTTCGACTTGAACAGGGT-3'; miR-705, forward primer 5'-AGTATTGGTGGAGTGGTGGTGAGGC-3' and reverse primer 5'-AGGGAGTAGGGAGGACTGC-3'. β-actin was used as the internal control: Forward primer, 5'-CCTGATATGCCGCTGGBCG-3'; reverse primer, 5'-GCGTAAACCTCCTAGATGAT-3'.

Western blotting. Brain tissues were isolated from different groups. Subsequently, 1 ml lysis buffer was added to extract the proteins. The quantity of proteins was determined by Bicinchoninic Acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). The protein samples were boiled for 5 min. A total of 30 μg of proteins were separated on 10%
SDS polyacrylamide gels, then transferred to polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The PVDF membrane was blocked with 5% non-fat milk for 1 h at room temperature, and immunoblotted with antibodies against BDNF (catalog no. sc-546, 1:500) or VEGF (catalog no. sc-152, 1:500) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), respectively overnight at 4°C. Subsequently, the membranes were washed three times with Tris-buffered saline with Tween-20 (20 mM Tris, pH 7.5; 150 mM NaCl; 0.1% Tween-20), and incubated with the horseradish peroxidase-labeled secondary antibody (1:5,000, catalog no. LK-GAR007, MultiSciences Biotechnology Corporate Ltd., Hangzhou, Zhejiang, China) for 1 h at room temperature. The bands were visualized by Enhanced Chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) and analyzed by Quantity One software version 4.4 (Bio‑Rad Laboratories, Inc.). β-actin (1:3,000, catalog no. A5441; Sigma‑Aldrich) was used as the internal control.

Hematoxylin eosin and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. The brains were removed and postfixed in freshly prepared ice 4% paraformaldehyde, dehydrated with graded alcohols and embedded in paraffin. The paraffin-embedded sections with 3-4 μm were deparaffinized with xylene and rehydrated with graded alcohol, then stained with hematoxylin and eosin (HE). The images of slices at x400 magnification were analyzed by a light microscope. Three fields were randomly selected from the damaged area.

TUNEL staining was performed to visualize apoptotic cells according to the manufacturer's protocols (In Situ Cell Death Detection kit; POD; Roche Diagnostics, Basel, Switzerland). Briefly, subsequent to being deparaffinized, the sections were incubated with proteinase K and permeabilized with 0.5% Triton X-100. Then the sections were incubated with TUNEL reaction mixture for 60 min. For each sample, five non-overlapping fields at x400 magnification in sections were randomly captured using a fluorescence inverted microscope. The apoptotic cells were stained in a green color.

Statistical analysis. All data were expressed as the mean ± standard deviation, and were analyzed using SPSS 20 software (IBM Corp., Armonk, NY, USA) with a one-way analysis of variance followed by the Least Significant Difference post hoc test. A value of P<0.05 was considered to indicate a statistically significant difference. Each experiment was repeated for a minimum of three times. For RT-qPCR and western blotting experiments, each mouse sample was expressed as a ratio to its corresponding β-actin value.

Results

A lentiviral expression vector for miR-705 was successfully constructed. To examine the validity of the plasmid, the recombinant plasmid (pCDH-CMV-MCS-EFI-copGFP-miR-705) was digested with two restriction enzymes. Approximately 960 bp of the fragment of interest was detected as presented in Fig. 1A. Combined with the DNA sequencing results, it was concluded that the overexpression vector for miR-705 was successfully constructed. To verify the transfection efficiency, 293T cells were transfected with pCDH-CMV-MCS-EFI-copGFP-miR-705. GFP was clearly expressed in 293T cells (Fig. 1B) and BMSCs cells (Fig. 1C). These results indicated that a lentiviral expression vector for miR-705 had been successfully constructed.

BMSCs-Ad-miR-705 mitigates the neurological impairment in ischemic brain injury mice. The effect of different treatments on nerve function were evaluated by neurological deficit scores (Nd), according to a previous study (14). As presented in Table I, the sham operation has no impairment on nerve function. The Nd of PBS group were the highest, suggesting that nerve function was severely damaged. The Nd of BMSCs group was reduced, indicating that BMSCs transplantation partly reduced the neurological impairment. In addition, compared with PBS and BMSCs groups, the Nd of BMSCs-Ad-miR-705 were the lowest with a significant difference (P<0.05). These results indicated that BMSC transplantation mitigated the neurological impairment, and that Ad-miR-705 infection improved the repair capacity of BMSCs.

BMSCs-Ad-miR-705 suppresses neuronal cell apoptosis in MCAO-induced cerebral infarction. To examine the effect of BMSC transplantation on neuronal morphology, brain tissues were isolated from different groups and HE staining was conducted. In Fig. 2, normal neuronal structure and morphology were observed in the sham group. In the PBS group, MCAO resulted in marked neuronal injury with loose nerve fibers, and certain neocortical neurons were clearly damaged with characteristic vacuolation. Transplantation with BMSCs or BMSCs-Ad-miR-705 markedly improved neurological recovery, for example the number of neurons was increased and the neuronal morphology became normal. In addition, BMSCs-Ad-miR-705 enhanced the effect of BMSCs on neuronal morphology recovery.

The TUNEL assay was used to further detect the effect of BMSCs on neuronal cell apoptosis. Few apoptotic cells were identified in the sham group, and different apoptotic rates were observed in the three other groups in Fig. 3. The apoptotic rate of the PBS group was the greatest at ~50%. BMSC transplantation partly reduced the apoptotic rate (27%). And the apoptotic rate of BMSCs-Ad-miR-705 was the lowest at ~13%. These results indicated that BMSCs-Ad-miR-705 significantly inhibited cell apoptosis caused by MCAO.

Table I. Neurological deficit scores from different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Neurological deficit scores</th>
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</thead>
<tbody>
<tr>
<td>Sham group</td>
<td>0</td>
</tr>
<tr>
<td>Phosphate-buffered saline group</td>
<td>9.34±1.21</td>
</tr>
<tr>
<td>BMSCs-Ad</td>
<td>6.32±1.33</td>
</tr>
<tr>
<td>BMSCs-Ad-microRNA-705</td>
<td>2.13±1.14</td>
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Data are presented as the mean ± standard deviation. BMSC, bone mesenchymal stem cell; Ad, adenovirus.
BMSCs-Ad-miR-705 improves BDNF and VEGF expression on mRNA and protein levels. To investigate the mechanism of BMSCs-Ad-miR-705 stimulating neurological recovery, the expression of two factors, BDNF and VEGF, were examined in brain tissues. Compared with the sham, PBS and BMSCs groups, the BMSCs-Ad-miR-705 group significantly increased the expression of miR-705, reconfirming the successful construction of Ad-miR-705. In Fig. 4, low and stable levels of BDNF, VEGF and miR-705 were detected in the sham group. Compared with the sham group, the expression levels of BDNF, VEGF and miR-705 were upregulated with significance (P<0.05). However, the levels of BDNF, VEGF and miR-705 in BMSCs-Ad and BMSCs-Ad-miR-705 groups were increased compared with those in the PBS group. The levels of BDNF, VEGF and miR-705 in BMSCs-Ad-miR-705 group were the greatest. In agreement, similar results were detected when protein expression levels of BDNF and VEGF were measured using western blotting (Fig. 5). This suggested that BMSC transplantation stimulated the expression of BDNF, VEGF and miR-705, and Ad-miR-705 infection more significantly enhanced the mRNA and protein levels of BDNF and VEGF.

Discussion

The present study demonstrated that administration of BMSCs can effectively improve functional recovery in animal models of cerebral ischemia, which is in agreement with that of a previous study (15). The current study identified that BMSCs-Ad-miR-705 significantly ameliorated neurological deficit, suppressed neuronal apoptosis and improved the expression of BDNF and VEGF in mice with ischemic brain injury. Taken together, these results indicate that injection of BMSCs-Ad-miR-705 is a potential and effective therapy for neurological recovery following cerebral ischemic injury.

In the present study, a lentiviral recombined expression vector was constructed for miR-705 overexpression. GFP was detected in plasmid-transfected 293T cells and lentiviral particle-infected BMSCs, indicating successful construction of Ad-miR-705. In Fig. 4, the highest expression level
of miR-705 was observed in BMSCs-Ad-miR-705-injected mice, suggesting that miR-705 was successfully overexpressed directed by Ad-miR-705. Chen et al (16) identified that Ad-VEGF could infect BMSCs and direct the expression of VEGF in BMSCs. Combined with the results of the present study, it is suggested that this method of lentivirus-mediated overexpression of target genes in BMSCs provides novel insight for cellular therapy.

miR-705 was first identified to be significantly expressed in BMSCs from osteoporosis bone marrow. TNFα and ROS have been observed to mediate the overexpression of miR-705 to regulate the cell lineage commitment of BMSCs through NF-κB signaling (12). The present study focused on investigating the function of miR-705 in cerebral ischemic damage. In mice with cerebral ischemia, intravenous BMSC therapy reduced cell apoptosis and promoted endogenous cell proliferation (17), and stimulated functional recovery for brain repair (18). In the current study, compared with the PBS group, the NDs of the BMSCs-Ad group were decreased, the neurological morphology was improved and the apoptosis rate of neurons were declined. These results were in agreement with the abovementioned previous research. At present, two targets of miR-705 have been reported: HOXA10 and FoxO1. HOXA10 is a member of the family of homeodomain-containing transcription factors, and its overexpression inhibits adipocyte differentiation of BMSCs (12). However, the decrease of FoxO1 leads to oxidative damage and inhibition of BMSC differentiation (13). According to the results of the current study, it was suggested that HOXA10 is the main target gene of miR-705 in improving BMSC therapy. However, further experiments are required.

Previous studies have indicated that administration of BMSCs facilitates the release of neurovascular trophic factors from activated astrocytes, including BDNF, nerve growth factor, VEGF and basic fibroblast growth factor through activating astrocytic phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase pathways (19,20). During the process of neurological recovery, these factors reduced
neuronal apoptosis and enhanced neuronal regeneration and cellular differentiation (21). It was observed that BMSC transplantation increased the expression of BDNF and VEGF. Mature BDNF is crucial in the protection of the neonatal or developing brain from ischemia injury (5). Administration of BMSCs-BDNF into mice significantly promoted functional recovery and reduced the numbers of TUNEL-positive apoptotic cells (22). VEGF promotes the formation of new cerebral blood vessels, and injection of VEGF improves neurological function and protects the brain against ischemia (23). VEGF-BMSCs combination therapy ameliorates ischemic damage by the activation of the PI3K/protein kinase B/glycogen synthase kinase 3β signaling pathway (16). VEGF, in addition to BDNF, has been implicated in the regulation of brain neurogenesis by promoting the proliferation and differentiation of neuronal precursors (24). The current on the effect of BMSCs on the expression of BDNF and VEGF was in agreement with the previous data and it was observed that BMSCs-Ad-miR-705 enhanced the outcome of BMSCs transplantation on NDs improvement, neuronal apoptosis and the expression VEGF and BDNF. Thus, these results provided novel insight into that the role of miR-705 in function neuronal recovery via induction of the secretion of growth factors, and indicated miR-705 as a potential therapeutic target for stem cell-mediated regenerative medicine for cerebral ischemia.

In summary, BMSCs-Ad-miR-705 promoted the secretion of VEGF and BDNF, suppressed the neuronal apoptosis and stimulated the neuronal regeneration, in turn functioning in the impairment of ischemic brain damage. However, the mechanism by which miR-705 functioned in the BMSCs-mediated signaling pathway requires further investigation.

References