Full Length Research Paper

Protective effects of bone marrow mesenchymal stem cells with stable expression of Bcl-2 on ischemia-hypoxia of hippocampal neurons

Fan He¹,², Zhi-Yi He¹*, Lei Li¹, Xue Qin¹, Shu-Min Deng¹ and Fang Liu¹

¹Department of Neurology, The First Affiliated Hospital, Chinese Medical University, Shenyang 110001 China.
²Department of Neurology, General Hospital of Shenyang Military Command, Shenyang 110016, China.

Accepted 24 May, 2011

The protective effect of bone marrow mesenchymal stem cells (MSCs) with stable expression of Bcl-2 on the in vitro ischemia-hypoxia of hippocampal neurons was investigated in the present study. Mouse Bcl-2 cDNA was amplified by RT-PCR followed by Lentivirus-mediated gene transfection into MSCs. Puromycin resistance was detected to screen MSCs undergoing successful transfection, and the mRNA expression of Bcl-2 was determined by RT-PCR. Serum and oxygen deprivation was performed in normal MSCs and those undergoing transfection aiming to mimic ischemia injury in vitro, and flow cytometry was used to detect the apoptosis rate of these cells. Neurogenic induction of Bcl-2 expressing MSCs was carried out followed by detection of neuron markers. The rat hippocampal neurons were isolated from neonate rats. The Bcl-2 expressing MSCs and hippocampal neurons were cocultured, and the protective effect of Bcl-2 expressing MSCs on the ischemia-hypoxia of hippocampal neurons was observed. Following transfection, MSCs had stable and efficient expression of Bcl-2 after screening. When compared with normal MSCs, the number of apoptotic Bcl-2 expressing cells was significantly decreased and that of survival cells markedly increased after serum and oxygen deprivation. In addition, the Bcl-2 expressing MSCs had the potent differentiation into neurons. In the coculture system, Bcl-2 expressing MSCs could remarkably reduce the apoptosis rate of hippocampal neurons after ischemia-hypoxia (P < 0.01). The MSCs undergoing Bcl-2 transfection had stable Bcl-2 expression, which can protect them from serum and oxygen deprivation induced apoptosis and protect hippocampal neuron against ischemia-hypoxia induced apoptosis in vitro. Furthermore, the Bcl-2 transfection does not affect the differentiation of MSCs into neurons.

Key words: B cell lymphoma/leukemia gene 2, gene transfection, mesenchymal stem cells.

INTRODUCTION

In recent years, stem cell transplantation, a new strategy different from conventional treatment of neurological diseases, has been developed to remodel the impaired neuronal pathways and improve neurological dysfunction. This method has been the focus of research on ischemic cerebrovascular diseases (ICVD) (Dharmasaroa, 2009). Stem cells used in the treatment of ICVD include neural stem cells (NSCs) and bone marrow-derived mesenchymal stem cells (MSCs). NSCs are derived from the brain and the embryonic stem cells, and not suitable for autologous transplantation due to ethical constraints. Therefore, MSCs are preferable for the treatment of ICV (Himes et al., 2006; Lee et al., 2006; Gregory et al., 2005). However, the low survival rate of MSCs after transplantation limits its wide application in clinical practice. Studies have demonstrated that MSCs injected to the ischemic lesions in several ways can reduce the infarct volume and improve the neurological function (Wong et al., 2005; Wei et al., 2005; Shen et al., 2006). Chen et al. (2003) revealed the proportion of MSCs differentiating into neurons was as high as 80% in vitro, but that was only 3 to 10% in vivo. The mechanisms
underlying the low differentiation rate in vivo are complex and may be attributed partially to the apoptosis of MSCs after transplantation. Therefore, it is imperative to reduce the apoptosis and increase the survival rate of MSCs after transplantation and improve the differentiation of these cells into neurons in the stem cell transplantation. In the present study, lentivirus mediated transfection of Bcl-2 into MSCs was performed achieving MSCs with stable Bcl-2 expression aiming to improve the long-term expression of Bcl-2 in MSCs and the subsequent survival of MSCs in vivo and prolong the protective effects of MSCs on the ischemia-hypoxia of neurons. Our study provides basis for clinical application of transplantation with MSCs.

MATERIALS AND METHODS

Gateway® BP Clonase™ II Enzyme Mix, Gateway® LR Clonase™ II Plus Enzyme Mix, Lipofectamine™ 2000, Opti-MEM I Reduced Serum Medium (Invitroge, USA), TRI REAGENT (MRC, USA), RQ1 RNase-Free DNase (Promega, USA), RevertAid™ First Strand cDNA Synthesis Kit, Taq DNA Polymerase, dNTP Mix, GeneRuler™ 100 bp DNA Ladder, RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA), Puromycin, Polybrene, B-ME (Sigma, USA), DNA Gel Extraction Kit, Plasmid Mini Kit (TIANGEN), Neomycin, D/F12 (Gibco), Transwell Permeable Supports (Corning), F344 rat MSCs complete medium, PBS, trypsin, Neurobasal medium+2% B27 (Cyagen), Annexin V-FITC apoptosis detection kit (Beyotime Institute) and F344 rat MSCs (Cyagen) were used in the present study.

Construction and identification of Bcl-2 expressing lentiviral vectors

According to mouse Bcl-2 gene sequence, attB1 and attB2 restriction sites were designed in the primers. The primers were as follows: 5'-GGGGACAAGTTTGTACAAAAAAAGCAGGCTGCCACCATGGCGCAAGCCGGGAGAACGGGGACCACTTTGTACAAGAAAGCTGGGTTCACTTGTGGCCCA-3'; 5'-GGGGACACCTTTTACCAAAAGAAGTCTTGTTGTTCACTTGGCCCA-3'. The cDNA was used as template for amplification by PCR followed by determination of mRNA quality by gel electrophoresis and subsequent mRNA extraction and purification. BP binding reaction was performed to construct vector pDOWN-mBcl-2. The reactant from BP binding reaction was transformed into competent One Shot OmniMAX™ 2 T1 Phage-Resistant Cells Escherichia coli. LR binding reaction was carried out to construct vector pLV/EXPN2-Puro-EF1a-mBcl2, and the reactant from LR binding reaction was transformed into competent One Shot® MACH1™ T1R Chemically Competent E. coli. After screening recombinants, the plasmids were extracted followed by sequencing.

Transfection of rat MSCs with lentivirus carrying Bcl-2

MSCs were seeded into 6-well plate at an appropriate density. When the cell confluence reached 30 to 50%, transfection was performed. The lentivirus carrying mBcl-2 in concentrate was added into the 6-well plate. Polybrene (6 μg/ml) was supplemented to the medium to help viral infection. The medium was refreshed after transfection. The mRNA expression of Bcl-2 was measured by RT-PCR.

Anti-apoptotic potency of Bcl-2 expressing MSCs

The F344 MSCs and Bcl-2/F344 MSCs were maintained in the T75 flask. When the cell confluence reached 80 to 90%, these cells were transferred into a 6-well plate and incubated in at 37°C in a humidified atmosphere with 5% CO₂ for 24 h. Then, the medium was removed and the medium containing Puromycin (4 μg/ml) was supplemented to screen MSCs with successful transfection, and incubation was performed overnight in a humidified atmosphere with 5% CO₂ at 37°C. The mRNA expression of Bcl-2 was measured by RT-PCR.

Neurogenic induction of Bcl-2 expressing MSCs

The frozen-thawed Bcl-2 F344 MSC was passaged at a ratio of 1:2 followed by neurogenic induction. Then, these MSCs were seeded into a 6-well plate at an appropriate density. When cell confluence reached 50 to 60%, 1 mmol/L β-ME (DMEM-20% FBS) was added to induce the MScs for 6 h. When cell confluence reached 90%, serum and oxygen deprivation (SOD) was performed as previously reported (Zhu et al., 2006). The apoptosis was detected by flow cytometry according to manufacturer's instructions. The cells in blank control, Annexin-FITC labeled cells, PI labeled cells, and Annexin-FITC plus PI labeled cells were detected and experiment was repeated three times.

Isolation, culture and identification of hippocampal neurons

The hippocampal neurons were isolated and seeded into 6-well plate followed by incubation for 2 days. When cell adherence was observed, coculture of neurons and Bcl-2 expressing MSCs was carried out. The F344 MSCs or F344 MSCs-mBcl2/eGFP were added to the upper chamber (about 2×10⁵/well) in duplicates followed by addition of DMEM/10% FBS. Then, the cells in the upper chamber were transferred into hippocampal neurons in the 6-well plate. SOD was done to mimic ischemic injury. Apoptosis rate was determined by flow cytometry.

RESULTS

Screening Bcl-2 expressing MSCs and detection of mRNA expression of Bcl-2

The screening of Bcl-2 expressing MSCs was carried out based on Puro resistance. Three days after screening, cell death was observed. When compared with wild type MSCs, the number of survival Bcl-2 expressing MSCs was significantly increased. Eight days after screening, obvious proliferation was noted, and the number of viable cells with Bcl-2 transfection was markedly higher than that of wild-type MSCs. After 8 days of treatment with Pro, the medium was refreshed with complete medium.
and the screening was discontinued. Then, the mRNA expression of Bcl-2 was determined by RT-PCR (Figure 1).

Detection of apoptosis after SOD by flow cytometry

The apoptosis rate in Bcl-2/F344 MSCs and F344 MSCs was 18.95±2.57% and 30.87±5.69%, respectively, showing significant difference (P < 0.01) (Figure 2).

mRNA expressions of NSE and Vimentin after neurogenic induction

After pre-induction, some spherical cells were observed, but a majority of cells were short spindle-shaped cells and adherent to the wall with favorable growth. After induction, these cells became spherical and contracted significantly. In addition, these cells had the tendency to suspend in the medium. Results showed cells had expressions of NSE and Vimentin regardless of the induction. Furthermore, the mRNA expression of NSE in MSCs after induction was significantly higher than that in cells without induction, but there was not significant difference in the mRNA expression of Vimentin between cells with and without induction (Figure 3).

Coculture of MSCs and hippocampal neurons

After 3 days of culture, the cell body of neurons was spindle-shaped or triangular and these cells aggregated to be a network with favorable refraction. With the prolongation of cell culture (after 5 days of culture), apoptosis was observed and the cell outline was unclear accompanied by poor refraction. In addition, some black granules were noted in the cell body and the protrusion was discontinued or suspended. However, in the coculture of Bcl-2 expressing MSCs and neurons, the morphology of Bcl-2 expressing MSCs and neurons, the morphology of neurons was not markedly changed. After 8 days of culture, apoptosis of neurons was determined and six wells were included in each group. When compared with control group, the apoptosis rate in the Bcl-2/F344 MSCs group and F344 MSCs group was significantly reduced (P < 0.01). In addition, the apoptosis rate of neurons in Bcl-2/F344 MSCs group was lower than that in F344 MSCs group (P < 0.01). (Figures 4 and 5; Table 1).

DISCUSSION

MSCs have the characteristics of adherent growth and can continuously differentiate and divide with stable hereditary. These cells are suitable for transfection of exogenous gene and subsequent protein expression (Kurozumi et al., 2004). Bcl-2 is an oncogene isolated from the follicular lymphoma by Tsujimoto et al. (1885). Bcl-2 can counteract with apoptosis of a lot of causes, and has been regarded as an anti-apoptotic gene. Although Bcl-2 gene is initially regarded as an oncogene, it does not affect the proliferation and differentiation of normal cells, and not cause the malignant transformation.

Figure 1. mRNA expressions of Bcl-2 in different cells: 1: water; 2: wild-type MSCs (F344 MSCs); 3: Bcl-2 expressing MSCs; 4. pLV/EXPN2-puro-EF1A-mBcl-2; 5: 100 bp marker.
and subsequent cancer formation. Therefore, Bcl-2 gene can be introduced into MSCs aiming to perform gene therapy and stem cell therapy. In recent years, Li et al. (2007) have transplanted the MSCs with Bcl-2 gene.

Figure 2. Flow results for apoptosis. (a) F344MSC-mBcl2/Egfp, (b) F344 MSCs.)
Figure 3. mRNA expressions of NSE and Vimentin after induction. 1: empty; 2: water; 3: NSE expression in Bcl-2 expressing F344 MSCs without neurogenic induction; 4: NSE expression in Bcl-2 expressing F344 MSCs after neurogenic induction; 5: NSE expression in the brain from the 2-day old rat; 6: marker; 8: water; 9: Vimentin expression in Bcl-2 expressing F344 MSCs without neurogenic induction; 10: Vimentin expression in Bcl-2 expressing F344 MSCs after neurogenic induction; 11: Vimentin expression in the brain from the 2-day old rat; 12: marker.

Figure 4. Features of MSCs and hippocampal neurons in the coculture system at different time points (400×). Mock means hippocampal neurons under simple normal culture.

modification into rats with myocardial infarction, and their results showed Bcl-2 did not affect the multipotentiality of MSCs. Furthermore, the capillary density in the peri-

lesion area was increased by 15% and the infarct volume
Figure 5. Apoptosis of neurons determined with flow cytometry. (a) Bcl-2/F344 MSCs; (b) F344 MSCs; (c) control.

Table 1. Apoptosis rate of neurons in the coculture system (% ±S).

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptosis rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2/F344 MSCs</td>
<td>18.3±2.16*, **</td>
</tr>
<tr>
<td>F344 MSCs</td>
<td>28.45±4.35</td>
</tr>
<tr>
<td>control</td>
<td>42.15±6.12</td>
</tr>
</tbody>
</table>

N = 6; *P < 0.01 vs. control; **P < 0.01 vs. F344 MSCs group.

decreased by 17% accompanied by significantly improved myocardial function. This study provides theoretical evidence for the treatment of cerebral ischemia with Bcl-2 expressing MSCs. In the study of Li et al. (2007), the Bcl-2 was not integrated into the genome of MSCs and the expression of Bcl-2 is transient. Therefore, the in vivo survival of transplanted MSCs was not improved and the long-term efficacy is still poor. Presently, strategies of cell transfection mainly include lipofection and virus mediated transfection. The frequently used viruses include adenovirus, herpes simplex virus, retrovirus and lentivirus. Transfection with retrovirus or lentivirus has relatively high efficiency and
target gene can be integrated into the genome of vector cells with stable and sustained expression of target gene. Transfection with retrovirus depends on the cell cycle, which was not found in transfection with lentivirus. Therefore, lentivirus was employed for transfection of MSCs achieving high and stable expression of Bcl-2.

In the treatment of diseases of the nervous system with MSCs transplantation, the cerebral ischemia is the most frequently investigated. The post-ischemic detrimental pathophysiology can result in apoptosis and/or necrosis of neurons and also harmful for the survival of transplanted MSCs and subsequent therapeutic efficacy. In the present study, serum and oxygen deprivation was performed according to previous report (Wei et al., 2005). Our results showed SOD could induce the apoptosis of MSCs. But the apoptosis rate in Bcl-2 expression MSCs was markedly reduced accompanied by increased survival of these cells. This result implies transfection of Bcl-2 is protective on ischemia induced injury, and the anti-apoptotic capacity of Bcl-2 expressing MSCs is more potent than that of normal MSCs. Therefore, it may be possible for the treatment of cerebral ischemia with Bcl-2 gene modified MSCs transplantation with improved efficacy.

Bone marrow derived MSCs are non-hematopoietic stem cells with the characteristics of self-renew and multipotent. These MSCs can differentiate into not only bone, cartilage cells, lipocyte and other mesenchymal cells, but neurons and glial cells under appropriate conditions (Woodbury et al., 2000; Sanchez-Ramos et al., 2000; Kohyama et al., 2001; Deng et al., 2001). MSCs can be obtained easily and have potentials of in vitro differentiation and division with stable hereditary. MSCs have been used in the transfection of exogenous genes and for expression of exogenous protein (Kurozumi et al., 2004). In addition, MSCs can be collected autologously and expanded in vitro. After genetic modification, these cells can be transfused autologously without concern of immune rejection. Moreover, these cells can promote the proliferation and differentiation of embryonic stem cells and neural stem cells, and play important role in the post-injury repair in nervous system, gene therapy, hematopoietic reconstitution, etc. These advantages render the MSCs an important vector in the gene therapy of a lot of diseases (Himes et al., 2006; Gregory et al., 2005; Lee et al., 2006). In vivo studies showed the transplanted MSCs in the animals with cerebral ischemia could express neuronal nuclei (NeuN), microtubule associated protein 2 (MAP-2) and glial fibrillary acidic protein (GFAP). But the expressions of these markers of neurons and glial cells can not confirm that the MSCs have differentiated into mature neurons and glial cells (Chen et al., 2001). Usually, mature neurons have 1 axon and multiple dendrites. These neuritis can form synapses with other cells through which the action potential is transmitted. Therefore, the synapse formation and generation of action potential are the true markers of physiological activities of neurons. Bae et al. (2007) found that, after transplanting Niemann-Pick disease into rats, MSCs could differentiate into Purkinje neurons with electrical activity and synapse network was also noted. Our results showed Bcl-2 expressing MSCs still had the potency of differentiation into neurons. This finding suggests that the introduction of Bcl-2 into MSCs does not alter the stem cell characteristics of MSCs. In the present study, only the differentiation of F344-MSC-mBcl2/eGFP into neurons was detected and statistical analysis was not performed. The high expression of NSE in Bcl-2 expressing MSCs may be related to the enhanced anti-apoptotic capability and prolonged survival time of these MSCs. Our results showed Bcl-2-MSCs or MSCs could protect hippocampal neurons against apoptosis in the coculture system. The apoptosis rate of neurons in the coculture system was significantly lower than that in control group (P < 0.01). Furthermore, the apoptosis rate of neurons in the coculture with Bcl-2-MSCs was lower than that in the coculture with MSCs (P < 0.01). These findings suggest the protective effects of Bcl-2 expression MSCs may be associated with the prolonged survival time and stable and efficient expression of Bcl-2 leading to gene therapy and stem cell therapy. Our study provides evidence for the treatment ICVD with stem cells.

REFERENCES


