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Research article

Bone marrow mesenchymal stem cells decrease CHOP expression and neuronal apoptosis after spinal cord injury

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HIGHLIGHTS

- Traumatic injury increased CHOP expression and apoptosis in rat spinal cord.
- BMSCs transplantation decreased CHOP expression, apoptosis, and increased the locomotor function.
- Co-culture with BMSCs decreased OGD induced CHOP expression and apoptosis in motor neurons.
- Co-culture with BMSCs-CM restored the viability of post-OGD motor neurons.

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ABSTRACT

Spinal cord injury (SCI) leads to irreversible neuronal loss and ultimately leads to paralysis. Bone marrow derived mesenchymal stem cells (BMSCs) have been demonstrated to be an effective approach to treat SCI. The present study was designed to investigate the role of BMSCs in rats with spinal cord injury and in oxygen-glucose deprivation (OGD) treated motor neurons. The results demonstrated that BMSCs could improve locomotor function and decrease expression of pro-apoptotic transcription factor C/EBP homologous protein (CHOP) and apoptosis after SCI. Furthermore, co-culture with BMSCs or conditioned medium from BMSCs could also decrease the expression of CHOP and apoptosis in post-OGD motor neurons, supporting that BMSCs exerts protective effects by decreasing the expression of CHOP in injured motor neurons. Our findings provide a potential novel mechanism for BMSCs treatments in patients with SCI.

1. Introduction

Spinal cord injury (SCI) leads to irreversible loss of neurons and impairments of motor and sensory function below the injury [1]. Recently, studies demonstrated that bone marrow mesenchymal stem cells (BMSCs) are an effective approach to treat SCI by supporting the cell survival, promoting axonal regeneration, and improving functional outcome [2,3]. BMSCs have the potential benefit for differentiating neurons, and related trophic factors can indirectly trigger endogenous survival signaling pathways to protect injured neurons and promote nerve regeneration after SCI [4]. However, the exact mechanism underlying BMSCs' protective effects against SCI is still elusive.

Endoplasmic reticulum (ER) is an intracellular organelle responsible for the synthesis and proper folding of proteins to maintain cellular homeostasis [5]. One of the main components of ER stress-mediated apoptosis pathway is C/EBP (CCAAT enhancer binding protein) homologous protein (CHOP), a transcription factor [6]. Suppressing CHOP expression can reduce apoptosis [7]. Previous studies have proved that CHOP plays a direct functional role in the early stage of traumatic SCI and attenuating ER stress response could improve functional recovery after SCI [8].

In the present study, we used modified Allen's weight-drop SCI rat model and oxygen glucose deprivation (OGD) treated VSC4.1

Abbreviations: BMSCs, bone marrow mesenchymal stem cells; BMSCs-CM, conditioned medium of BMSCs; CHOP, C/EBP homology protein; ER, endoplasmic reticulum; H&E, hematoxylin and eosin; OGD, oxygen–glucose deprivation; SCI, spinal cord injury.

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motor neurons to investigate effects of BMSCs on CHOP expression and apoptosis in injured neurons after SCI.

2. Materials and methods

2.1. Primary BMSCs culture and characterization

The experimental procedures were approved by the Animal Ethics Committee of Zhejiang University and were carried out in accordance with institutional guidelines.

Rat primary BMSCs were isolated according to our previous study [9]. Cells were routinely characterized by flow cytometry analysis. Cells were labeled for 30 min using antibodies against CD34, CD44, CD90 and CD105, conjugated with FITC (1:200 v/v, Boster Biotechnology, China). To track the BMSCs *in vivo* after injection into the spinal cord, green fluorescent protein (GFP) tagged adenovirus vector was used. Some BMSCs at 80% confluence were transfected with a GFP-encoding adenovirus backbone vector, pHBAd-CMV-IRES-GFP. The transfection efficiency of GFP in BMSCs was >99%, which was confirmed under a fluorescent microscope (FluoView FV1000; Olympus Corporation, Tokyo, Japan).

2.2. Spinal cord injury mode

Sprague-Dawley male rats (200–220 g) were divided into 3 groups: sham operation group, SCI group and BMSCs treatment SCI group ($n = 20$/group). After 40 mg/kg, i.p. sodium pentobarbital anesthesia, the vertebral column of the rats was exposed and a laminectomy was carried out at T10 level. A weight of 10 g was dropped from a height of 5 cm on the exposed spinal cord, and the impounder was left for 20 s before being withdrawn to produce a moderate contusion in SCI group. The sham operation animals received the same surgical procedure except injury. The 5 μl GFP-positive or GFP-negative BMSCs (5×10^5) were injected into the epicenter of the injured spinal cord using an electrode microneedle in BMSCs treatment rats while the same quantity PBS were injected into the sham rats after contusion immediately.

Some rats were sacrificed for pathological, immunohistochemistry and RT-qPCR analysis 24 h after transplantation. The locomotor function was assessed by the Basso, Beattie and Bresnahan (BBB) locomotor rating scale within 7 days after transplantation ($n = 10$/group). The BBB scale is to evaluate the functional recovery of locomotor capacity in rats after spinal cord contusion [2].

The survival of BMSCs in the spinal cord was confimed by Western blot assay to detect the expression of GFP in our preliminary experiment. After 1 week transplantation, a spinal cord segment (4 mm length, $n = 3$) at the contusion epicenter was dissected and homogenized for protein assay. Spinal cord segment without BMSCs transplantation was set as control. The primary antibodies were as follows: GFP antibody (M1004; 1:300; Hua'an Biotechnology, China) and β-actin antibody (1:5000; Sigma, USA). See Supplementary Fig. 1.

2.3. H&E staining and in situ TUNEL staining

Five rats per group were re-anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused with 4% paraformaldehyde in phosphate-buffered saline 24 h after transplantation. The lesion epicenter (4 mm length) of the spinal cord was removed and the transverse sections (8 μm) were used for H&E Staining and TUNEL staining. Some sections (20 μm) were stained with the DAPI staining solution (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride, DAPI, Sigma) to show the nuclear and GFP-positive BMSCs were visualized under fluorescent microscopy.

TUNEL staining was used to detect the apoptotic cells in spinal cord according to the manufacturer's protocol (Roche Diagnostics Corporation, Indianapolis, IN, USA) as previously reported [10]. Cells with blue granules in the nucleus were TUNEL-positive cells. Quantitative analysis was performed blindly by counting the number of TUNEL-positive cells in five microscopic fields from six cross sections of the injured spinal cord.

2.4. Immunohistochemistry

The sections from rats ($n = 5$/group) were incubated overnight at 4 °C with CHOP antibody (1:100, Santa Cruz Biotechnology, USA) followed by incubation with biotinylated horse-anti-mouse IgG (1:200, Boster Biotechnology, China) for 2 h at room temperature. Then, avidin–biotin–peroxidase complex solution (ABC, 1:100, Boster Biotechnology, China) was added and incubated for another 2 h. Subsequently, the sections were visualized with 3′3-diaminobenzidine solution (DAB kit) (Gene Tech) under an optical microscope.

2.5. RT-qPCR

The lesion epicenter of spinal cord was removed (about 4 mm), and total RNA was obtained using the RNA extraction kit (Qiagen, Hilden, Germany) ($n = 5$/group). Primers were used for the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) in RT-PCR to amplify GAPDH (forward, 5′-AGTTCAACGGCACAGTCAAG-3′; reverse, 5′-TACTCAGCACCAGCATCACC-3′) as an internal control of CHOP (forward, 5′-CGGAGTGTACCCAGCACCATCA-3′; reverse, 5′-CCCTCTCCTTTGGTCTACCCTCA-3′). Fold changes in gene expression were estimated using the CT comparative method normalizing to GAPDH CT values and relative to control samples. $\Delta CT = CT\ CHOP - CTGAPDH$, $\Delta\Delta CT = \Delta CT - \Delta CT$ control. Fold difference $= 2 - (\Delta\Delta CT)$ and the expression of sham operation rats was set as 1.

2.6. Oxygen–glucose deprivation (OGD)

The ventral spinal cord 4.1 (VSC4.1) motor neuron cells were grown in RPMI1640 media with fetal bovine serum (10%, v/v; Gibco, Invitrogen) and 1% penicillin and streptomycin at 37 °C with 5% CO_2 in a fully humidified incubator as previously reported [11].

Induction of cell death and apoptosis *in vitro* by OGD and re-oxygenation model, was initiated as previously reported with slight modification [12]. Briefly, VSC4.1 motor neurons were incubated in glucose-free Hanks' balanced salt solution in a sealed hypoxic GENbag fitted with a catalyst (BioMèrieux, Marcy l'Etoile, France) to scavenge free oxygen. Cells cultured in Hanks' balanced salt solution containing normal concentration of glucose with 5% CO_2 in a fully humidified incubator were used as the Non-OGD control. Cells were returned to original medium, and placed in a normoxic chamber (re-oxygenation, 37 °C, 5% CO_2) for 20 h. In preliminary experiments, a time-course study for VSC4.1 motor neurons involving in the time period of OGD for 2, 4, 8, 12, 16 or 20 h was carried out and a 8 h OGD period was determined as the optimum time point for co-culture of BMSCs and 8, 12, 16 h OGD periods were used for co-culture of conditioned medium of BMSCs.

2.7. Co-culture of post-OGD VSC4.1 motor neurons with BMSCs

BMSCs (5×10^5/well) were seeded to the insert chamber of the 6-well 0.4 μm transwell system and co-cultured with 5×10^5/well VSC4.1 motor neurons for 48 h. Then the insert chambers were removed and VSC4.1 motor neurons were exposed to 8 h OGD and