Cytotherapy, 2014; 16: 1336–1344

International Society for Cellular Therapy
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ORIGINAL PAPERS

Mechanism of mesenchymal stem cell–induced neuron recovery and anti-inflammation

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Abstract

Background aims. After ischemic or hemorrhagic stroke, neurons in the penumbra surrounding regions of irreversible injury are vulnerable to delayed but progressive damage as a result of ischemia and hemin-induced neurotoxicity. There is no effective treatment to rescue such dying neurons. Mesenchymal stem cells (MSCs) hold promise for rescue of these damaged neurons. In this study, we evaluated the efficacy and mechanism of MSC-induced neuro-regeneration and immune modulation. *Methods.* Oxygen-glucose deprivation (OGD) was used in our study. M17 neuronal cells were subjected to OGD stress then followed by co-culture with MSCs. Rescue effects were evaluated using proliferation and apoptosis assays. Cytokine assay and quantitative polymerase chain reaction were used to explore the underlying mechanism. Antibody and small molecule blocking experiments were also performed to further understand the mechanism. *Results.* We showed that M17 proliferation was significantly decreased and the rate of apoptosis increased after exposure to OGD. These effects could be alleviated via co-culture with MSCs. Tumor necrosis factor-α was found elevated after OGD stress and was back to normal levels after co-culture with MSCs. We believe these effects involve interleukin-6 and vascular endothelial growth factor signaling pathways. *Discussion.* Our studies have shown that MSCs have anti-inflammatory properties and the capacity to rescue injured neurons.

Key Words: *immune modulation, MSC, neuro-regeneration, stroke*

Introduction

Stroke is among the leading causes of death in the United States (1). The incidence of stroke is closely related to risk factors such as hypertension, cigarette smoking, diabetes mellitus and atrial fibrillation (2–5). Ischemic stroke results from cerebral artery occlusion with subsequent drastic reduction in oxygen and glucose levels in the affected brain areas.

Secondary brain injury following the ischemia includes a cascade of events such as inflammation and clot-induced thrombin activation. Thrombin can affect many cell types, including brain endothelial cells, leading to disruption of the blood-brain barrier and formation of brain edema (6), with injury and death to neurons (7,8), microglia (9) and astrocytes (10). Leukocytes also contribute to secondary brain injury after infiltration into the injury site through production of reactive oxygen species, pro-inflammatory cytokines, chemokines, and matrix metalloproteinases (MMPs) (11,12). Previously, Liesz *et al.* compared humoral neuroinflammation and showed a profound and dynamic increase in the expression of interleukin (IL)-1, tumor necrosis factor (TNF)α, interferon (IFN)γ, IL-6, transforming growth factor (TGF)β and IL-10 (13). Because inflammatory cascades contribute to secondary injury after stroke, there is a strong need to identify a reliable cell therapy to modulate this pathologic process.

We chose to study bone marrow (BM) mesenchymal stem cells (MSCs) because they are the most promising of all stem cell types based on pre-clinical and clinical studies (14–16). MSCs are multipotent cells that can differentiate into different cell lineages and have emerged as a promising tool for cell-

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(Received 10 January 2014; accepted 2 May 2014)

http://dx.doi.org/10.1016/j.jcyt.2014.05.007

targeted therapies and tissue engineering in animal models and clinical trials. In addition, MSCs are immunosuppressive, which can ultimately modulate inflammatory reactions following intracerebral hemorrhage (ICH). A previous report demonstrated that the immunosuppressive function of MSC was induced by IFNγ in the presence of any of three other proinflammatory cytokines, TNFα, IL-1α and IL-1β (17). The combination of these cytokines stimulates MSCs to express high levels of chemokines and inducible nitric oxide synthase (iNOS), both of which drive T-cell migration into proximity with MSC. These properties make MSC therapy a good candidate for stroke treatment.

Although several studies address the efficacy of MSCs in ischemic and hemorrhagic cerebral hypoxia models (18–20), the molecular mechanism of their possible benefit is still unclear. We therefore proposed to investigate the mechanism of MSC induced neuro-protection and neuro-regeneration using an OGD model. To simulate ischemic stroke *in vitro*, neuronal cells were subjected to stress through OGD and then co-cultured with MSCs.

In our current study, we investigated the mechanism of MSC-induced rescue of neuronal cells from OGD stress. First, we characterized the MSCs isolated from donor marrow to confirm whether the cells meet International Society for Cellular Therapy (ISCT) criteria for MSCs. Second, we co-cultured the neuronal cell line M17 and MSCs in a non-contact system after OGD stress; MSCs exhibited a neuro-protective effect through release of IL-6 and vascular endothelial growth factor (VEGF) and alleviation of inflammation by decreasing TNFα released from stressed neurons. This study provides new insights into the mechanisms of MSC-based therapy and the effects of MSC-induced immunoregulation in response to neuronal injury.

Methods

Cell culture

An M17 human neuroblastoma cell line was obtained from American Type Culture Collection. The cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) (catalog 11965126; Invitrogen, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (catalog S11550, lot: K11050; Atlanta Biologicals, Flowery Branch, GA, USA). MSCs were isolated from BM from a healthy donor using Histopaque-1077 (catalog 10771; Sigma, St. Louis, MO, USA) following density gradient protocols. The MSCs were generated after culture in alpha minimum essential medium (αMEM) (catalog 12561072; Invitrogen) supplemented with 16.5% fetal bovine serum (designated CCM medium) according to a previously reported method (21–23).

MSC characterization

Characterization of human BM-MSCs via MSC markers: MSC from human BM sample could be expanded in an adherent growth manner. MSC exhibited typical fibroblast-like morphology (Supplementary Figure 1A). These cells were capable of differentiating into osteoblasts and adipocytes (Supplementary Figure 1C–F). Additionally, they expressed MSC markers (CD73, CD90 and CD105) but were negative for hematopoietic stem cell marker (CD34) and pan-leukocyte marker (CD45). Therefore, the characteristics of our BM-MSC met the criteria set by ISCT for MSCs (Supplementary Figure 1G).

Colony-forming units (CFU) assay. 200 MSC cells were seeded at passage 2 to one 10-cm diameter culture dish in CCM and cultured in 37°C, 5% CO_2 incubator for 14 days. The CCM medium was removed, and cells were washed with phosphate-buffered saline and then stained with 3% crystal violet in 100% methanol. Stained colonies were counted under an inverted microscope. CFU was calculated as number of colonies divided by cells plated × 100 (21). The percent of CFUs for early passage MSC expanded at low density were found to be >40% (Supplementary Figure 1B).

Osteogenic and adipogenic differentiation assay. MSCs were seeded at 1×10^5 cells/well in six-well plate at passage 1 and grown to 100% confluence. CCM was replaced with either bone differentiation medium (CCM supplemented with 1 nmol/L dexamethasone (catalog D2915), 20 mmol/L β-glycerolphosphate (catalog G9422), and 50 μg/mL L-ascorbic acid 2-phosphate (catalog A8960)), or fat differentiation medium (CCM supplemented with 0.5 μmol/L dexamethasone, 0.5 μmol/L isobutylmethylxanthine (catalog I5879), and 50 μmol/L indomethacin (catalog I7378)) to induce bone differentiation and fat differentiation. Cells induced under osteogenesis were stained for calcium with 1% alizarin red S (catalog A5533). Cells induced under adipogenesis were stained for lipid droplets with 0.3% oil red O (catalog O0625) (22,23). All differential media supplements and stains were obtained from Sigma.

OGD stress model

M17 cells were grown in 24-well or 6-well plates coated with rat collagen I (catalog 08115; Millipore,