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hucMSC derived exosomes promote functional recovery in spinal cord injury mice via attenuating inflammation

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ABSTRACT

The exploration of effective spinal cord injury (SCI) healing still remain a great challenge due to the high morbidity, complex pathology and unclear targets. Human umbilical cord mesenchymal stem cells (hucMSC) play an important role in tissue regeneration. However, transplanting stem cells has a potential risk of teratogenicity. Recent studies have suggested that exosomes secreted by stem cells may contribute to tissue injury repair. We hypothesized that the application of hucMSC derived exosomes may be a potential way for SCI treatment. Our studies showed the hucMSC derived exosomes with a mean particle size of 70 nm could effectively trigger the bone marrow derived macrophage (BMDM) polarization from M1 to a M2 phenotype. In vivo studies demonstrated that the hucMSC derived exosomes could improve the functional recovery after SCI through down-regulation of the inflammatory cytokines, such as TNF-α, MIP-1α, IL-6 and IFN-γ. Collectively, our findings indicated that hucMSC derived exosomes could facilitate spinal cord injury healing via attenuating the inflammation of the injury region. Our results provided a new perspective and therapeutic strategy for the use of hucMSC derived exosomes in soft tissue repair.

1. Introduction

Spinal cord injury (SCI) and traumatic brain injury (TBI) are complex and devastating clinical conditions characterized by neuronal loss, axonal destruction and demyelination during the secondary injury cascade [1,2]. Under physiological conditions, immunocytes can't pass the blood-brain barrier (BBB) in the spinal [3]. After disruption of the BBB by a mechanical force that directly destroys neural tissue and endothelial cell membranes, immunocytes accumulate and infiltrate into the lesion site to drive inflammatory immune responses [4]. The inflammatory response is the most important pathological process, which plays vital roles in the prognosis of SCI. Many cells participate in the innate immune response after SCI, including inherent within the nervous tissue of microglia and blood-borne neutrophils, mononuclear macrophage, etc. During the early stage of SCI, inflammatory cytokines exhibit cytotoxicity to the nervous system and aggravate tissue injury [5]. After SCI, some cytotoxic cytokines such as TNF-α, IL-1β, and IL-6 are produced. These pro-inflammatory cytokines are involved in recruiting leukocytes and activating macrophages and microglia, and such cytokines are upregulated early in the inflammatory response [6]. Therefore, these cytokines may become potential targets for medication therapy in SCI [7–9].

In the central nervous system (CNS) lesions, influenced by local microenvironment of the different inflammatory response signal, macrophages have heterogeneous phenotypes that range along a continuum from the “classically-activated” proinflammatory, cytotoxic M1 cells, to the “alternatively-activated” anti-inflammatory, prorepair M2 cells [10]. Increasing evidence suggests that the M1 macrophages dominant around the blood vessels of the central nervous system, and can secrete inflammatory cytokines, like IL-1β, IL-6, TNF-α and IFN-γ, which further damage to the cells of the host [11,12]. Activated M2 macrophages can substantially inhibit immune inflammatory reaction by producing IL-4 and IL-10, remove necrotic tissue fragments, promote angiogenesis and tissue repair [13,14]. Therefore, targeting macrophages can be applied for treating SCI at several levels that is, stopping inflammatory monocyte recruitment, inhibiting macrophage proliferation, blocking M1 activation pathway, reprogramming macrophages towards the M2 phenotype, and transplantation of beneficial macrophages [15,16].

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Exosomes are small (40–100 nm) membrane vesicles which are released from various cell types, including reticulocytes, dendritic cells, B cells, T cells, mast cells, epithelial cells, and tumor cells [17]. When exosomes are released into extracellular environment, they are still in proximity of the cell of origin or may enter the biological fluids such as plasma, urine, milk, cerebrospinal fluid, amniotic fluid and tumor effusions to allow the long-range exchange of exosome/microvesicle-mediated biological information [18]. These exosomes are extracellular vesicles that transport different molecules between cells [19]. Several mechanisms have been advanced to describe exosome-recipient cell interactions: cellular binding via conventional receptor-ligand interactions, similar to cell-cell communication; attaching/fusing with target cell membrane; internalization by recipient cells by endocytosis in a transcytotic manner [20]. Exosomes usually mediate the local cell-cell communication by transferring coding RNA [17], noncoding RNA [21], antigen presentation molecules [22], DNA [23] and proteins [24]. With the ability of transferring functional components, exosomes have multiple functions of stimulating target cells, transferring membrane receptors, delivering proteins, and inducing epigenetic changes in recipient cells [18]. They play important roles in intercellular communication in CNS, where they can mediate neuronal and glia communication [25], promote neuronal repair and growth, regulate the immune response [26].

Mesenchymal stem cell (MSC) derived exosomes have been reported to protect limbs from ischemic injury via the promotion of angiogenesis [27]. MSCs communicate with brain parenchymal cells and may regulate neurite outgrowth by transfer of miR-133b to neural cells via exosomes [28]. MSCs derived exosomes promote muscle regeneration by enhancing myogenesis and angiogenesis [29]. Mesenchymal stem cell derived exosomes effectively trigger the macrophage polarization from M1 to a M2 phenotype; and they have potent exosome-mediated anti-inflammatory effect through down-regulation of the inflammatory cytokine IL-6 accompanied by the concomitant upregulation of IL-10 [30].

Herein, we hypothesized that exosomes secreted by hucMSC have a positive role in the functional recovery after SCI. To address this hypothesis, hucMSC derived exosomes were prepared and used to treat the mouse with spinal cord injury through the tail vein injection. We found that exosomes derived from hucMSC could attenuate the inflammation of the injury region through down-regulation of the inflammatory cytokines, like TNF-α, MIP-1α, IL-6 and IFN-γ. Ethology and morphology experiments suggested that hucMSC derived exosomes significantly improved the functional recovery after spinal cord injury. These results suggested that hucMSC derived exosomes have promising potential for clinical application in SCI therapy.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were females, aged 7–8 weeks old and weighed 17–22 g at the time of surgery. All animals were maintained under specifical pathogen-free (SPF) conditions in the animal facility of Jinan University (Guangzhou, China). The experimental procedures were approved by the Jinan University's Institutional Lab Animal Care and Use Committee. Maximum care was taken to limit the number of animals used in this study.

2.2. Reagents

The following reagents were used: Dulbecco's Modified Eagle Medium (DMEM, Gibco, C11995500BT), low glucose Dulbecco's Modified Eagle Medium (LG-DMEM, Gibco, C11885500BT), heat-inactivated horse serum (HS, Gibco, 26050-070), fetal bovine serum (FBS, Gibco, 10270-106), penicillin/streptomycin (P/S, 10,000 μg/ml, Sigma, P0781), phosphate buffered saline (PBS, Hyclone, SH30256.02), enhanced chemiluminescence (Millipore, WBKLS0500), anti-CD9 antibody (Abcam, ab92726), anti-CD63 antibody (Abcam, ab193349), anti-β-tubulin antibody (Sigma Aldrich, T3952), anti-LaminA antibody (Abcam, ab26300), horseradish peroxidase-coupled secondary antibody (Invitrogen, SA1-100), horseradish peroxidase-coupled secondary antibody (Invitrogen, G-21040), DAPI (Abcam, ab104139), F4/80 antibody (mouse) (Abcam, ab6640), anti-iNOS antibody (rabbit) (Genetex, GTX74171), anti-Arg1 antibody (rabbit) (Genetex, GTX109242), Alexa Fluor 488 goat anti-mouse IgG (H + L) antibody (Invitrogen, A32723) and Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibody (Invitrogen, A-11034), Alexa Fluor 594 donkey anti-mouse secondary antibodies (Invitrogen, A-21203) and Alexa Fluor 594 donkey anti-rabbit secondary antibodies (Invitrogen, A-21209).

2.3. Preparation of hucMSC

Fresh human umbilical cords were collected from informed, consenting mothers. Moreover, the fresh umbilical cords were processed within the optimal processing period of 6 h. The cords were rinsed twice by PBS in penicillin and streptomycin, the cord blood being removed during this process. The washed cords then were cut into 1–2 mm pieces and floated in LG-DMEM containing 10% FBS, 5% HS and 1% P/S (v/v). The pieces of cord were subsequently incubated at 37 °C in humid air with 5% CO_2. Non-adherent cells were removed by washing with PBS. The medium was replaced every 3 days after the initial plating. When well developed colonies of fibroblast-like cells appeared after 10 days, the tissue cultures were trypsinized and passaged (without dilution) into a new plate for further expansion and the medium was changed every 3 days [31].

2.4. Isolation of exosomes derived from hucMSC

The 70%–80% of the confluent hucMSCs cultures were washed twice with PBS and then incubated in serum-free LG-DMEM for 48 h. The conditioned medium was collected and centrifuged at 1000 ×*g* for 10 min to remove cell debris, followed by centrifugation at 2000 ×*g* for 10 min and 10,000 ×*g* for 20 min. The supernatant was collected and concentrated using 100KDa MWCO (Millipore) at 1000 ×*g* for 30 min. The concentrated supernatant was loaded upon 5 ml PBS and then ultracentrifuged at 120,000 ×*g* for 60 min (Optimal-90K, Beckman Coulter). The microvesicles-enriched fraction was harvested and diluted with PBS and then centrifuged at 1000 ×*g* for 30 min using 100 kDa MWCO. Finally, the purified exosomes were collected and subjected to filtration on 0.22 μm pore filter (Millipore) and stored at −80 °C.

2.5. hucMSC derived exosomes characterization

Purified exosomes were identified by transmission electron microscopy (TEM, JEM-2100F, Japan Electronics Co., Ltd.). In brief, a drop of exosomes (20 μl) was pipetted onto a grid which was coated with formvar and carbon, standing for 5 min at room temperature. The excess fluid was removed with a piece of filter, and the sample was negatively stained with 3% (w/v) phosphotungstic acid (pH 6.8) for 5 min. After air drying under an electric incandescent lamp, the sample was analyzed by TEM.

Exosomes size distribution and concentration were examined by a Malvern Zeta sizer Nano ZS90 (Malvern, UK). Exosome preparations were thawed from −80 °C and diluted in deionized water to concentration of 1 mg/ml then injected into the sample chamber. Shutter and gain were kept at the same settings for all samples.

2.6. Culture and exosomes treatment of bone marrow derived macrophages

Bone Marrow Derived Macrophage (BMDM) were isolated from C57BL/6 mice and were cultured for 7 days in 10 cm dishes using