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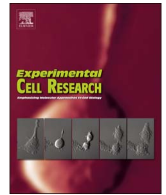

Research article

Conditioned medium of periodontal ligament mesenchymal stem cells exert anti-inflammatory effects in lipopolysaccharide-activated mouse motoneurons

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ABSTRACT

Conditioned medium derived from mesenchymal stem cells (MSCs) shows immunomodulatory and neuroprotective effects in preclinical models. Given the difficulty to harvest MSCs from bone marrow and adipose tissues, research has been focused to find alternative resources for MSCs, such as oral-derived tissues. Recently, we have demonstrated the protective effects of MSCs obtained from healthy human periodontal ligament tissue (hPDLSCs) in murine experimental autoimmune encephalomyelitis model. In the present in vitro study, we have investigated the immunomodulatory and neuroprotective effects of conditioned medium obtained from hPDLSCs of Relapsing Remitting- Multiple sclerosis (RR-MS) patients on NSC34 mouse motoneurons stimulated with lipopolysaccharide (LPS). Immunocytochemistry and western blotting were performed. Increased level of TLR4 and NFκB, and reduced level of IκB-α were observed in LPS-stimulated motoneurons, which were modulated by pre-conditioning with hPDLSC-conditioned medium. Inflammatory cytokines (TNF-α, IL-10), neuroprotective markers (Nestin, NFL 70, NGF, GAP43), and apoptotic markers (Bax, Bcl-2, p21) were modulated. Moreover, extracellular vesicles of hPDLSC-conditioned medium showed the presence of anti-inflammatory cytokines IL-10 and TGF-β. Our results demonstrate the immunosuppressive properties of hPDLSC-conditioned medium of RR-MS patients in motoneurons subjected to inflammation. Our findings warrant further preclinical and clinical studies to elucidate the autologous therapeutic efficacy of hPDLSC-conditioned medium in neurodegenerative diseases.

1. Introduction

In recent years, extensive research has been done on the therapeutic efficacy of mesenchymal stem cells (MSCs) [1]. Thanks to their unique abilities of self-renewal and multilineage differentiation, such as neuronal, glial, and mesodermal [2–5], numerous pre-clinical studies have reported the protective role of MSCs in regenerative medicine [6]. Nevertheless, factors including immune incompetency, carcinogenicity, requirement for ex vivo cell expansion, and costs, have made the limitations for stem cell based therapies at translational level [7]. From a functional point of view, it has been demonstrated that MSCs exert their protective functions, including immunomodulation and neuroprotection, in a paracrine manner by synthesis and secretion of a variety of cytokines, neurotrophic factors, and growth factors [8–10], and that these MSCs-derived biologically active molecules, also called as secretome, may circumvent the limitations associated with stem cell therapy [11]. Indeed, many studies have explored the potential beneficial applications of MSCs-conditioned media in different pathological models with the ability to regenerate neural, osteogenic, and myocardial cells [12].

Among diverse adult tissues, bone marrow and adipose tissues serve as major resources for MSCs [13]. However, considerable difficulty to harvest MSCs from these tissues and their low yield have led the researchers to find alternative tissue resources, such as oral-

Abbreviations: ANOVA, Analysis of variance; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 Associated X protein; DMEM, Dulbecco's modification of Eagle's medium Dulbecco; ELISA, Enzyme linked immunosorbent assay; FBS, Fetal bovine serum; GAP43, Growth Associated Protein 43; HRP, Horse radish peroxidase; hPDLSCs, Human periodontal ligament mesenchymal stem cells; IκB-α, Inhibitor of kappa B alpha; IL-10, Interleukin 10; LPS, Lipopolysaccharide; MSCs, Mesenchymal stem cells; NGF, Nerve growth factor; NFL70, Neurofilament 70; NFκB, Nuclear factor kappa B; RR-MS, Relapsing Remitting-Multiple sclerosis; TLR4, Toll-like receptor 4; TGF-β, Transforming growth factor- beta; TNF-α, Tumor necrosis factor-alpha

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derived tissues and placenta [14,15]. More recently, we have reported that MSCs of human periodontal ligament tissue (hPDLSCs) isolated from healthy donors showed immunosuppressive and neuroprotective effects in a mouse model of experimental autoimmune encephalomyelitis [16] and they are able to differentiate into neurogenic lineage [17]. In the present in vitro study, we have investigated whether conditioned media derived from hPDLSCs (hPDLSC-CM) of Relapsing Remitting-Multiple Sclerosis (RR-MS) patients may provide protection against inflammatory insult. To this end, we have preconditioned NSC34 mouse motoneuronal cells with hPDLSC-CM and stimulated with lipopolysaccharide (LPS) to induce inflammatory damage. Mediators of LPS-stimulated Toll-like receptor 4 pathway, inflammatory cytokines, neuronal growth factors, and apoptotic factors were investigated. In addition, extracellular vesicles present in hPDLSC-CM were examined for the presence of potential immunosuppressive cytokines.

2. Materials and methods

2.1. Ethic statement

The protocol and informed consent from human periodontal ligament biopsies were carried out in accordance with the Declaration of Helsinki and approved guidelines of Medical Ethics Committee at the Medical School, "G. d'Annunzio" University, Chieti, Italy (no. 266/17.04.14). The formal consent form was signed by all subjects before samples collection. The Department of Medical, Oral and Biotechnological Sciences and the Laboratory of Stem Cells and Regenerative Medicine are certified according to the quality standard ISO 9001:2008 (certificate no. 32031/15/S).

2.2. hPDLSCs culture establishment

Human periodontal ligament biopsies were carried out from healthy donors and patients selected by RR-MS (n=5 for each). Sample tissues derived from horizontal fibers of periodontal ligament of premolar teeth during root scaling. Each patient was pre-treated with chlorhexidine 0.2% solution for 1 min. The specimens of periodontal tissue were grinded cut into small pieces and after washed several time with PBS (LiStarFish, Milan, Italy), subsequently cultured with medium for the growth of human Mesenchymal Stem Cells chemically defined (MSCGM-CD) (Lonza, Basel, Switzerland) (TEC). The medium was changed twice a week, and cells spontaneously migrating from the explants after reaching about 80% of confluence, were trypsinized (LiStarFish), and subcultured. Cells at passage 2 were used for the experiments [18].

2.3. Cytofluorimetric characterization of hPDLSCs from RR-MS patients

hPDLSCs and hPDLSCs derived from RR-MS patients, at the second passage were collected; 5×10^5 cells per sample were incubated with 1 μg of the specific antibody, conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), phycoerythrin-cyanine 5.5 (PE Cy5.5), or Alexa Fluor 488 for 30 min at 4 °C in the dark. Human PDLSCs were stained using the following antibodies: anti-CD13, anti-CD29, anti-CD44, anti-CD45, anti-CD105, anti-CD166 (Ancell, MN, USA), anti-CD14, anti-CD133 (BergischGladbach, Germany), anti-CD73, anti-CD90, anti-CD117, anti-CD146, anti-CD271, anti-Sox2, anti-SSEA4, anti-OCT3/4 (Becton Dickinson, BD, San Jose, CA, USA); anti-CD144 (Acris Antibodies, Herford, Germany), anti-CD34 (Beckman Coulter, Fullerton, CA, USA). After incubation, cells were acquired with a flow cytometer (FACS Calibur; BD). Data were analyzed by the FlowJo software v8.8.6 (TreeStar, Ashland, OR) [19]. The immunophenotyping analysis was performed for each donor cell primary culture.

2.4. Morphological investigations of hPDLSCs

Glass-adherent hPDLSCs at P2 were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 2 h, subsequently were stained with toluidine blue and observed by light microscopy. All sections were observed with a Zeiss Axiophot apparatus (Zeiss, Jena, Germany), and images captured using a Nikon digital camera Digital Sight.

2.5. Cell growth and viability of hPDLSCs

Ex vivo expanded hPDLSCs and RR-MS hPDLSCs were seeded at 2×10^3 cells/well in triplicate using a 96-well flat-bottom plate and maintained in MSCGM-CD medium for 24, 48, 72 h and 1 week. After the incubation period, 20 μl/well of MTT were added to culture medium and cells were incubated for 3 h at 37 °C. The supernatants were read at 650 nm wavelength using an ND-1000 NanoDrop Spectrophotometer microplate reader (Synergy HT, BioTek Instruments, Vermont, USA). Moreover, the doubling-time of the trypan blue harvested cells, at 24, 48, 72 h and 1 week of culture, was calculated by using an algorithm available online (http://www.doubling-time.com).

2.6. Mesengenic differentiation ability of hPDLSCs

To assay the ability of hPDLSCs, obtained from RR-MS donors, to differentiate into mesengenic lineages, cells were cultured in osteogenic and induction/maintanance medium (Lonza) respectively. Osteogenic and adipogenic capability was assayed by histocnemical staining and biochemical evaluation: Alizarin Red S (ARS) and Oil Red staining respectively, as previously described by Manescu et al. [20]. The mesengenic differentiation process was performed in three separate experiments, osteogenic differentiation ability was evaluated after 7 and 21 days of culture, while cell adipogenic capability was assessed after 28 days of culture. Moreover, osteogenic and adipogenic markers were evaluated by real-time PCR. In particular for osteogenic induction was analyzed the expression of Runt-related transcription factor-2 (RUNX-2) and AlkalinPhospatase (ALP) after 21 days of differentiated culture, and for adipogenic induction was evaluated the expression of Fatty Acid Binding Protein 4 (FABP4) and Peroxisome Proliferator-Activated Receptor γ (PPARγ) after 28 days of differentiation. To this end, total RNA was isolated and Real-Time PCR was carried out as previously described by Cianci et al. [21]. Commercially available TaqMan Gene Expression Assays (RUNX-2 Hs00231692_m1; ALP Hs01029144_m1; FABP4 Hs01086177_m1; PPARγ Hs011155 13_m1) and the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were used according to standard protocols. Beta-2 microglobulin (B2M Hs99999907_m1) (Applied Biosystems, Foster City, CA, USA) was used for template normalization. RT-PCR was performed in three independent experiments, duplicate determinations were carried out for each sample.

2.7. Preparation of CM

CM from RR-MS patients hPDLSCs (15×10^3 cells/cm^2) cultured in xeno-free MSCGM-CD was collected after 72 h of incubation, and centrifuged at 1200 rpm for 5 min. The supernatants were recentrifuged at 3000 rpm for 3 min, followed by collection of the secondary supernatants from CM-hPDLSCs. Subsequently, 1 mL of secondary supernatants was resuspended in 3 mL of ice aceton and maintained over night at 4 °C, and after centrifuged at 16.000 rpm for 12 min at 4 °C (Centrifuge 5804 R, Eppendorf, Milan, Italy). The suspension was lysated in RIPA and quantified by means Breadford assay. Total proteins obtained were 125 μg/μl.

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