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Role of human amnion-derived mesenchymal stem cells in promoting osteogenic differentiation by influencing p38 MAPK signaling in lipopolysaccharide -induced human bone marrow mesenchymal stem cells

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ABSTRACT

Periodontitis is a chronic inflammatory disease induced by bacterial pathogens, which not only affect connective tissue attachments but also cause alveolar bone loss. In this study, we investigated the anti-inflammatory effects of Human amnion-derived mesenchymal stem cells (HAMSCs) on human bone marrow mesenchymal stem cells (HBMSCs) under lipopolysaccharide (LPS)-induced inflammatory conditions. Proliferation levels were measured by flow cytometry and immunofluorescence staining of 5-ethynyl-2′-deoxyuridine (EdU). Osteoblastic differentiation and mineralization were investigated using chromogenic alkaline phosphatase activity (ALP) activity substrate assays, Alizarin red S staining, and RT-PCR analysis of HBMSCs osteogenic marker expression. Oxidative stress induced by LPS was investigated by assaying reactive oxygen species (ROS) level and superoxide dismutase (SOD) activity. Here, we demonstrated that HAMSCs increased the proliferation, osteoblastic differentiation, and SOD activity of LPS-induced HBMSCs, and down-regulated the ROS level. Moreover, our results suggested that the activation of p38 MAPK signal transduction pathway is essential for reversing the LPS-induced bone-destructive processes. SB203580, a selective inhibitor of p38 MAPK signaling, significantly suppressed the anti-inflammatory effects in HAMSCs. In conclusion, HAMSCs show a strong potential in treating inflammation-induced bone loss by influencing p38 MAPK signaling.

1. Introduction

Periodontitis is a group of inflammatory process most commonly caused by Gram-negative bacterial infections. It not only results in a loss of supporting alveolar bone surround the teeth, but also spontaneous if left untreated [1]. Lipopolysaccharide (LPS), a component of the outer membranes of Gram-negative bacteria, is capable of inducing bone resorption and inhibiting osteoblast differentiation in vivo or in vitro [2–4]. Moreover, various studies have demonstrated that LPS could increase the oxidative stress and promote excessive production of reactive oxygen species (ROS), which influence osteoclast-induced superoxide generation and bone deficiency [5,6]. Therefore, attenuating immune response is essential for the treatment of bone deficiency associated with bacterial infection.

Several approaches like laser-assisted therapy, guided bone regeneration membranes and herbal medicines are used in the treatment of periodontitis [7–9]; however, these approaches are associated with various disadvantages, such as high traumatic response, remarkable individual differences, and limited availability. Recently, tissue engineering, using appropriate seed cells that mimics the natural healing process to treat inflammatory diseases, has shown great potential in treating bone deficiency. Human amnion-derived mesenchymal stem cells (HAMSCs), obtained from discarded amniotic membrane, have low anti-inflammatory properties and fewer ethical concerns as compared to other sources of stem cells [10]. Previous study has demonstrated that HAMSCs are capable of driving osteogenic differentiation in human bone marrow mesenchymal stem cells (HBMSCs) [11,12], which led us to hypothesize that HAMSCs play a role in LPS-induced bone deficiency. In this study, a transwell co-culture system was used to determine the in vitro effects of HAMSCs on osteogenic differentiation in LPS-induced HBMSCs. Interestingly, we found that HAMSCs promoted proliferation, enhanced the expression of osteogenic marker proteins, activated alkaline phosphatase activity (ALP), and stimulated mineralized matrix deposition; thus, confirming that HAMSCs are capable of providing an anti-inflammatory environment for reversing the LPS-induced suppression of osteogenic differentiation.

The osteogenic differentiation of mesenchymal stem cells, as well as the LPS-induced inflammatory response, is the consequence of activa-

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tion of key intracellular pathways, including mitogen-activated protein kinases (MAPKs) pathway [13–16]. P38 MAPK, involved in inflammatory responses, cell cycle control, apoptosis, and cell fate specification, was found to be activated at an early stage during primary osteogenic differentiation [17,18]. Previous studies have shown that Runt-related transcription factor 2 (RUNX2) is modulated by various extracellular signaling pathways, containing p38 MAPK, and the transcriptional activity of RUNX2 is stimulated by these signaling pathways [19–21]. To clarify the underlying mechanisms, we further investigated the role of HAMSCs on p38 MAPK signaling pathway and RUNX2 in LPS-induced HBMSCs.

2. Material and methods

2.1. Chemicals and reagents

Trypsin-EDTA, phosphate-buffered saline (PBS), fetal bovine serum (FBS), penicillin G-streptomycin sulfate, and α-minimum essential medium (αMEM) were purchased from Gibco® Life Technologies. The bicinchoninic acid (BCA), ALP, and xanthine oxidase assay kits were purchased from the Jiancheng Corp (Nanjing, China). β-glycerophosphate, ascorbic acid, dexamethasone, and 2,7-dichlorodihydro-fluoresceindiacetate (DCFH-DA) were purchased from Sigma–Aldrich (St. Louis, MO). Six-well culture plates and transwells (6-Well Millicell Hanging Cell Culture Inserts, 0.4 μm, PET) were purchased from Millipore® (Bedford, MA, USA). The TRIzol reagent and polymerase chain reaction (PCR) primers were purchased from Invitrogen (Carlsbad, CA, USA), and reverse transcriptase (RT)-PCR kit was purchased from TaKaRa Bio (Otsu, Japan). The goat anti-rabbit IgG, phospho-p44/42 (p-ERK1/2) MAPK rabbit mAb, p44/42 MAPK (ERK1/2) rabbit mAb, phospho-p38 MAPK (Thr180/Tyr182) (D3F9) rabbit mAb, p38 MAPK (D13E1) rabbit mAb, RUNX2 (D1L7F) rabbit mAb, and SB203580 (p38 inhibitor) were purchased from Cell Signaling Technology (Danvers, MA, USA). The PE Mouse anti-phospho- Jun kinase (JNK) (pT183/pY185) was purchased from BD Phosflow™ (Franklin Lakes, NJ, USA). The Cell-Light™ EdU Apollo®488 In Vitro Imaging Kit was purchased from RiboBio (Guangzhou, China). The protein assay kit, RIPA buffer, and DAPI were purchased from Beyotime (Shanghai, China). Other reagents used were of the highest commercial grade available.

2.2. Cell culture

The HBMSCs were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HAMSCs were collected from discarded amniotic membrane samples obtained within 24 h using the pancreatin/collagenase digestion method as described previously [22–24]. Third-passage adherent amniotic cells were collected from a flask using ethylenediaminetetraacetic acid (EDTA) treatment and characterized by flow cytometric analysis (BD Biosciences, Franklin Lakes, NJ, USA) of staining with monoclonal antibodies specific for human CD29, CD44, CD73, CD90, CD105, CD45, CD34, and HLA-DR [25,26] (Fig. 1). Both the cell types were cultured in 60-mm plates in αMEM supplemented with 100 U/L penicillin, 100 mg/L streptomycin, and 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C. Cells from passages 3–5 were used and culture medium was changed every 3 days. The study protocols were approved by the Ethics Committee of the School of Stomatology, Nanjing Medical University, China (NO.PJ2013-037-001). Informed consent was obtained from all the participants enrolled in this study.

2.3. The co-culture system

A transwell co-culture system was used to investigate the effects of HAMSCs on the proliferation and osteogenic differentiation of LPS-induced HBMSCs. HBMSCs were seeded at an initial cell density of 5×10^4 cells/cm^2 in 6-well culture plates. Transwells were placed in other 6-well culture plates and HAMSCs were seeded at increasing HBMSCs: HAMSCs ratios (5×10^4 cells/transwell, 10×10^4 cells/transwell and 15×10^4 cells/transwell). Following the attachment of the cells (approximately 24 h), HBMSCs were subjected to a 24 h treatment with serum-free medium or LPS (1 μg/mL) to induce inflammatory responses as previously described [27]. After washing with PBS, transwells containing HAMSCs were transferred into the corresponding wells of the 6-well culture plate containing HBMSCs to create the HAMSC/HBMSCs transwell co-culture system. HBMSCs in wells with transwells served as the treatment groups, while HBMSCs without transwells were used as the control groups.

2.4. Analysis of cellular proliferation

The effect of HAMSCs on LPS-induced HBMSCs proliferation was determined by flow cytometry at 3, 5, and 7 days and via immunofluorescence staining of EdU at 5 days. Briefly, after starvation in serum-free medium or LPS (1 μg/mL) for 24 h, HBMSCs were washed with PBS. Transwells containing HAMSCs were moved into the corresponding wells of the 6-well culture plate containing HBMSCs, and the medium was replaced with culture medium containing 10% FBS. At 3, 5, and 7 days, HBMSCs were harvested and fixed with 75% ice-cold ethanol at 4 °C for 30 min in the dark. Cell cycle fractions (G0, G1, S, and G2 M phases) were determined by a FACScan flow cytometer (BD Biosciences, USA). The EdU levels in each group at 5 days was investigated by a Cell-Light™ EdU Apollo®488 In Vitro Imaging Kit as previously described [12]. EdU levels were examined by immunofluorescence staining after incubating for 30 min. DAPI was used to stain the cell nuclei.

2.5. In vitro *osteogenic differentiation*

After incubating in serum-free medium or LPS (1 μg/mL) for 24 h, HBMSCs were washed with PBS. Transwells containing HAMSCs were moved into the corresponding wells of the 6-well culture plate containing HBMSCs. Both the cell types were cultured in osteogenic medium (OS) containing 10 mM β-glycerophosphate, 100 nM ascorbic acid, and 100 nM dexamethasone. HBMSCs co-cultured with HAMSCs were analyzed for ALP activity and mineralized matrix formation, which was followed by Western blotting analysis, RT-PCR, and flow cytometry. SB203580, a highly selective inhibitor of p38 signaling, was prepared in DMSO and used in the signaling inhibition assay at the concentration of 10 μm as previously described [17,28]. To eliminate the influence of SB203580 on HAMSCs, LPS-induced HBMSCs were treated with SB203580 for 24 h after incubating in LPS (1 μg/mL) before co-culture with HAMSCs.

2.6. Assessment of ALP activity and mineralized matrix formation

After the end of the 14-day co-culture period, transwells containing HAMSCs were removed. HBMSCs in each group were then washed twice with PBS and lysed with Triton X-100 (0.5%) for 15 min. BCA assay kit was used to determine the protein concentration [29]. ALP activity was determined using the ALP assay kit (Jiancheng Corp, Nanjing, China) according to the manufacturer's instructions and estimated based on the absorbance at 405 nm as previously described [30,31]. The enzyme activity was expressed as micromoles of reaction product per minute per total protein.

HBMSCs co-cultured with HAMSCs for 21 days were stained with Alizarin red S to assess mineralized matrix deposition for bone nodule formation. Cells were washed twice with PBS, fixed with 75% dehydrated alcohol, and then stained with 40 mM alizarin red S (pH 4.4) for 10 min at room temperature. Red staining of mineralized matrix deposition was observed using a microscope and the area of stained extracellular matrix relative to the total culture surface was measured