The International Journal of Biochemistry & Cell Biology 51 (2014) 1–9

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Salvianolic acid B promotes osteogenesis of human mesenchymal stem cells through activating ERK signaling pathway

Daohua Xu[a,b,1], Liangliang Xu[b,c,f,1], Chenhui Zhou[a], Wayne Y.W. Lee[b,c,e,f], Tie Wu[a], Liao Cui[a,*], Gang Li[b,c,d,e,f,**]

[a] Department of Pharmacology, Guangdong Medical College, Dongguan, PR China
[b] Department of Orthopaedics & Traumatology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong, China
[c] Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong, China
[d] MOE Key Laboratory of Regenerative Medicine, School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong, China
[e] The CUHK-ACC Space Medicine Centre on Health Maintenance of Musculoskeletal System, The Chinese University of Hong Kong Shenzhen Research Institute, Shenzhen, PR China
[f] Lui Che Woo Institute of Innovative Medicine, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China

ARTICLE INFO

Article history:
Received 12 August 2013
Received in revised form 18 February 2014
Accepted 10 March 2014
Available online 19 March 2014

Keywords:
Mesenchymal stem cell
Osteogenesis
Salvianolic acid B
Osteoporosis

ABSTRACT

Salvianolic acid B, a major bioactive component of Chinese medicine herb, Salvia miltiorrhiza, is widely used for treatment of cardiovascular diseases. Our recent studies have shown that Salvianolic acid B can prevent development of osteoporosis. However, the underlying mechanisms are still not clarified clearly. In the present study, we aim to investigate the effects of Salvianolic acid B on viability and osteogenic differentiation of human mesenchymal stem cells (hMSCs). The results showed Salvianolic acid B (Sal B) had no obvious toxic effects on hMSCs, whereas Sal B supplementation (5 μM) increased the alkaline phosphatase activity, osteopontin, Runx2 and osterix expression in hMSCs. Under osteogenic induction condition, Sal B (5 μM) significantly promoted mineralization; and when the extracellular-signal-regulated kinases signaling (ERK) pathway was blocked, the anabolic effects of Sal B were diminished, indicating that Sal B promoted osteogenesis of hMSCs through activating ERK signaling pathway. The current study confirms that Sal B promotes osteogenesis of hMSCs with no cytotoxicity, and it may be used as a potential therapeutic agent for the management of osteoporosis.

1. Introduction

Osteoporosis is the most widespread bone disease, characterized by low bone mineral density and the deterioration of bone microarchitecture, leading to bone fragility fractures (Kanis et al., 2009; Raisz, 2005). Currently, the therapeutic agents of osteoporosis are mainly inhibitors of bone resorption including bisphosphonates, calcitonin and selective estrogen receptor modulators (Marie and Kassem, 2011; Sandhu and Hampson, 2011). Although these agents are effective in stabilizing bone mass, they do not increase bone formation. Mesenchymal stem cells (MSCs) are non-hematopoietic cells which can be easily isolated from bone marrow and other tissues, such as adipose, umbilical cord and peripheral blood. MSCs have multi-potent capacity to differentiate into a variety of other cell types, including osteoblasts, adipocytes, chondrocytes, myoblasts and neurons (Jiang et al., 2002; Pittenger et al., 1999). MSCs have also been shown to be immunosuppressive and anti-inflammatory, as they do not express MHC-II, CD80, CD86 and CD40, and minimally express MHC-I on the cell surface (Deans and Moseley, 2000; Pittenger et al., 1999; Tse et al., 2000). In recent years, MSCs have been used to promote healing with promising results, such as bone fracture and segmental bone defect (Kumar et al., 2010; Shekkeris et al., 2012; Undale et al., 2011). Most recently, Guan et al. have found that directing MSCs to bone could augment bone formation and increase bone mass (Guan et al., 2012). In osteoporotic patients, studies have shown that the osteogenic differentiation potential of MSCs is reduced (Benisch et al., 2012; Dalle Carbonare et al., 2009; Rodriguez et al., 2000). Therefore, enhancing osteogenesis of MSCs is thought to be a useful therapeutic strategy for bone diseases such as osteoporosis (Pino et al., 2012).

* Corresponding author. Tel.: +86 769 22896547; fax: +86 769 22896547.
** Corresponding author at: Room 904, 9/F, Li Ka Shing Institute of Health Institute, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, China. Tel.: +852 3763 6153; fax: +852 2646 3020.
E-mail addresses: cuiliao@163.com (L. Cui), gangli@cuhk.edu.hk (G. Li).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.biocel.2014.03.005
1357-2725/

MSCs differentiation into mature functional osteoblasts is a complex process involving many transcriptional factors and signaling pathways. Runx2 (Runt-related transcription factor 2) is the central control gene of the osteoblast phenotype. Mice with homozygous mutation in Runx2 gene show a complete lack of ossification (Komori et al., 1997). The ERK signaling pathway has been intensively investigated in regulating MSCs differentiation. ERK1/2 is constantly activated during osteogenic differentiation, and the study by Jaiswal et al. has suggested that the commitment of hMSCs into osteogenic or adipogenic lineages is governed by activation or inhibition of ERK1/2, respectively (Jaiswal et al., 2000). ERKs activation can increase the phosphorylaion and transcription potential of Runx2 (Park et al., 2010).

Salvianolic acid B (Sal B), a major bioactive component of traditional Chinese medicine, Salvia miltiorrhiza, is widely used for treatment of cardiovascular diseases (He et al., 2008; Joe et al., 2012). Studies have shown that Sal B exerts neuroprotective effects (Kim et al., 2011; Lee et al., 2013) and also could alleviate liver fibrosis (Wang et al., 2012). Recently we have investigated the role of Sal B on bone metabolism in glucocorticoid induced osteoporosis in rats and found that Sal B was able to prevent osteoporosis induced by glucocorticoid (Cui et al., 2012). However, the effect of Sal B on osteogenic differentiation of MSCs was not studied and the underlying mechanisms of Sal B on bone metabolism were still not clarified. In the present study, we have investigated the effect of Sal B on osteogenic differentiation in hMSCs. The results show that Sal B can promote the osteogenic differentiation of hMSCs by activating ERK signaling pathway, which may partially explain our previous finding that Sal B prevents osteoporosis induced by glucocorticoid.

2. Materials and methods

2.1. Reagents and cell culture

Sal B was purchased from Paipai Limited Technology Corporation (Guangzhou, China). Human fetal bone marrow-derived MSCs (hMSCs) were donated from the Stem Cell Bank in the Prince of Wales Hospital. Human ethics approval was obtained from the Joint CUHK-NTEC Clinical Research Ethics Committee of the Chinese University of Hong Kong (Reference No. CRE-2011.383). Informed written consent form was approved by the Clinical Research Ethics Committee and signed by donor before sample collection. The hMSCs were kept in Modified Eagle's Medium of Alpha (a-MEM) (Gibco) supplemented 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco).

2.2. Plasmid construction, transfection, lentivirus production and infection

The shRNA used for silencing ERK1 and ERK2 was designed as published (Hong et al., 2009). ERK1, ERK2 shRNA and scrambled shRNA sequence templates were inserted into pLL3.7 plasmid and pseudo-lentiviruses were produced by transfection of 293FT packaging cells (Invitrogen, USA) using the calcium phosphate method. For transduction, 1×10^5 cells were seeded into 6-well plate and incubated with lentiviruses and 8 μg/mL polybrene in the incubator for 24 h (Xu et al., 2012).

2.3. Phenotypic characterization of hMSCs

After reaching 80% confluence, the cells were rinsed twice with phosphate buffered saline (PBS) and treated with 0.05% trypsin–EDTA for 2 min. Then, serum-containing medium was immediately added to the culture to end trypsinization. Then, the fluid was collected and centrifuged ($800 \times g$ for 5 min). After discarding the supernatant, the precipitate was resuspended in staining buffer and incubated with fluorochrome-conjugated primary antibodies against CD34, CD44, CD45, CD73, CD90, CD105, or corresponding isotype control (BD Biosciences, USA) at 4 °C for 30 min. The stained cells were immediately detected using Flow Cytometry (BD Biosciences, USA).

2.4. Cell viability assay

Samples (5×10^3 per well) were subcultured in a 96 flat-bottomed well plate. After 24 h of incubation, the medium was changed into Sal B containing media at different concentrations. Cells were incubated at 37 °C for 1 and 3 days. The cell proliferation was determined using methyl thiazolyl tetrazolium (MTT) reduction assay. After incubation, cells were treated with the MTT solution (final concentration, 0.5 mg/ml) for 4 h at 37 °C. The dark blue formazan crystals formed in intact cells were solubilized with 150 μL DMSO and the plate was shaken for 10 min. The absorbance at 570 nm was measured with a microplate reader.

2.5. ALP activity assay

After MSCs were treated with or without OIM (osteogenic induced medium) and Sal B (5 μM) for 7 days, the plate was washed with PBS and the cells were lysed by lysis buffer consisting 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100. The ALP activity was determined using p-nitrophenylphosphate as the substrate. Absorbance at 405 nm was measured and the protein concentration of cell lysates was measured using the Bradford assay at 595 nm on a microplate spectrophotometer (Bio-Rad, USA). ALP activity was normalized according to the total protein concentration.

2.6. ALP staining

After MSCs were treated with or without OIM and Sal B for 7 days, the cells were washed with PBS twice and fixed with 70% ethanol for 10 min. The cells were equilibrated by ALP buffer (0.15 M NaCl, 0.15 M Tris–HCl, 1 mM $MgCl_2$, pH 9.0) twice, incubated with ALP substrate solution (5 μL BCIP and 10 μL NBT in l mL ALP buffer) at 37 °C in dark for 60 min. Then the reaction was stopped by distilled water and the plate was dried before taking photo.

2.7. Mineralization assay

After 14 days of osteogenic induction, cells were fixed with 70% ethanol for 10 min. Then the fixed cells were stained with 0.5% alizarin red S (pH 4.1) for 10 min at room temperature and washed three times with deionized water. Orange red staining indicated the position and intensity of calcium deposits. The calcium deposition was extracted with 10% cetylpyridinium chloride (CPC, Sigma) and quantified by measuring the OD of the extract at 550 nm.

2.8. RNA extraction and real-time PCR

Total RNA was extracted from cultured cells with RNeasy Mini Kit (Qiagen, USA) and first-strand cDNA was synthesized using M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions. Real-time PCR was performed using the Step One Plus Real-Time PCR System (Applied Biosystems, USA). The reaction conditions consisted of 15 μl reaction volumes with diluted cDNA template 3 μL, 7.5 μL SYBR-Green Master Mix (2×), 3.9 μL PCR-Grade water and 0.3 μL of each primer (10 μM). Amplification conditions were as follows: first at 95 °C for 5 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Primer sequences were as follows: osteopontin (OPN) forward: 5′gtaccctgatgctacagacg-3′, reverse: