Biomedicine & Pharmacotherapy 90 (2017) 835–841

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Original article

Influence of sinomenine upon mesenchymal stem cells in osteoclastogenesis

Biao Zhou[a,1], Xiaolong Lu[b,1], Zhi Tang[a,1], Dan Liu[a], Youliang Zhou[a], Peng Zeng[b], Hui Xiong[a,*]

[a] Hunan University of Chinese Medicine, China
[b] The 2nd affiliated Hospital of Hunan University of Chinese Medicine, China

ARTICLE INFO

Article history:
Received 6 December 2016
Received in revised form 23 March 2017
Accepted 26 March 2017

Keywords:
Sinomenine
Mesenchymal stem cells
Osteoclastogenesis
RANKL
OPG
PGE2

ABSTRACT

With the advent of aging, the morbidity rates of such diseases as osteoarthritis, rheumatoid arthritis, and osteoporosis has witnessed a significant increase. As a common rattan drug, sinomenine (SIN) has been widely applied for the treatment of various arthritic diseases in traditional Chinese medicine (TCM) clinics. Given that SIN has been reported to inhibit the expression of Prostaglandin E2 (PGE2) in several types of cells, in this study, the influence of SIN treatment on PGE2 expression in mesenchymal stem cells (MSCs), thereby changing the osteoprotegerin (OPG) receptor/activator for the nuclear factor-κ B ligand (RANKL) ratio, was investigated. Our results showed that, when compared with the untreated cells, treatment with 0.25 mM SIN can down-regulate the mRNA and protein expression levels of the Prostaglandin E synthase 3 (PTGES3) or PGE2 and RANKL, while the OPG was up-regulated. After being cultured with SIN treated MSC-conditioned medium (stMSC-CM), the amount of TRAP-positive multinucleated osteoclasts differentiated from RAW264.7 cells was reduced. Also, the expression levels of specific markers for active osteoclasts were decreased when incubated with stMSC-CM. Moreover, these changes were able to be recovered when the exogenous RANKL was added to the MSC-CM culture. This indicates that the increased OPG/RANKL ratio can reduce the osteoclastogenesis of RAW264.7 cells. Our results demonstrated that SIN has an inhibitory effect on osteoclast differentiation through mechanisms involving the inhibition of the PGE2-induced OPG/RANKL ratio. SIN can also serve as a proinflammatory mediator to regulate the MSC immunosuppressive effects. Our findings suggest that SIN can be useful for the treatment of bone diseases associated with over-activity of osteoclasts.

1. Introduction

Joint diseases, such as osteoarthritis (OA), gouty arthritis and rheumatoid arthritis (RA), are a major cause of disability [1]. OA is a chronic disease which progressively degrades the articular cartilage of a joint [2,3]. RA is a symmetric autoimmune disease with symptoms like panarthritis and arthrosis structure damage [4]. In both cases, changes in the subchondral bone and synovium are closely associated with these symptoms [3]. OA and RA differ in their etiologies, however, common mechanisms contribute to joint damage and pain in both conditions [3]. Osteoclasts (OC) play an important role in causing bone damage, and come from monocytes in peripheral blood [5]. Under the induction of a series of cytokines from macrophage colony stimulating factors (M-CSF), IL-6, IL-11, IL-1, tumor necrosis factor α (TNF-α), and IL-17, OC can activate receptor activators for the nuclear factor-κ B ligand (RANKL) system, and then progressively differentiate into multinuclear and erosive mature osteoclasts [5,6]. The over-activity of osteoclasts serves as the main factor for bone erosion, playing a key role in RA bone damage. RANKL serves as the starting signal of osteoclast generation and can promote osteoclastogenesis mainly through interacting with RANK [5,6]. As a soluble receptor of RANKL, osteoprotegerin (OPG) can compete with RANL to bind with RANKL and hinder the differentiation and activation of osteoclasts, hence inhibiting bone resorption [5,6]. Research has shown that the expression levels of RANKL/RANK/OPG can be used as a key index in determining the degree of bone resorption mediated by osteoclasts [5–7].

Mesenchymal stem cells (MSCs) are the multipotential stem cells derived from mesoblasts with high self-updating abilities and

* Corresponding author at: No. 300, Bachelor Road, Hanpu Science and Education Garden, Yuelu District, Changsha City, Hunan Province, China.
E-mail address: xionghuihn@126.com (H. Xiong).
[1] These authors contribute equally to the work.

http://dx.doi.org/10.1016/j.biopha.2017.03.084
0753-3322/

multiple differentiable potentials. MSCs have strong immunosuppression and immunologic tolerance induction functions. These functions suppress proliferation and inflammation, and are able to ameliorate chronic inflammations caused by immune system disorders [8]. Several kinds of inflammatory factors like TNF-α, IL-1, and IL-6 can induce RANKL expression of mesenchymal stem cells, promoting the formation of osteoclasts [9]. Meanwhile, Koichi et al. [10] found that MSCs can secrete a large quantity of OPG and inhibit the expression of the nuclear factor of activated T-cells cytoplasmic-1 (NFATc1) and cathepsin K. This hinders the differentiation of osteoclasts. On the other hand, some soluble molecules secreted by MSCs, such as NO, IDO, PGE2, IL-10, and TGF-β also can participate in the mediation of immunosuppression [8,9]. Jarvinen et al. [11] discovered that a Prostaglandin E2 (PGE2) inhibitor can lower the induction ability of MSCs in T-cell proliferation. Thus, MSCs can directly or indirectly inhibit the proliferation of T cells, enhance the function of Treg cells, and hinder the secretion of multiple inflammatory factors such as IL-1 and IL-7. These responses then further interdict the activation of RANKL and inhibit the differentiation of precursor osteoclast cells, thus reducing the formation of mature osteoclasts and alleviating bone damage.

As a common rattan drug, SIN has been widely applied in the treatment of various arthritic diseases in traditional Chinese medicine (TCM) clinics [12]. In treating bone related diseases, TCM is effective, has few side effects, and easy to administer. However, the physical foundation and mechanisms in TCM require further research. SIN, as a kind of monosomy alkaloid extracted from the rattan lotus of caulis sinomenii and sinomeniumacutum, enjoys diverse pharmacological effects of anti-inflammation, anti-arthritis, and immunosuppression [12,13]. Related pharmaceuticals have been widely used in treating various rheumatic diseases with effectiveness and few side effects [12]. Research has shown that SIN can improve the damaged bones in a type-II collagen induced rat arthritis model, and inhibit the activation of T cells through reducing the TRACP in peripheral serum [14,15]. SIN also inhibits the gene expression of synovial cell inflammatory factors [16]. These functions indicate that SIN can slow down the development of bone damage. Also, it was reported that SIN can inhibit the expression of PGE2 in several types of cells [17,18]. Whether SIN can directly influence PGE2 expression in MSCs, thereby changing the OPG/RANKL ratio, has yet to be determined. Therefore, we propose that SIN can regulate the expression of PGE2 in MSCs, thus inhibiting the expression of RANKL and the formation of osteoclasts.

2. Materials and methods

2.1. Isolation and culture of mesenchymal stem cells (MSCs)

The study was approved by the Second Affiliated Hospital of the Hunan University of Chinese Medicine Medical Ethics Committee. The procedures were designed based on institutional guidelines. The isolation and culture of MSCs from human bone marrow (BM) was processed as previously reported [19]. The BM cells were seeded uniformly into culture flasks containing DMEM/F12 medium supplemented with 20% FBS, 100 units/ml of penicillin-streptomycin, and 2 ng/ml of epidermal growth factor (EGF) in a humidified atmosphere of 37 °C with 5% CO_2. The medium was completely replaced every 3 days, and the non-adherent cells were discarded. Cells were sub-cultured at a 1:3 dilution into culture dishes using 0.25% Trypsin (Hyclone) when they reached a confluence of over 80%. For differentiation, MSCs were cultured in an osteogenic or adipogenic differentiation medium (Lonza, Switzerland). After 3 weeks, osteogenically differentiated cells were stained using Alizarin Red (Sigma–Aldrich, 2.0% in ddH2O for 5 min), and adipogenically differentiated cells were stained using Oil Red O (Sigma–Aldrich, 0.5% in isopropanol for 30 min). Cells were observed and photographed using an Olympus microscope (DSX110, Japan).

2.2. TRAP staining

To confirm osteoclast formation, cells were stained with the tartrate-resistant acid phosphatase (TRAP) staining kit according to the manufacturer's instructions (Sigma, USA). Osteoclast formation was assessed by counting the number of TRAP-positive cells. Osteoclasts were counted on ten fixed microscopic fields in each well under the Olympus microscope (DSX110, Japan).

2.3. SIN treatment

MSCs at passage 3 were digested and re-suspended in 100 ml of PBS with a density of 1×10^5 cells/well in a 96-well plate overnight. Then, SIN (Aladdin Reagents, China) was added at concentrations of 0 μM, 100 μM, 250 μM, 500 μM, 1 mM, and 2.5 mM. Each group had three duplicates. After 24 h of incubation, the cell characterization and viability tests were performed. The optimal concentration of SIN for the MSCs was confirmed. To obtain a SIN treated MSC-conditioned medium (MSC-CM), cells were cultured with SIN for approximately 48 h and the medium was collected. Then, the detached MSCs in the MSC-CM were removed via centrifugation at $800 \times g$ for 5 min. MSC-CM was stored at −80 °C for further use, and was diluted (1:1) in RAW264.7 osteoclast precursor culture medium prior to incubation.

2.4. Flow cytometry and cell characterization

The cells were harvested via treatment with 0.1% trypsin-EDTA, and detached cells were washed with cold PBS (pH 7.3). The cells were labeled as FITC-conjugated antibody (Abcam, USA), CD34, or phycoerythrin (PE)-conjugated antibodies (Abcam, USA), including CD44, and CD105. FITC- and PE-conjugated mouse immunoglobulin Gs (IgGs) were used for background non-specific binding. The cells were examined using a FACSCanto II cytometer (BD Biosciences, Germany), and the data were analyzed using FlowJo software (FlowJo, USA).

2.5. Cell viability assay

MSCs were digested and seeded into 96-well plates with a density of 1×10^5 cells/well. Cell viability was measured by MTT assay at day 2 after seeding MSCs with SIN treatment. The optical density (OD) value was measured at 490 nm, and a cell viability curve was drawn.

2.6. Real-time quantitative PCR (RT-qPCR)

Cells were washed with PBS, and RNA was extracted using the RNeasy Plus Micro Kit (QIAGEN, USA). Then, cDNA was synthesized with the SuperScript® IV First-Strand Synthesis System (Invitrogen, USA). RT-qPCR was performed in Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, USA), using a 20 ng template in a 25 μl reaction volume with 2 × Power SYBR® Green PCR Master Mix (Invitrogen, USA) and gene specific primer pairs for RANKL, OPG, and Prostaglandin E synthase 3 (PTGES3) in MSCs and TRACP, matrix metalloproteinases-9 (MMP9), and Cathepsin K in osteoclast precursors (Table 1). Relative gene expression levels in SIN treated MSCs were compared with those in untreated MSCs. All data are displayed as the mean ± SD of three independent experiments.