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Original article

Icariin influences adipogenic differentiation of stem cells affected by osteoblast-osteoclast co-culture and clinical research adipogenic

Shuncong Zhang[a,b,*], Pengbo Feng[a,*], Guoye Mo[a], Daxing Li[a], Yongxian Li[a], Ling Mo[b], Zhidong Yang[b], De Liang[b]

[a] The First Clinical Medical College, Guangzhou University of Chinese Medicine, Guangzhou, 510406, China
[b] Department of Spine surgery, The first affiliated hospital of guangzhou university of chinese medicine, Guangzhou, 510405, China

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ABSTRACT

Objective: To build mouse osteoblast MC3T3-E1 and mouse osteoclast RAW264.7 co-culture system and to study the effect of icariin on the activity of osteoblasts and osteoclasts in the co-culture system.
Methods: In vitro acquisition and cultivation of mouse osteoblasts MC3T3-E1 and mouse RAW264.7 cells were conducted. Osteoblast and osteoclast activities of cells were detected by CCK-8 staining experiment, alizarin red staining and tartaric-resistant acid phosphatase (TRAP) staining. We used different concentrations of icariin to interfere in osteoblast-osteoclast co-culture system. The effects of icariin on various genes were detected by PCR and Western blot methods The correction between the expression of PPARγ and BMD was analyzed in patients with osteoporosis.
Results: Mouse osteoblast-osteoclast co-culture system was built, and the osteogenic differentiation effect was enhanced. Icariin can improve the MC3T3-E1 osteogenic differentiation activity, enhance the expression of OPG and RANKL gene protein, reduce the NF-κb gene and protein expression, increase of ALP, TGF-b1 and RANKL gene expression level and reduce RANK gene expression. Icariin can act on MC3T3-E1 cells-RAW264.7 cells co-culture system, and promote the osteogenic activity of MC3T3-E1 cells, inhibit the osteoclast activity of RAW264.7 cells and reduce the level of BMSCs adipogenic differentiation. The expression level of PPAR-γ gene was negatively correlated with the level of BMD.
Conclusions: Mouse MC3T3-E1 cells and mouse RAW264.7 cells could be co-cultured in vitro, and icariin could improve the osteogenic activity of MC3T3 cells-RAW264.7 cells and decrease the osteoclast activity. Icariin could inhibit adipogenic differentiation of BMSCs in the osteoblast-osteoclast co-culture, promoting osteogenic differentiation and inhibiting osteoclast differentiation.
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1. Introduction

As an important function of human body structure, bone tissue is of great significance to human body movement. Osteoporosis is the most common bone lesions. It was reported that nearly 20% of people over 50 years old had osteoporosis and the incidence rate has been rising [1]. It increases exponentially in the aging population [2].The incidence of osteoporosis in Singapore over the age of 65 is expected to increase from 6% to 19% (from 1990 to 2030) [3]. Osteoporosis is easy to cause osteoporotic fracture. The physiological function of healthy bone requires osteoclasts and osteoblasts. At present, there are some limitations in the use of anti-osteoporosis drugs [4,5]. Therefore, it is necessary to understand the mechanism of osteoporosis.

Bone marrow stromal stem cells, osteoclasts and osteoblasts play an important role in the normal growth and metabolism of bone tissue. Bone resorption by osteoclasts and bone formation by osteoblasts are particularly important [6]. There were three stages of bone modeling: osteoclasts were absorbed on the bone surface to absorb a small amount of bone. The osteoblasts form new bone into depression. Bone matrix mineralization, the newly formed bone is not equivalent to the absorption of bone. These processes are regulated by estrogen, RANKL/OPG pathway, ROS, various growth factors and other related kinase signal transduction pathways [7,8]. Therefore, it is important to study the mechanism

* Corresponding authors at: Department of Spine surgery, The first affiliated hospital of guangzhou university of chinese medicine, NO.16 Jichang Road, Guangzhou, 510405, China.
E-mail addresses: zhangshunncong@163.com (S. Zhang), fengpenggbo@163.com (P. Feng).

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of the interaction between osteoblasts and osteoclasts for the understanding of the pathological changes of osteoporosis.

Osteoporosis has a poor prognosis and its pathological process is complex. A number of new drugs have been studied for the treatment of osteoporosis. Chinese traditional medicinal plant extract of Epimedium has long been used to promote bone tissue growth [9]. It has been reported that about nine prenyl flavonoid of Epimedium extract play role in bone marrow mesenchymal stem cells, osteoblast, osteoclast cell line, targeted regulation of the estrogen signaling pathway and other bone regeneration pathway [10], including icariin and epimedium prime. Therefore, it is necessary to further clarify the specific pharmacological mechanism of icariin (molecular structure shown in Fig. S1), which is of great significance to the further understanding of the drug metabolism and the specific effect of the drug.

The occurrence of osteoporosis may be related to the imbalance of BMSCs [11]. The dynamic balance of BMSCs differentiation is that BMSCs can differentiate into osteoblasts and adipocytes, and the two differentiation directions can be regulated by each other, and they can be transformed into each other [12]. The differentiation of BMSCs to osteoblasts and adipocytes is regulated by a variety of cytokines, such as CCAAT/enhancer binding protein alpha (C/EBPα) and peroxisomal increase son activation receptor gamma (PPARγ). C/EBPβ plays an important role in the early stage of fat production, which can directly induce the expression of C/EBPα and PPARγ [13]. Runx2 is considered to be the key factor regulating the differentiation of BMSCs into osteoblasts [14]. To elucidate the relationship between various regulatory factors may contribute to understanding the mechanisms of BMSCs adipogenic differentiation.

In addition, as an important factor in the fat differentiation, whether PPARγ changes in the clinical osteoporosis patients and its correlation with bone mineral density have not been fully clarified. Therefore, in this study, we used qRT-PCR and Western blot methods to analysis the correlation between expression of PPARγ and osteoporosis bone mineral density (BMD) in experimental group and control group.

2. Methods

In vitro acquisition and cultivation of mouse osteoblasts MC3T3-E1 and mouse RAW264.7 cells were conducted. When it is necessary to induce differentiation, the culture medium is added into osteogenic differentiation medium (Fig. S1). After RANKL induced mouse RAW264.7 cells differentiation into mature osteoclasts, we built osteoblast-osteoclast co-culture system in Transwell chamber (Fig. S2). Osteoblast and osteoclast activities of cells were detected by CCK-8 staining experiment, alizarin red staining and tartaric-resistant acid phosphatase (TRAP) staining. The effects of co-culture system on the OPG and TGF-b1 of mouse osteoblast MC3T3-E1 and RANK and NF- nuclear factor kappa B (NF-κb) gene of mouse osteoclast were detected by PCR and Western blot methods. The selection of primers is shown in Table S1.

After that, we used different concentrations of icariin to interfere in osteoblast-osteoclast co-culture system, and CCK-8 detection, alizarin red staining and TRAP staining were conducted to detect cell activity. The effects of icariin on OPG and TGF-b1 of mouse osteoblast MC3T3-E1 and RANK andNF-κb gene of mouse osteoclast in co-culture system were detected by PCR and Western blot methods. The selection of primers is shown in Table S2.

We again built osteoblast-osteoclast-stem cell co-culture system in Transwell chamber and used different concentrations of icariin to interfere it. Then we used oil red O staining and HE staining of stem cells to detect the differentiation of their fat and so on. MC3T3-E1 of mouse osteoblast cell and OPG, RANKL, ALP, TGF-b1, RANK and NF-κb mRNA genes of induced osteoclast mixed co-cultured in the co-culture system, as well as the PPARγ, C/EBPα, C/EBP b and RUNX2 mRNA genes of BMSCs were detected by PCR method for their expression and changes. After that, the protein expressions of OPG, NF-κb and PPARγ were detected by Western blot method.

We collected data from 16 patients with osteoporosis between Dec 2015 and Mar 2016. Clinical characteristics of each patient were collected, including age, BMD, sex, body mass index (BMI), weight and length. BMD of lumbar spine (1–4) was measured by QDR-4500W dual energy X-ray bone density instrument. In this study, osteoporosis was diagnosed according to T value, which was based on the diagnostic criteria of osteoporosis by WHO. The diagnostic criteria were as follows: (1) $T > -1$, normal range; (2) $-2.5 < T < -1$, patients have osteopenia; (3) $T < -2.5$, patients have osteoporosis; (4) $T < -2.5$ and the occurrence of one or more fractures, patients have severe osteoporosis. According to the above criteria, the patients were divided into non-osteoporosis group ($-2.5 < T < -1$) and osteoporosis group ($T < -2.5$). The expressions of PPARγ gene and protein in clinical patients were detected by PRC and Western blot, and the selection of primers is shown in Table S3. The correction between the expression of PPARγ and BMD was analyzed.

The quantitative data obtained by PCR and Western blot were expressed as $\bar{x} \pm SD$, and the statistical analyses were performed using SPSS 13.0 software. Independent sample T test was used to compare means in two different groups. One-Way ANOVA was used to compare multiple groups at the same time point. The variance analysis of repeated measures was used to compare the data of the same group at different time points. Data were first tested by Levene test to determine whether they meet the homogeneity of variance. The correlation between PPARγgene and protein expression and BMD was analyzed using Pearson correlation coefficient, and the linear curves were drawn. P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Establishment of mouse osteoblast-osteoclast co-culture system in vitro

After RANKL induced differentiation, RAW264.7 cells showed a single nuclear macrophage like change, with large colony like distribution due to the strong ability to proliferate, and the cells gradually extended into irregular shape, with lots of cells showed spindle shape (Fig. S3). Mouse osteoblast-osteoclast co-culture system was built. Light microscopy showed that MC3T3-E1 cells in the co-culture system were in good agreement with the morphological characteristics of MC3T3-E1 cells in general, as well as the RAW264.7 cells. During the co-culture period, the osteogenic differentiation effect was more obvious, and the cell formation was in the order of arrangement. The number of osteoclasts was increased, and the colony distribution was enhanced (Fig. S4).

CCK-8 detection results showed that the absorbance of cells was 0.615, 0.670, 0.795, 0.619, 0.685, 0.745 and 0.688, and the mean value was 0.688 ± 0.071, indicating the activity was good. Alizarin red staining showed that MC3T3-E1 cells can form dark alizarin red staining change and have osteogenic activity. TRAP staining results showed that RAW264.7 cells can differentiate into mature osteoclasts, which contain a number of nuclei (Fig. S5).

In co-culture system, the OPG and ALP gene expression levels of MC3T3-E1 cells decreased significantly, while the expression of TGF-b1 and RANKL genes increased significantly. The RANK and NF-κb genes levels of RAW264.7 in co-culture system were decreased significantly (Fig. 1). The OPG protein expression level of

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