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Original Article

Effects of icariin on the proliferation and differentiation of MC3T3-E1

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Abstract: Icariin (ICA), the main active flavonoid glucoside isolated from Herbaepimedii (HEF), is an anabolic agent in bone that has been reported to prevent bone loss in ovariectomized rats and postmenopausal women. However, the understanding of its mechanism of this anabolic action of ICA in osteogenesis remains incomplete. Here, we found that Icariin could promote MC3T3-E1 osteoblastic cell proliferation, associated with increased protein levels of the proliferation markergene cyclin D1 and, CDK4. Icariin also enhanced MC3T3-E1 cell differentiation and mineralization demonstrated by increased the expression of differentiation markers, alkaline phosphatase (ALP) and collagen type I (Col I), osteocalcin (OCN) and bone nodule formation via Alizarin red S staining. In addition, the ratio of OPG/RANKL also could be enhanced by ICA. Therefore, in the present study, we investigate the effects of ICA on osteoblast differentiation from at least three perspectives, so as to obtain an understanding of its mechanism of action and elucidate the molecular mechanisms of this drug, which might provide an insight into the rationality and in-depth theoretical basis for the clinical application of ICA in preventing osteoporosis.

Keywords: Osteoporosis, icariin, MC3T3-E1 cells, proliferation, differentiation, mineralization

Introduction

Osteoporosis, the most common bone disease in humans has become a well-known major public threat accompanying with increasing social-economic burden in our aging society [1]. It is characterized by low bone mass, deterioration of bone tissue and disruption of bone architecture, compromised bone strength, and an increase in the risk of fracture [2]. Hormone replacement therapy (HRT) has been demonstrated to prevent bone loss and commonly includes a combination of estrogen, progesterone and progestin [3]. However, long-term HRT results in adverse side effects, including hypocalcemia, worsening of renal impairment and osteonecrosis of the jaw [4], and therefore novel therapeutic strategies for the treatment of osteoporosis are required.

Epimedium is a traditional medicinal herb that has been used to treat fractures, bone and joint diseases, and gonad dysfunctions in Asia for thousands of years [5]. It was reported as an effective enhancer of bone healing [6] that could be prescribed for treating osteoporosis. Icariin ($C_{33}H_{40}O_{15}$; molecular weight: 676.67), the main active flavonoid glucoside isolated from Epimedium pubescens, was found to have various kinds of pharmacological effects such as anti-osteoporosis, improvement of cognition, anti-depression, cardiovascular protection, anti-tumor, improvement of sexual dysfunction, anti-inflammation and immunoprotection [7-11]. A further study on its mechanism reveals that icariin could promote bone formation and inhibit bone resorption in various cell lines as well as experimental animal models [12-15].

Although there are ample studies concerning the potential effects of ICA on osteoblast proliferation and differentiation, the definite conclusive results have yet to be elucidated, but the detailed molecular mechanisms underlying these effects remain unclear. In the present study, we further examined the detailed molecular mechanisms of the effect of icariin in MC3T3-E1 cells.

Material and methods

Cell lines

MC3T3-E1 cells (an osteoblast-like cell line from C57BL/6 mouse calvaria) were purchased from obtained from the Cell Center of the Chinese Academy of Medical Sciences. The cells were cultured at 37°C in a 5% CO_2 atmosphere in α-minimum essential medium (α-MEM) (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Icariin was obtained from the Institute of Pharmaceutical Research (Beijing, China) with a purity of 99%. Stock solutions of icariin were prepared in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA) and stored at -20°C. Assay kits of ALP and BCA were obtained from Jiancheng Biotech (Nanjing, China). ELISA kits for COL I and OCN assays were purchased from R&D Co (USA).

Cell proliferation assay

MC3T3-E1 cells were seeded in 96-well plates at 0.8-1 × 10^3 per well. After 24 h, the medium was replaced with a medium containing various test agents. During the experiment, the treatments (including medium and icariin) were changed every day, and fresh icariin was added. Four hours before the end of the incubation, Cell proliferation was evaluated using Cell Counting Kit-8 (Beyotime, China) according to the manufacturer's instructions. Briefly, 10 μl of CCK-8 solution was added to culture medium, and incubated for 2 h. The absorbance at 450 nm wavelength was determined with a reference wavelength of 570 nm.

COL I and OCN contents and ALP activity

COL I and OCN contents of MC3T3-E1 cells were measured according to literature [16, 17]. A 1.0 mL portion of the cell suspension at a concentration of 1.0 × 10^5 cells per mL was added to each well of a 24-well plate. After 24 h, diluted ICA (5 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL, respectively) was added to the culture. The medium containing ICA at same concentrations was changed once every 3 days and the culture supernatant was collected through growth and differentiation of the MC3T3-E1 cells and stored at -70°C. After 7 days and 14 days, the culture supernatant was combined mixed, and then the COL I and OCN contents were assayed with a sandwich ELISA assay kit (R&D Systems Inc.). ELISA assays were performed for COL I and OCN according to manufacturer's instruction and their absorbance was measured on a microplate reader at a wavelength of 450 nm. The supernatant was used for ALP activity determination with ALP assay kits. Total protein contents were measured by BCA assay kit. ALP activity was represented as U/g·prog.

Bone nodule formation assay

Bone nodule formation was determined by an Alizarin red S assay. Briefly, the MC3T3-E1 cells (2 × 10^4 cells/well) were seeded in a 24-well plate and and incubated for 24 h, and then treated with ICA (5 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL respectively) for 21 days. The differential medium was changed once every two days during the incubation period. The supernatant was removed and the cells were fixed with 4% neutral formaldehyde for 15 min, then the cells were washed twice with PBS, stained with 0.1% (w/v) Alizarin red-Tris solution for 30 min. After washing thoroughly with deionized water, the images of the stained cells were photographed with an inverted microscope (IX51, Olympus, Tokyo, Japan).

The formation of bone nodules were further verified by semi-quantitative analysis. Cetylpyridinium chloride (500 μL, 100 mM) was added into each well to dissolve the bound dye. The absorbance of the solubilized Alizarin Red was determined at 570 nm using a microplate reader.

Western blot analysis

Protein extracts from MC3T3-E1 cells were subjected to 10% SDS-PAGE and subsequently transferred to a PVDF membrane. The membrane was blocked with 5% (w/v) nonfat milk and incubated sequentially with the primary antibodies against Cyclin D1, CDK4, OPG and RANKL, and then with horseradish peroxidase-conjugated secondary antibodies. Anti-β-actin antibody was used as an internal control. The protein bands were visualized using Odyssey software (Infrared Imaging System LI-COR Biosciences).

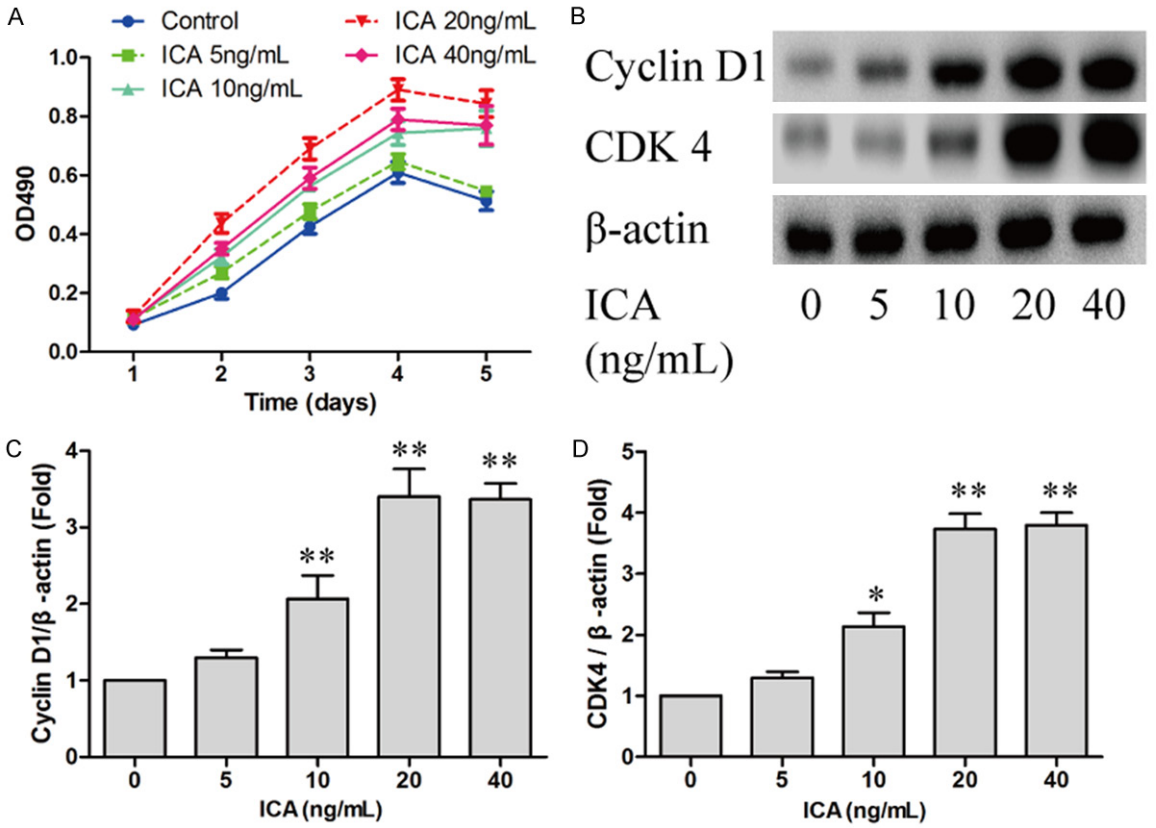

Figure 1. ICA promotes cell proliferation of MC3T3-E1 cells. The cells were treated with ICA (5-40 ng/ml) for 1 to 5 days respectively, and cell viability was measured by CCK-8 assay. A. Concentration-dependent effect of ICA on MC3T3-E1 cells growth. B. Cyclin D1 and CDK4 expression were significantly increased following ICA treatment. C and D. The optical densities of the bands were measured using Image-Pro Plus software. Data were shown as mean ± S.D. of three independent experiments. *P < 0.05, **P < 0.01 *vs* control.

Statistical analysis

Statistical analyses were performed with SPSS 13.0 software. The results were evaluated by χ^2 test and the other data were evaluated by Student's t-test and expressed as the mean ± SD from three independent experiments. A *P*-value of less than 0.05 was considered statistically significant.

Results

Effect of ICA on the cell proliferation of MC3T3-E1 cells

To investigate the effect of ICA on the growth of MC3T3-E1 cells, Firstly, we conducted CCK-8 assay to measure cell growth of MC3T3-E1 cells after the cells treated with ICA for 2 days and 3 days at various concentrations that range from 5 to 40 ng/mL. As shown in **Figure 1A**, the proliferation activity of MC3T3-E1 cells was significantly promoted by ICA in a dose-dependent manner. Furthermore, we also examined the expression of certain genes involved in proliferation regulation. As shown in **Figure 1B-D**, ICA increased the expression of Cyclin D1, CDK4. These results suggest that the treatment of ICA promotes the growth of MC3T3-E1 cells to some extent.

Effect of ICA on COL I, OCN content and ALP activity of MC3T3-E1 cells

ALP is known to be associated with bone metabolism and differentiation of osteoblasts and its activity is one of the most common indicators of osteoblast differentiation and osteogenic properties. As shown in **Figure 2A**, ALP activity was dose-dependently enhanced ALP activity in MC3T3-E1 over the 3 days, and the maximal effect was reached when cells were incubated with 20 ng/mL.

As the expression of OCN and Col I changes during the maturation and differentiation of

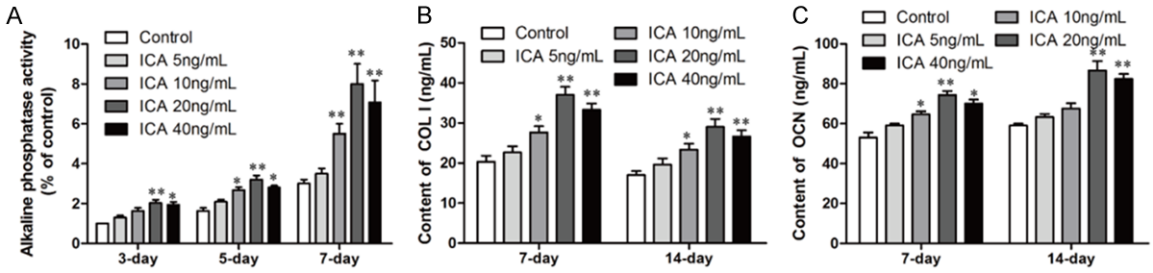

Figure 2. ICA promotes cell differentiation of MC3T3-E1 cells. MC3T3-E1 cells were incubated with various concentrations of ICA for 3 days, 5 days, 7 days or 14 days. The medium was collected for COL I and OCN content (7, 14 days), ALP activity (3, 5, 7 days) determination with ELISA kits. A. Effect of ICA on ALP activity of MC3T3-E1 cells. B. Effect of ICA on COL I secretion of MC3T3-E1 cells. C. Effect of ICA on OCN secretion of MC3T3-E1 cells. Data were represented as mean ± S.D. of three independent experiments. *P < 0.05, **P < 0.01 *vs* control.

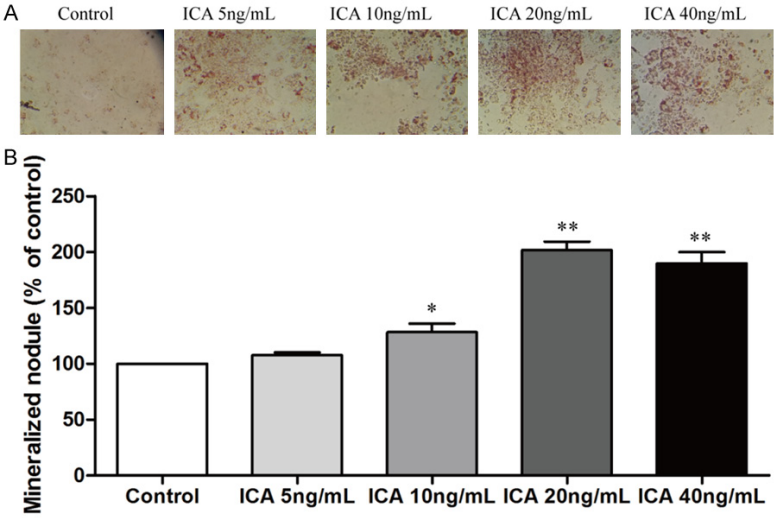

Figure 3. ICA promotes bone nodules formation of MC3T3-E1 cell. The cells were treated with differential medium for 21 days, and then collected for Alizarin red staining. A. Alizarin red staining of calcium deposits. B. Alizarin Red S quantification measured at 562 nm. Data were shown as mean ± S.D. of three independent experiments. *P < 0.05, **P < 0.01 *vs* control.

Effect of ICA on bone nodule formation of MC3T3-E1 cells

We next tested the effect of ICA on osteoblastic differentiation as evidenced by mineralization. As shown in **Figure 3A**, mineralization was clearly observed after 21 days of culture with ICA after staining with Alizarin S red (**Figure 3A**). In addition, ICA can stimulate the mineralization for all doses of ICA treatments group compared with control group ($P < 0.01$), and maximal and significant effects were observed at a concentration of 20 ng/mL (**Figure 3B**).

Effect of ICA on OPG and RANKL protein levels of MC3T3-E1 cells

osteoblasts, we examined the effect of ICA on their expression in MC3T3-E1 cells. Following treatment of the cells with for 7 days of culture, the expression of Col I was markedly enhanced for all doses of ICA treatments groups compared with the control group (**Figure 2B**), and significant differences were found among ICA treatment groups ($P < 0.05$). Similarly, ICA remarkably stimulated OCN secretion in a dose-dependent manner for 14 days of culture (**Figure 2C**). The maximal effect of ICA on Col I and OCN was reached when cells were incubated with 20 ng/mL.

The above data indicate that 20 ng/ml ICA was optimal to stimulate MC3T3-E1 differentiation.

Recent reports have found that the medium of osteoblast containing high ratio of OPG/RANKL markedly inhibited the formation of osteoclasts. In this study, we test the protein levels of OPG and RANKL using MC3T3-E1 cells cultured for 3 days. As shown in **Figure 4A**, OPG protein expression was largely enhanced by ICA in a dose-dependent mannercompared to the control group. In contrast, RANKL protein expression was largely reduced by ICA in a dose-dependent manner compared to the control group (**Figure 4B**). The OPG/RANKL ratio was significantly augmented by ICA. The result was consistent with the previous reports [18]. Taken together, the ratio of OPG/RANKL may be the ultimate determinant of bone resorption and

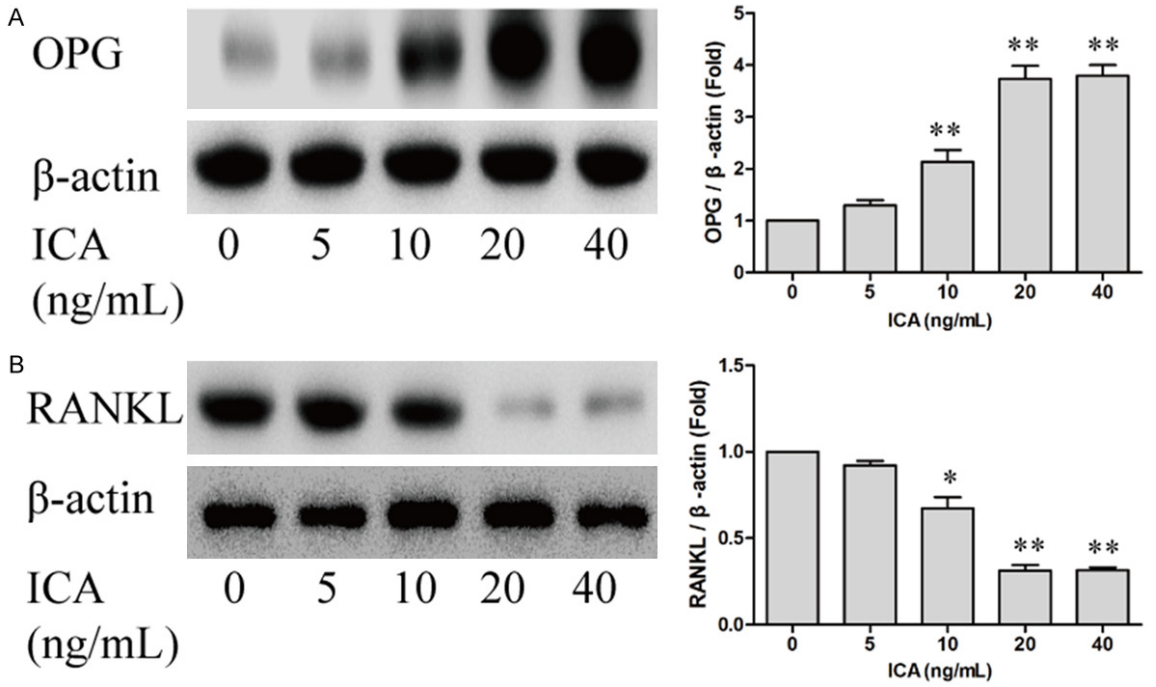

Figure 4. Effect of ICA on OPG (A) and RANKL (B) protein levels in MC3T3-E1 cells. Cells were harvested after treatment with various concentrations of ICA for 5 days, and lysed to gather proteins for Western blot analysis. Data were shown as mean ± S.D. of three independent experiments. *P < 0.05, **P < 0.01 *vs* control.

bone remodeling can be assessed by the relative ratio of OPG to RANKL.

Discussion

In this present study, our data demonstrated that ICA significantly promoted the proliferation, differentiation and mineralization of MC3T3-E1. Furthermore, our findings also indicated that ICA could significantly enhance osteogenesis by increasing OPG/RANKL ratio.

The main pathogenesis of osteoporosis is the imbalance in bone remodeling and resorption, which lead to destruction of the microstructure of bone tissue, bone fragility and susceptibility to fracture [19]. Currently, clinical treatment for osteoporosis is mainly focused on the inhibition of the osteoclasts activity and bone resorption, and treatment methods for improving the ability of osteoblast bone formation is still very rare. It is mainly due to the regulation mechanisms of osteogenesis cell bone-forming ability is a complex process, the drug targets for improving osteoblast bone formation have also poorly understood. Therefore, study the molecular mechanisms of osteoblast bone formation and find the drug targets, will provide new strategies and approaches to the treatment of osteoporosis.

Icariin (ICA) is believed to be the major active ingredient of Epimediiherba [20]. A large number of in vivo and in vitro studies indicated the extensive pharmacological effects of icariin. At the in vivo level, ICA demonstrated significant antiosteoporosis effects in OVX rat/mouse, glucocorticoid induced rat as well as osteoprotegerin (OPG) knockout mice [21]. In addition, a significant osteogeniceffect of ICA was observed in bone mesenchymal stem cells, osteoblasts and osteoblast like cells [22, 23]. However, the osteogenic effect of ICA on MC3T3-E1 is still unknown. In our study, we first investigated whether cell proliferation could be regulated by ICA in MC3T3-E1 cells. The CCK-8 assay results showed that ICA could increase the cell proliferation of MC3T3-E1 cells dose dependently.

ALP, Col I and OCN are the major biological markers in osteoblasts differentiation. Up-regulation of ALP, an enzyme serving as a marker of osteoblastic differentiation, occurs at the middle stage of differentiation [24]. Our study showed that ALP activity was significantly in-

creased by ICA. OCN is a later marker of osteoblastic differentiation that is closely related to osteoblastic maturation [24, 25]. We demonstrated ICA remarkably stimulated OCN secretion at 14 days of MC3T3-E1 cell culture. In addition, osteoblasts abundantly synthesize and secrete Col I, a major bone matrix constituent and extracellular macromolecule in osteoblast cultures. In our experiments, the expression of Col I was markedly enhanced for 7 days of MC3T3-E1cell culture. This indicates that ICA prompts the synthesis of bone collagen and upregulates osteoblastic differentiation.

Next, we found that ICA induced mineralized nodule formation at 14 days of MC3T3-E1 cell culture. This result supports the hypothesis that ICA promotes osteoblastic differentiation in vitro, through increased synthesis and secretion of matrix proteins. Osteoblasts produce RANKL and OPG, two cytokines with opposing effect on osteoclasts [26]. In this study, the stimulatory effect of ICA on osteoblast differentiation was further investigated by analyzing the amount of cytokine OPG and RANKL secreted into the cell culture supernatant by Western Blot. Our result found that the OPG/RANKL ratio was significantly augmented by ICA. Therefore, we speculate that the distinguished increase of OPG in MC3T3-E1 cells may lead to bone regeneration.

In conclusion, ICA, which could stimulate osteoblastic bone regeneration by up-regulating ratio of OPG/RANKL, is likely to be a promising drug or a lead compound in the prevention and treatment of osteoporosis. Besides, the influence of ICA on MC3T3-E1 cells only has in vitro evidences, which warrants further in vivo investigation. With further investigation, there is probability that new moieties based on ICA would be developed with higher bioactive capacities and moderate pharmacokinetic properties.

Disclosure of conflict of interest

None.

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