Effects of icariin on the regulation of the OPG-RANKL-RANK system are mediated through the MAPK pathways in IL-1β-stimulated human SW1353 chondrosarcoma cells

ZEMING WANG[1,2], LI DING[1,2], SIHAN ZHANG[1], TAO JIANG[3], YONGMIAN YANG[4] and RONGHENG LI[1]

[1]Department of Combination of Chinese and Western Medicine, [2]Laboratory Research Center, and [3]Department of Pneumology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016; [4]Department of Gynaecology, The Fourth Hospital of Shijiazhuang, Shijiazhuang, Hebei 050011, P.R. China

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Abstract. Arthrodial cartilage degradation and subchondral bone remodeling comprise the most predominant pathological changes in osteoarthritis (OA). Moreover, accumulating evidence indicates that the abnormal expression of osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL) and receptor activator of nuclear factor kappa-B (RANK) plays a vital role in the collapse of cartilage and subchondral bone. In the present study, the effects of icariin on the expression levels of these 3 factors in interleukin (IL)-1β-stimulated SW1353 chondrosarcoma cells were investigated. The SW1353 chondrosarcoma cells were cultured in the presence or absence of icariin and mitogen-activated protein kinase signaling pathway inhibitors, and were then stimulated with IL-1β. Cell viability was assessed by MTT assay. The mRNA and protein expression of OPG, RANKL and RANK was analyzed by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and ELISA, respectively. In addition, the levels of phosphorylated p38 (p-p38) and phosphorylated extracellular signal-regulated kinase (p-ERK)1/2 were detected by western blot analysis. The results from western blot analysis revealed that treatment with icariin decreased the levels of p-p38 and increased the levels of p-ERK1/2 in the IL-1β-stimulated SW1353 cells. In addition, treatment with icariin decreased the levels of RANK and RANKL. Furthermore, the suppressive effects of icariin on OPG and OPG/RANKL were greater than those exhibited by the p38 signaling pathway inhibitor (SB203580). The findings of the the present study suggest that icariin has therapeutic potential for use in the treatment of OA.

Correspondence to: Professor Rongheng Li, Department of Combination of Chinese and Western Medicine, The First Affiliated Hospital of Chongqing Medical University, No. 1 You Yi Road, Chongqing 400016, P.R. China
E-mail: Lrongheng1231@163.com

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Introduction

Arthrodial cartilage degradation, subchondral bone remodeling and osteophyte formation are involved in the development of osteoarthritis (OA) and are the result of mechanical and biological factors (1). OA is divided into 2 categories, primary and secondary OA. The prevalence of primary OA in women increases with age. Glucocorticoids and non-steroidal anti-inflammatory drugs are the recommended treatments for OA; however, these drugs are associated with elevated risks, such as gastrointestinal disorders and osteoporosis and have thus failed to obstruct the progression of OA. Due to the economic burden and limited efficacy of these drugs, safer and more well tolerated agents are required for the treatment of OA.

Although the exact mechansisms involved in the pathogenesis of OA remain to be elucidated, cartilage degradation and subchondral bone remodeling are the most commonly observed changes. Both of these processes are involved in the progression of OA and are interconnected and cannot be separated from each other; however, there is controversy as to which of these processes is the initial etiological factor in OA (2,3). In particular, OA is characterized by an imbalance in bone metabolism that results in subchondral bone osteoporosis at the early stages of OA and bone sclerosis at the late stages (2,4,5), in which the osteoprotegerin (OPG)-receptor activator of nuclear factor kappa-B ligand (RANKL)-receptor activator of nuclearfactor kappa-B (RANK) system has been proven to play a critical role (6). OPG, as a secreted member of the tumor necrosis factor receptor family, inhibits bone resorption through competitive binding to RANKL and prevents it from binding to its receptor, RANK, on the surface of osteoclasts. Therefore, the OPG/RANKL ratio is a decisive factor in bone metabolism (7). It has also been demonstrated that OPG and RANKL are detected in the superficial zone of normal articular cartilage, but extend to the middle zone of OA cartilage (8). Previous studies have found that OPG/RANKL and OPG levels are significantly decreased in chondrocytes in knee OA and in patients with OA, but are increased at the late

stages of OA (8-11). Generally, OPG exerts dual effects based on its ability to suppress chondrocyte apoptosis (12) and alleviate growth plate cartilage destruction (13); it stimulates the production fo 2 catabolic factors, protease-activated receptor-2 (PAR-2) and matrix metalloproteinase (MMP)-13 (11); on the contrary, RANKL aggravates cartilage degradation (14). Thus, maintaining the OPG/RANKL ratio within a normal range represents a strategy for protecting cartilage. The OPG-RANKL-RANK system has been shown to be a common downstream target in OA and is inappropriately activated by interleukin (IL)-1β (15) during OA and downregulated by $1\alpha,25(OH)_2D_3$ (16,17). In addition, pro-inflammatory cytokines, most prominently IL-1β and $1\alpha,25(OH)_2D_3$, have been shown to contribute to the progression of OA by inducing the expression of OPG, RANKL and RANK. Various pathways have also been shown to mediate the expression of OPG, RANKL and RANK during the progression of OA, and these include mitogen-activated protein kinases (MAPKs) (18,19), EP2/EP4 receptor (20) and the Wnt/β-catenin signaling pathway (7).

Plant-derived agents have received considerable attention for their potential for use as substitute therapies for the treatment of OA in recent years, as they have fewer complications and multiple curative effects (21,22). For instance, icariin, a prenylated flavonol glycoside, is the cardinal active constituent of the crude extract of the herb *Epimedium pubescens* which has been traditionally used in the treatment of age-related diseases in Asia (23). Icariin exhibits both proliferative and anti-inflammatory (24,25) properties. It has also been reported that icariin protects chondrocytes from inflammatory and apoptotic responses and promotes extracellular matrix synthesis (22,26,27), during OA, thus preventing bone loss (28,29). Taken together, these data suggest that icariin has therapeutic potential for use in the treatment of OA.

In the present study, we aimed to determine the effects of icarrin on the expression of OPG, RANKL and RANK in SW1353 chondrosarcoma cells stimulated with IL-1β. We decided to use the SW1353 cell line in our experiments as SW1353 cells have a similar phenotype with human chondrocytes and they have been successfully used in previous scientific studies (30-33). The expression of transcription factors possibly involved in the upregulation of OPG, RANKL and RANK during the development of OA, including phosphorylated p38 (p-p38) and extracellular signal-regulated kinase (ERK)1/2 was also analyzed.

Materials and methods

Cell culture and study design. SW1353 human chondrosarcoma cells (Institute of Biochemistry and Cell Biology, Shanghai, China) were cultivated in Dulbecco's modified Eagle's medium/nutrient (Gibco BRL, Rockville, MD, USA) containing 10% (v/v) fetal bovine serum (Gibco BRL) and 100 U/ml penicillin-streptomycin solution (Beyotime Institute of Biotechnology, Jiangsu, China) at 37˚C in a humidified atmosphere under 5% CO_2. The cells were seeded at a density of 10^6 cells in one tissue culture flask and pre-treated in the presence or absence of p38 inhibitor (SB203580; 10 μmol/l), ERK1/2 inhibitor (PD98059; 20 μmol/l) (both from Beyotime Institute of Biotechnology), or icariin (12 μg/ml) (Cayman Chemical Co., Ann Arbor, MI, USA) for 1 h, then stimulated with IL-1β (10 ng/ml) (Pepro Tech, Rocky Hill, NJ, USA) for 48 h.

MTT assay. The SW1353 cells were cultured in 96-well plates ($1x10^4$ cells/well; 3 plates) with icariin (0, 1.5, 3, 6, 12 and 24 μg/ml) and incubated for 24, 48 and 72 h. Subsequently, MTT solution was added to each well for another 4 h at the final concentration of 5 mg/ml. The supernatants were then removed, and dimethylsulphoxide was added to the wells to dissolve the formazan crystals. The A-values of each well were recorded at 570 nm using an enzyme-labeled meter (Thermo Fisher Scientific, Waltham, CA, USA) after the 96-well plates were shaken for 10 min.

Western blot analysis. Total proteins from the cells were extracted using a phosphorylated protein extraction kit (Keygen Biotech Co., Ltd., Nanjing, China). The protein concentration was determined using the bicinchoninic acid protein assay (Beyotime Institute of Biotechnology) and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis; subsequently, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by electroblotting. The membranes were sealed at room temperature with 5% bull serum albumin (Sigma, San Antonio, TX, USA) for 2 h and then washed 3 times with Tris-buffered saline and Tween-20 (TBST) (Tris, 4.84 g; NaCl, 17.6 g; HCl, 2 ml; 0.1% Tween-20, 2 ml). The PVDF membranes were probed with primary antibodies against the p-p38 (1:1,000), p-ERK1/2 (1:2,000) (Cell Signaling Technology, Inc., Beverly, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (ImmunoWay, Biotechnology Co., Newark, DE, USA) at 4˚C overnight. The membranes were then washed with TBST and incubated with peroxidase-conjugated second antibody (1:2,000) (Cell Signaling Technology) at 37˚C for 2 h. These protein bands were subsequently analyzed using an enhanced chemiluminescence (ECL) Plus kit (Beyotime Institute of Biotechnology).

Quantitative reverse transcriptionpolymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen, Burlington, ON, Canada) according to the recommendations of the manufacturer. Reverse transcription was performed using the PrimeScript RT Reagent kit (Promega Madison, WI, USA) and first-strand cDNA was synthesized using the RNA PCR kit (Takara Bio, Inc., Shiga, Japan). The cDNA mixtures were diluted 1:10 in sterile distilled water (dH_2O), and 5 μl aliquots were subjected to quantitatvie (real-time) PCR using 2X SYBR-Green I dye (Takara Bio) in 20 μl reactions containing dH_2O 4 μl, 2X SYBR-Green I 10 μl and 0.5 μl primers (sense and antisense), as shown in Table I. The PCR primers were designed by Geneseed Biotech (Guangzhou, China). Quantitative (real-time) PCR was performed using an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with 40 cycles of 95˚C for 5 min, 95˚C for 15 sec and 60˚C for 30 sec; measurements were made at the end of a 60˚C annealing step. Data were analyzed using SmartCycler software (version 2.0). The $2^{-\Delta\Delta CT}$ method was used to calculate relative fold changes in mRNA expression. All real-time PCR experiments were performed in triplicate. The calculated values for gene expression were normalized

Table I. Primers used for PCR analysis.

Target	Primer sequence (5'→3')	GenBank accession no.
RANKL	F: GGATCACAGCACATCAGAGCAGAG R: CCAGATGGGATGTCGGTGGCATT	NM_003701.3
RANK	F: GGCACTGGATCAATGAGGCTTGT R: CATGCTCCCTGCTGACCAAAGT	NM_003839.3
OPG	F: CGGGAAAGAAAGTGGGAGCAG R: CTTCAAGGTGTCTTGGTCGCCAT	U94332.1
18SrRNA	F: CCTGGATACCGCAGCTAGGA R: GCGGCGCAATACGAATGCCCC	NR_003286

RANKL, receptor activator of nuclear factor kappa-B ligand; RANK, receptor activator of nuclear factor kappa-B; OPG, osteoprotegerin; 18SrRNA, 18S ribosomal RNA; F, forward; R, reverse.

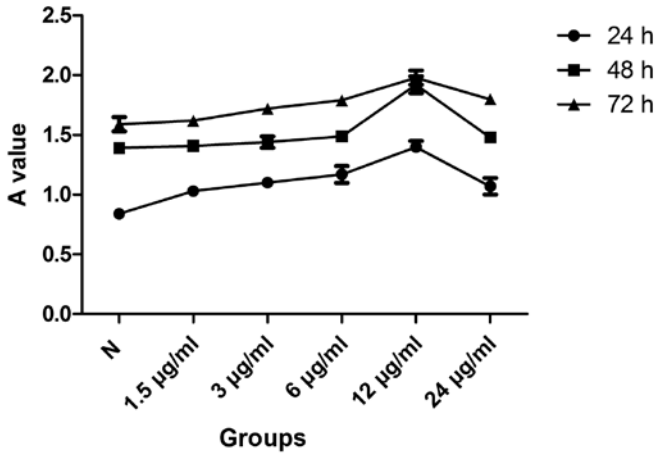

Figure 1. Effects of various concentrations of icariin on the viability of SW1353 cells. Human SW1353 chondrosarcoma cells were treated with various concentrations of icariin for 3 consecutive days. Cell proliferation was analyzed by MTT assay. Treatment of the cells with 1.5, 3, 6 and 12 μg/ml icariin induced a dose-and time-dependent increase in proliferation. However, when the cells were treated with the concentration of 24 μg/ml of icariin, cell viability was dereased. N, normal.

against the levels of *Homo sapiens* RNA, 18S ribosomal 1 (RN18S1) ribosomal RNA.

ELISA. For the quantification of OPG and RANKL protein levels in the culture medium, the SW1353 cells were treated as described above. After 48 h, the supernatants were collected and stored at -80˚C until analysis with ELISA. Human OPG and RANKL ELISA kits were purchased from Uscn Life Science Inc. (Wuhan, China). The experiments were performed in accordance with the manufacturer's instructions.

Statistical analysis. Data are expressed as the means ± standard deviation. All data were analyzed with variance analysis. Statistical significance was assessed by one-way analysis of variance (ANOVA) or the Games-Howell test according to the homogeneity of variance, and a value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of icariin on cell viability. As shown by MTT assay, treatment of the SW1353 cells with icariin (1.5, 3, 6 and 12 μg/ml) had a dose-dependent proliferative effect. However, the treatment of the SW1353 cells with 24 μg/ml icariin was associated with adverse effects. In a subsequent experiment, SW1353 cells cultured with icariin for 3 consecutive days revealed a time-dependent increase in proliferation. According to our results, the pro-mitogenic effect of 12 μg/ml of icariin at 48 h was superior to the effect of other doses at other time points and, therefore, this concentration and time period were used in subsequent experiments (Fig. 1).

Effects of icariin treatment on p-p38 and p-ERK1/2 expression in IL-1β-stimulated SW1353 cells. To analyze the effects of icariin on the activation of p38 and ERK1/2, the SW1353 cells were incubated in the presence or absence of 12 μg/ml of icariin, in addition to a p38 inhibitor (SB203580, 10 μM) and a ERK1/2 inhibitor (PD98059, 20 μM). After 1 h, the cells were stimulated with 10 ng/ml IL-1β. After 48 h, p-p38 and p-ERK1/2 were extracted and analyzed by western blot analysis. The expression levels of these 2 proteins were significantly elevated when the SW1353 cells were only treated with IL-1β (Fig. 2). By contrast, the levels of p-p38 decreased (Fig. 2A), while the p-ERK1/2 levels increased following treatment with icariin (Fig. 2B). In addition, western blot analysis revealed that the levels of these 2 proteins were inhibited to a greater extent by treatment with the corresponding signaling pathway-specific inhibitors than with icariin (Fig. 2). Furthermore, the difference in the expression levels was statistically significant ($P<0.05$).

mRNA expression of OPG, RANKL and RANK following treatment with signaling pathway inhibitors and/or icariin in IL-1β-stimulated SW1353 cells. In order to investigate the effects of icariin on OPG, RANKL and RANK expression, the SW1353 cells were treated with or without 12 μg/ml icariin for 1 h and then stimulated with 10 ng/ml IL-1β. Total RNA and cell extracts were collected 48 h later and the mRNA expression levels of OPG, RANKL and RANK levels determined

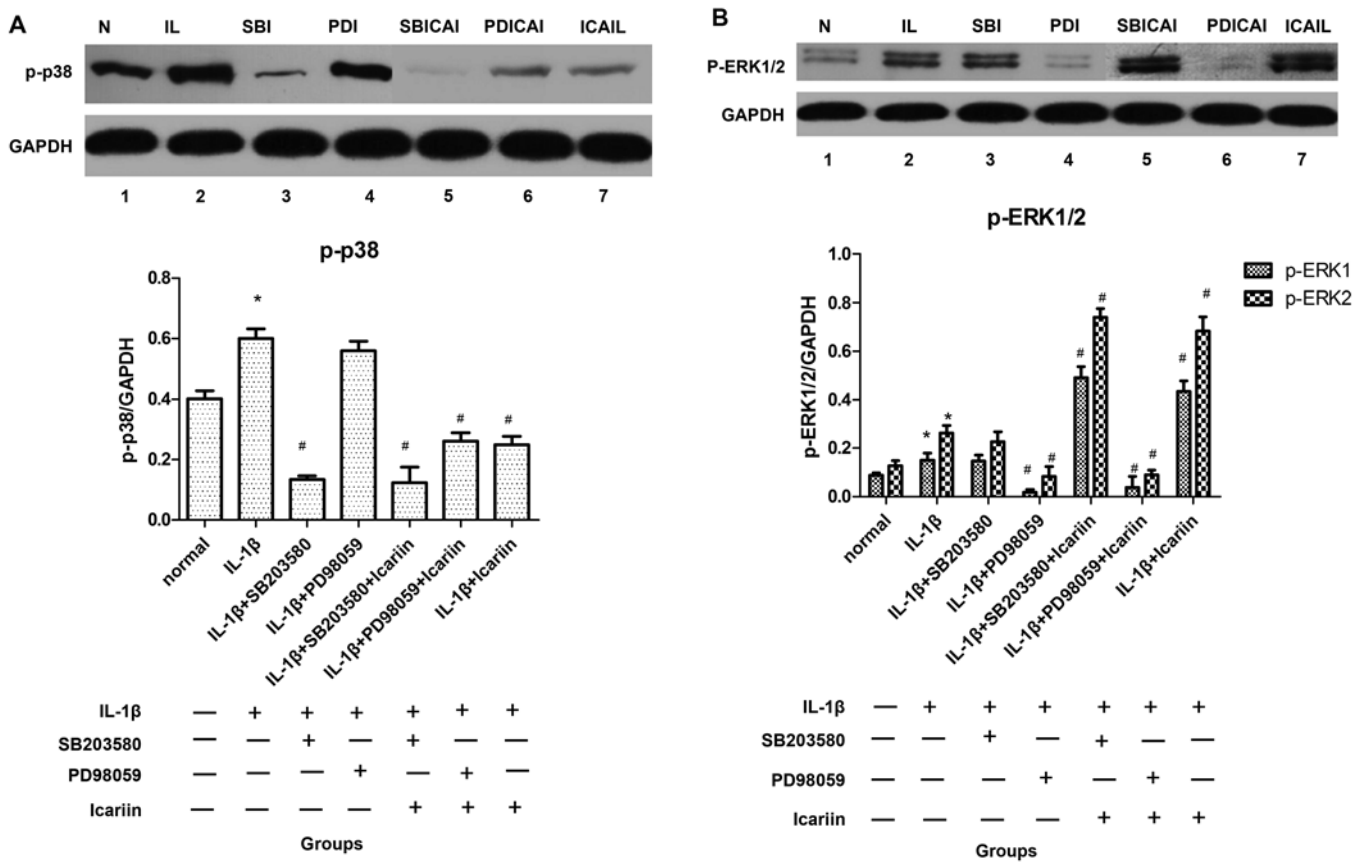

Figure 2. The use of signaling pathway-specific inhibitors to investigate the icariin-mediated protein expression of phosphorylated (p)-p38 and phosphorylated extracellular-signal-regulated kinase (p-ERK)1/2. Densitometric analysis of the protein bands detected revealed that the expressio of (A) p-p38 and (B) p-ERK1/2 was significantly inhibited by the relevant signaling pathway-specific inhibitor compared with the levels of these 2 proteins in interleukin (IL)-1β-stimulated cells treated only with icariin (P<0.05). Moreover, icariin treatment was associated with (B) an increased p-ERK1/2 level, but (A) a decreased p-p38 level (P<0.05). *P<0.05 compared with normal (N) groups, #P<0.05 compared with IL-1β group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

detected in the cell extracts by RT-qPCR. Treatment with icariin inhibited the increase in the mRNA expression of OPG, RANKL and RANK and the OPG/RANKL ratio which was induced in response to pre-treatment with IL-1β. To further investigate the possible regulatory mechanisms, inhibitors of MAPK signaling pathways were used. Treatment with icariin induced the most significant decrease in the mRNA expression of OPG, RANKL and RANK and in the OPG/RANKL ratio, followed by the p38 inhibitor (SB203580) and ERK1/2 inhibitor (PD98059). Treatment with icariin was associated with a more prominent inhibition of OPG, RANKL, RANK expression and the OPG/RANKL ratio than treatment with the p38 signaling pathway-specific inhibitor alone (Fig. 3).

Protein expression of OPG and RANKL following treatment with signaling pathway inhibitors and icariin in IL-1β-stimulated SW1353 cells. To investigate the effects of icariin on OPG and RANKL protein expression, the supernatants were collected and analyzed using the ELISA kit. Treatment with SB203580 (p38 inhibitor) markedly decreased OPG expression and the OPG/RANKL ratio and increased RANKL protein expression, whereas treatment with PD98059 (ERK inhibitor) had the opposite effect. Consistent with the aforementioned findings, it was demonstrated that treatment with icariin inhibited the increase in OPG and RANKL protein expression and the OPG/RANKL ratio which was induced in response to pre-treatment with IL-1β. In addition, icariin treatment was associated with a more prominent inhibition of OPG expression and the OPG/RANKL ratio than treatment with the p38 signaling pathway-specific inhibitor alone (Fig. 4).

Discussion

In the present study, the effects of treatment with icariin on OPG, RANKL and RANK expression in IL-1β-stimulated SW1353 chondrosarcoma cells were investigated. In addition, these effects were associated with those on the p38 and ERK1/2 signaling pathways induced by specific signaling pathway inhibitors. As a result, it was shown that the inhibition of the increase in the expression of OPG, RANKL and RANK and the OPG/RANKL ratio by icariin treatment was partly mediated by the downregulation in p38 and the upregulation of ERK1/2.

OA is a gradual and degenerative disease, characterized by inflammation and erosion of articular cartilage and subchondral bone remodeling. Moreover, available anti-osteoarthritic drug treatments seem to be limitedly effective in treating OA, apart from surgical therapie. One reason for this failure may be the multi-factorial and organic pathogenesis of this disease (34,35). It has been previously demonstrated that agents with multiple properties derived from natural resources (36) offer new treatment opportunities and are recommended for the treatment of OA (37).

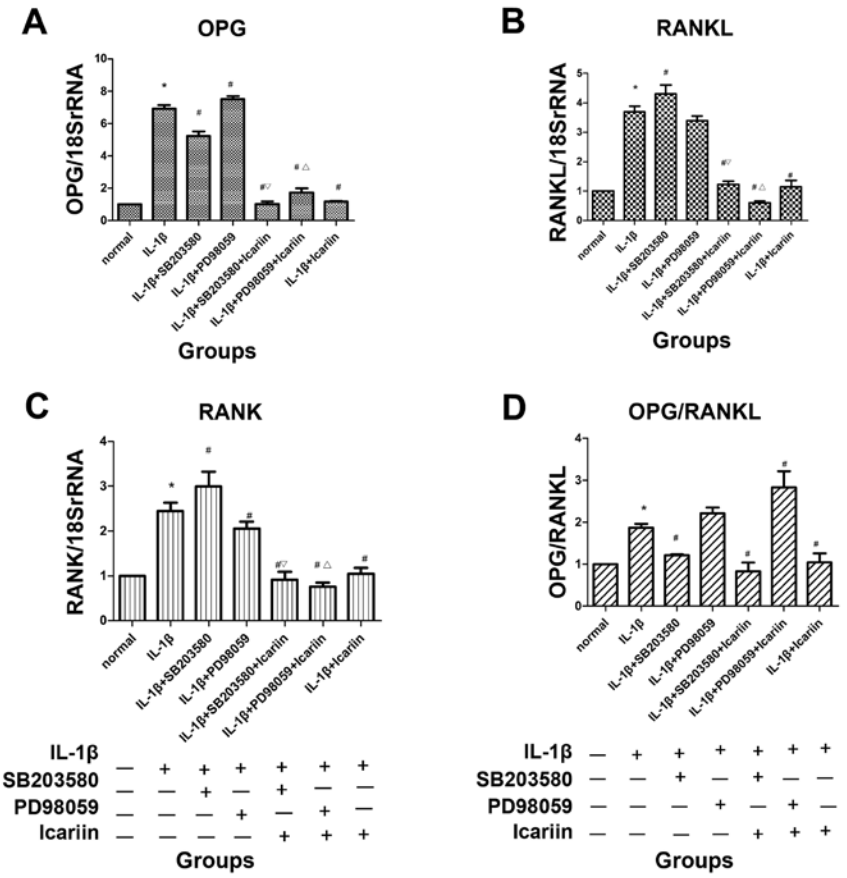

Figure 3. The use of signaling pathway-specific inhibitors to investigate the icariin-mediated mRNA expression of osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL) and receptor activator of nuclear factor kappa-B (RANK). Human SW1353 cells were pre-treated with 12 μg/ml icariin, 10 μM SB203580 (p38 inhibitor) or 20 μM PD98059 (extracellular signal-regulated kinase 1/2 inhibitor) for 1 h. Interleukin (IL)-1β (10 ng/ml) was then added to each set of cells. After 48 h, the mRNA levels of OPG, RANKL and RANK were detected by RT-qPCR. Data are expressed as the means ± standard deviation. *P<0.05, compared with the normal group; $^{\#}$P<0.05, compared with the IL-1β group; $^{\triangledown}$P<0.05, compared with the IL-1β + SB203580 group; $^{\triangle}$P<0.05, compared with the IL-1β + PD98059 group.

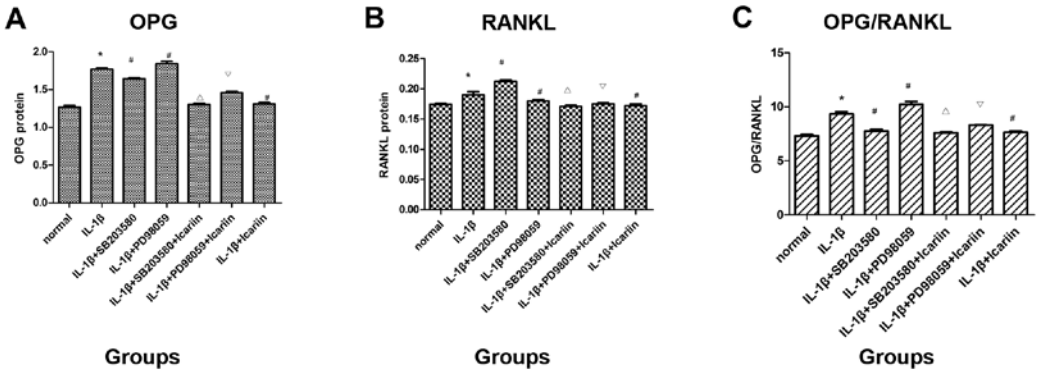

Figure 4. The use of signaling pathway-specific inhibitors to investigate the icariin-mediated protein expression osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B ligand (RANKL). Human SW1353 cells were pre-treated with 12 μg/ml icariin, 10 μM SB203580 (p38 inhibitor), or 20 μM PD98059 [extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor], for 1 h. Interleukin (IL)-1β (10 ng/ml) was then added to each set of cells. After 48 h, the levels of OPG and RANKL proteins were detected using ELISA. Data are expressed as the means ± standard deviation. *P<0.05, compared with the normal group; $^{\#}$P<0.05, compared with the IL-1β group; $^{\triangle}$P<0.05, compared with the IL-1β + SB203580 group; $^{\triangledown}$P<0.05, compared with the IL-1β + PD98059 group.

Epimedium pubescens is commonly used in traditional Chinese medicine for the nourishment of bone and the stimulation of gonadal functions. Icariin, as the main active compound, has been associated with anti-inflammatory, anti-apoptotic (22,26,27), anti-arthritic (in chondrocytes) (38) and anti-bone resorption (28,29) properties. It is widely accepted that icariin has estrogen-like and anti-osteoporotic activity and can be potentially used for the treatment of osteoporosis during the onset of primary OA (39). In a previous study, it was demonstrated that icariin protected chondrocytes from

damage by reducing the activity of nuclear factor (NF)-κB and increasing the expression of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (IκBα) (38). However, although it has been demonstrated that icariin suppresses the loss of bone mass and elevates the OPG/RANKL ratio (40), the regulation of OPG, RANKL and RANK expression by icariin in chondrocytes is not yet fully understood.

IL-1β, known as a pro-inflammatory cytokine and critical catabolic factor, induces the expression of other cytokines, such as IL-8 and prostaglandin E2, and plays an important role in OA (41). The pathological microenvironment of OA chondrocytes is usually mimicked *in vitro* with IL-1β (42). In previous studies, IL-1β was applied in order to create a cellular model of OA using SW1353 cells, and it was found to significantly increase the expression of OPG, RANKL and RANK and the OPG/RANKL ratio (15,43). By contrast, treatment with icariin was proved effective in conserving the chondrocytes *in vitro* by inhibiting the excessive increase in the expression of OPG, RANKL and RANK and the OPG/RANKL ratio.

It is well known that the OPG-RANKL-RANK system plays important roles in OA, with each factor appropriately expressed. The role of these these 3 factors in OA largely involves their ability to regulate bone metabolism. Their expression has have been found in chondrocytes (11). In particular, the overexpression of OPG induces an increase in MMP-13 and PAR-2 expression (11) rather than cell pro-proliferation under appropriate levels and induces OA with chondrocalcinosis (44). Additionally, RANKL stimulates osteoclastogenesis in growth plates (45). Of these factors, the OPG-RANKL-RANK system has been the most commonly studied, but OPG- and RANKL-specific inhibitors have not gained attention as regards cartilage and chondrocytes. Therefore, therapeutic strategies focusing on prophylactic agents applied for the regulation of the OPG-RANKL-RANK system in chondrocytes are required. The present study suggests that icariin treatment inhibits the expression of OPG, RANKL and RANK and the OPG/RANKL ratio induced by IL-1β in chondrocytes. Furthermore, this suppressive effect demonstrates the therapeutic potential of icariin for use in the treatment of OA.

The mechanisms through which icariin downregulates the expression of OPG, RANKL and RANK and the OPG/RANKL ratio remains unclear. However, an increasing number of studies has demonstrated that MAPK signaling pathways and the Wnt/β-catenin signaling pathway are involved in the IL-1β-induced expression of OPG, RANKL, and RANK and the OPG/RANKL ratio (7,46). Therefore, in the present study, the molecular mechanisms mediated by icariin that result in the inhibition of OPG, RANKL and RANK expression and the OPG/RANKL ratio were investigated. It was confirmed that MAPK signaling pathways are involved in chondrocytes stimulated with IL-1β and treated with icariin.

Consequently, the inhibition of cartilage degeneration and subchondral bone remodeling is a priority for the effective treatment of OA. Additionally, maintaining the OPG/RANKL ratio within the normal range is a main issue. In the present study, the p38 inhibitor, SB203580, was found to have a suppressive effect on elevated OPG levels and the OPG/RANKL ratio, but it increased RANK and RANK levels in IL-1β-stimulated SW1353 cells, while the ERK1/2 inhibitor, PD98059, had the opposite effect. However, while the inhibition of each pathway alone partly affected the expression of OPG, RANKL and RANK and the OPG/RANKL ratio, other pathways also participate in this process. Although treatment with icariin resulted in the marked activation of the ERK1/2 pathway and the inactivation of the p38 pathway, it also had a suppressive effect on the increased RANKL and RANK levels in IL-1β-stimulated SW1353 cells cultured with icariin. Therefore, apart from the p38 and ERK1/2 pathways, other pathways, such as the c-Jun N-terminal kinase (JNK) and Wnt/β-catenin signaling pathways, are involved in this process. The ability of icariin to modulate multiple pathways suggests that icarrin has synergistic effects, thus rendering it effective as an anti-arthritic herb extract compared to other specific inhibitors of signaling pathways. Furthermore, icariin has become an effective agent for pharmacological intervention against the progression of OA.

The results of this study indicate that icariin decreases the expression of OPG, RANKL and RANK and the OPG/RANKL ratio in IL-1β-stimulated chondrocytes. This is partly achieved by inhibiting the activation of the p38 and enhancing the activation of the ERK1/2 pathways. These findings provide valuable insight into the mechanistic details of OA, which may facilitate the development of original therapeutic strategies for this disease.

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