Luteolin Inhibits the Activity, Secretion and Gene Expression of MMP-3 in Cultured Articular Chondrocytes and Production of MMP-3 in the Rat Knee

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Abstract

We investigated whether luteolin affects the gene expression, secretion and activity of matrix metalloproteinase-3 (MMP-3) in primary cultured rabbit articular chondrocytes, as well as production of MMP-3 in the rat knee to evaluate the potential chondroprotective effects of luteolin. Rabbit articular chondrocytes were cultured in a monolayer and IL-1β-induced gene expression levels of MMP-3, MMP-1, MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS-4), ADAMTS-5 and type II collagen were measured by reverse transcription - polymerase chain reaction (RT-PCR). Effects of luteolin on interleukin-1β (IL-1β)-induced secretion and enzyme activity of MMP-3 in rabbit articular chondrocytes were investigated by western blot analysis and casein zymography, respectively. The effect of luteolin on MMP-3 protein production was also examined *in vivo*. The results were as follows: (1) luteolin inhibited the gene expression levels of MMP-3, MMP-1, MMP-13, ADAMTS-4 and ADAMTS-5. However, it increased the gene expression level of collagen in rabbit articular chondrocytes; (2) luteolin inhibited the secretion and activity of MMP-3; (3) luteolin inhibited *in vivo* production of MMP-3 protein. These results suggest that luteolin can regulate the gene expression, secretion and activity of MMP-3, by directly acting on articular chondrocytes.

Key Words: Osteoarthritis, Luteolin, Chondrocyte

INTRODUCTION

Osteoarthritis is the most common degenerative articular disease affecting millions of people, in particular the elderly. The synovial inflammation, formation of osteophyte, changes in subchondral bone and degeneration of articular cartilage are the major pathophysiologic features of osteoarthritis. The cause of osteoarthritis is unclear, and it involves multiple biochemical and mechanical factors. During the progression of osteoarthritis, the equilibrium between physiologic synthesis and degradation of articular cartilage is disrupted (Mankin, 1982; Aigner and McKenna, 2002).

It has been reported that the activation of degradative enzymes leads to the loss and degradation of proteoglycans and collagen in articular cartilage, and the matrix metalloproteinase (MMP) plays a pivotal role in the destruction of articular cartilage in patients who are suffering from osteoarthritis (Dean *et*

al., 1989; Kullich *et al.*, 2007). Among the various matrix metalloproteinase including collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9) and stromelysins (MMP-3, -7, -10 and -11) (Birkedal-Hansen *et al.*, 1993; Burrage *et al.*, 2006), MMP-3 degrades proteoglycans and activate procollagenase in articular cartilage (Garnero *et al.*, 2000; Lin *et al.*, 2004). In an experimental model of osteoarthritis, the role of MMP-3 in damage of articular cartilage was demonstrated. MMP-3 protein was reported to be expressed in the synovium and the surface of cartilage in the knee joints and in pannus-like tissue of patients with osteoarthritis (Okada *et al.*, 1992; Shibakawa *et al.*, 2003; Kobayashi *et al.*, 2005; Blom *et al.*, 2007).

Therefore, we suggest it is valuable to find the potential action of regulating (inhibiting) the activity, secretion and gene expression of MMP-3 by the natural products derived from medicinal plants used as empirical remedies for arthritis.

According to many reports, luteolin, a flavonoidal compound

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derived from diverse medicinal plants showing anti-inflammatory and anti-oxidative activities, has shown anti-inflammatory, anti-oxidative and anti-carcinogenic effects (Manju and Nalini, 2007; Jung *et al.*, 2012; Sun *et al.*, 2012). Luteolin and its analogues showed anti-arthritic effects in experimental models of rheumatoid arthritis and infectious arthritis (Lee and Kim, 2010; Lee and Han, 2011; Impellizzeri *et al.*, 2013). Furthermore, luteolin has been reported to affect the proliferation and function of synovial fibroblasts in rats (Hou *et al.*, 2009), and to suppress the activities of the other degradative enzymes, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 & 5 (Moncada-Pazos *et al.*, 2011). Choi and Lee reported that luteolin affected interleukin-1β (IL-1β)-induced production of MMPs and cytokines in the SW982 synovial sarcoma cell line (Choi and Lee, 2010).

However, to the best of our knowledge, there are no reports about the effects of luteolin on gene expression, secretion and activity of MMP-3, an articular cartilage-degradative enzyme that decomposes proteoglycans, in primary cultured rabbit articular chondrocytes or on production of MMP-3 in the rat knee. Therefore, we examined the effect of luteolin on IL-1β-induced gene expression, secretion and activity of MMP-3 *in vitro* and production of MMP-3 *in vivo*, in order to evaluate the potential chondroprotective effect of luteolin.

MATERIALS AND METHODS

Materials

All the chemicals and reagents used in this experiment including luteolin (purity: 95.0%) were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco BRL (Grand Island, NY, USA) and recombinant human IL-1β was purchased from R&D Systems (Minneapolis, MN, USA).

Primary cultures of chondrocytes from rabbit articular cartilage

Male New Zealand White Rabbits (Daehan Biolink, Seoul, Korea) were obtained from at 2 weeks of age. Animals were housed one per cage and provided with distilled water and food *ad libitum*. They were kept under a 12 h light/ dark cycle (lights on from 08:00 to 20:00) at constant temperature (22.5°C) and humidity (55%). Animals were cared through all of the experimental procedures in accordance with the Guide for the Care and Use of Laboratory Animals regulated by Chungnam National University, Daejeon, Korea. Rabbit articular chondrocytes were isolated from the tibial plateaus and femoral condyle in cartilage of the knee joints. Cartilage was washed in phosphate-buffered saline (PBS) and minced into pieces measuring about 2 mm³. Cartilage tissue was digested for 4 h with 0.2% type II collagenase at 37°C. After collection of individual cells by brief centrifugation, the cells were transferred to 100 mm culture dishes (seeding density: 10^5 cells/cm²) in 12 mL DMEM supplemented with 10% fetal bovine serum (FBS), in the presence of penicillin (100 units/mL) and streptomycin (100 μg/mL). Cells were cultured at 37°C in a humidified, 5% CO_2/95% air, water-jacketed incubator (Sanyo, Tokyo, Japan). The medium was replaced every other day.

Treatment of cells with luteolin and the other natural products

Chondrocytes were seeded on 6-well culture plates at a density of 10^5 cells/cm². After 2 days in monolayer culture, the cells were incubated for 2 h in growth medium with 1, 10, 50, or 100 μM of luteolin, fisetin, quercetin or ferulic acid, respectively, followed by incubation in the presence or absence of IL-1β (10 ng/mL) for 24 h. Luteolin, fisetin, quercetin or ferulic acid was dissolved in dimethylsulfoxide (DMSO), diluted in PBS, and administerted in culture medium (final concentrations of DMSO were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% DMSO in medium did not affect gene expression, secretion and activity of MMP-3 in primary cultured chondrocytes. The supernatant was collected, centrifuged, and both cell and supernatant fractions were stored at -80°C until use.

Cytotoxicity assay

Chondrocytes were seeded at a density of $2×10^5$/mL (0.1 mL/well) in a 96-well microtiter plate, and allowed to attach for 24 h to keep the log phase growth at the time of drug treatment. Luteolin, fisetin, quercetin or ferulic acid was dissolved in DMSO, and administered in DMEM supplemented with 10% FBS (final concentrations of DMSO were under 0.5%). 0.5% DMSO alone did not affect the proliferation of chondrocytes. After incubation with the indicated drug concentrations for 72 h, cell proliferation was determined using the sulforhodamine B (SRB) assay (Skehan *et al.*, 1990).

Isolation of total RNA and RT-PCR

Total RNA was isolated from chondrocytes using Easy-BLUE Extraction Kit (INTRON Biotechnology, Kyung-ki-do, Korea) and reverse transcribed by using AccuPower RT Premix (BIONEER Corporation, Daejeon, Korea) according to the manufacturer's instructions. 2 μg of total RNA was primed with 1 μg of oligo (dT) in a final volume of 30 μL (RT reaction). 2 μL of RT reaction product was PCR amplified in 20 μL using Thermoprime Plus DNA Polymerase (ABgene, Rochester, NY, USA). PCR was performed with the following primers: MMP-3 (5′ATG GAC CTT CTT CAG CAA 3′; 5′TCA TTA TGT CAG CCT CTC 3′); MMP-13 (5′AGG AGC ATG GCG ACT TCT AC 3′; 5′TAA AAA CAG CTC CGC ATC AA 3′); MMP-1 (5′TCA GTT CGT CCT CAC TCC AG 3′; 5′TTG GTC CAC CTG TCA TCT TC 3′); ADAMTS-4 (5′CAA GGT CCC ATG TGC AAC GT 3′; 5′CAT GTG CCA CCA CCA GTG TCT 3′); ADAMTS-5 (5′TGT CCT GCCAGC GGATGT 3′; 5′ACG GAA TTA CTG TAC GGC CTA CA 3′); type II collagen (5′AAC ACT GCC AAC GTC CAG AT 3′; 5′CTG ACG CAC GGT ATA GGT GA 3′). As quantitative controls, primers for GAPDH (5′ACT GGC GTC TTC ACC ACC AT 3′; 5′AAG GCC ATG CCA GTG AGC TT 3′) were used. The PCR products increased as the concentration of RNA increased. The amplified fragment sizes were 350 base pairs (bp) for MMP-3, 458 bp for MMP-13, 300 bp for MMP-1, 90 bp for ADAMTS-4, 110 bp for ADAMTS-5, 220 bp for type II collagen and 400 bp for GAPDH. After PCR, 15 μL of PCR products were subjected to 2% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator (Moon *et al.*, 2011).

Western blot analysis for measuring secretion level of MMP-3 in culture supernatant

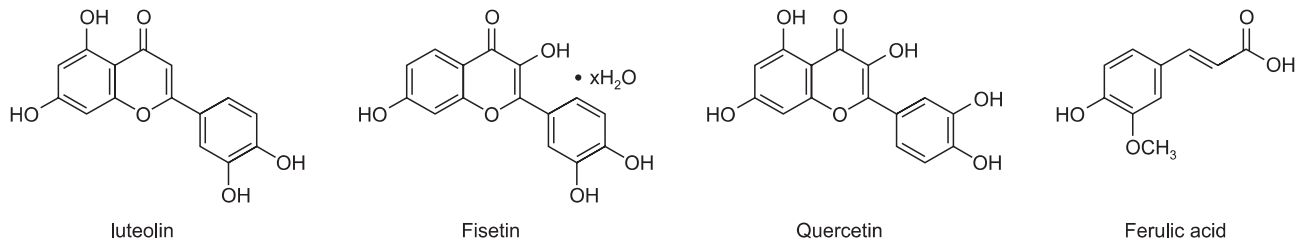The Bradford assay was used to measure protein concen-

Fig. 1. Chemical structure of luteolin and luteolin-related natural products.

trations in culture supernatants to ensure consistent weight of protein samples subjected to electrophoresis. Culture supernatant samples containing MMP-3 proteins (50 μg each) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Blots were blocked using 5% skim milk in Tris-buffered saline /Tween 20 (TBS-T) and probed overnight with MMP-3 antibody in blocking buffer at 4ᵒC. Antibody against MMP-3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were washed with TBS-T and then probed for 1 h with a secondary antibody conjugated with horseradish peroxidase (Calbiochem, La Jolla, CA, USA). After 4 intensive washes with TBS-T, immunoreactive bands were detected using an enhanced chemiluminescence kit (Pierce ECL western blotting substrate, Thermo Scientific, Waltham, MA, USA).

Casein zymography to measure the activity of MMP-3

A modified casein-substrate zymography was carried out using culture supernatants from chondrocytes pretreated for 2 h with luteolin and stimulated for 24 h with IL-1β in DMEM containing 0.5% FBS. The Bradford assay was used to measure protein concentrations in culture media to ensure consistency across samples. Samples were electrophoresed at 4ᵒC in a 10% SDS gel containing 0.1% casein. After the electrophoresis, gels were washed with 10 mM Tris-HCl (pH 8.0) containing 2.5 % Triton X-100. Next, the gels were incubated at 37ᵒC for 48 h in 50 mM Tris-HCl (pH 8.0) containing 1% Triton X-100, 0.2 M NaCl and 5 mM $CaCl_2$. Finally, gels were stained with 1% Coomassie Brilliant Blue, destained, and photographs were taken (Moon et al., 2011).

In vivo experiments

Male Sprague-Dawley rats (Daehan Biolink, Seoul, Korea) weighing 200-210 g were used to investigate the effect of luteolin on production of MMP-3 in articular cartilage in vivo. Animals were housed 5 per cage, and provided with distilled water and food ad libitum, and kept under a 12 h light/ dark cycle (lights on from 08:00 to 20:00) at constant temperature (22.5ᵒC) and humidity (55%). Animals were cared through all of the experimental procedures in accordance with the Guide for the Care and Use of Laboratory Animals regulated by Chungnam National University, Daejeon, Korea. Rats were randomly divided into 4 groups as follows: control, IL-1β only, 50 μM luteolin plus IL-1β, or 100 μM luteolin plus IL-1β. Rats were anesthetized with vaporized diethyl ether, and those from the 50 μM luteolin plus IL-1β and 100 μM luteolin plus IL-1β treatment groups received a 30 μL injection of 50 μM or 100 μM luteolin, respectively, into the right knee joint. After 3 h, rats from the IL-1β only group, the 50 μM luteolin plus IL-

1β group and the 100 μM luteolin plus IL-1β group received a 30 μL injection of 20 ng IL-1β in sterile PBS into the right knee joint. Rats from the control group were injected with 30 μL of sterile PBS (Hu et al., 2011). Rats were euthanized with CO_2 asphyxiation72 h after injections. Articular cartilage (tibial plateau and femoral condyle) was isolated from each animal, homogenized, and prepared for measuring the level of production of MMP-3 protein by western blot analysis. Tissue lysates from articular cartilage homogenates containing MMP-3 protein (50 μg each) were subjected to 10% SDS-PAGE and transferred onto a PVDF membrane. Blots were blocked with 5% skim milk in Tris-buffered saline/Tween 20 (TBS-T) and probed with MMP-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer overnight at 4ᵒC. Membranes were washed with TBS-T and probed for 1 h with a secondary antibody conjugated with horseradish peroxidase (Calbiochem, La Jolla, CA, USA). After 4 intensive washes with TBS-T, immunoreactive bands were detected using an enhanced chemiluminescence kit (Pierce ECL western blotting substrate, Thermo Scientific, Waltham, MA, USA).

RESULTS

Effect of luteolin, quercetin, fisetin, or ferulic acid on MMP-3 gene expression in rabbit chondrocytes

To compare the potency of activity on MMP-3 gene expression, effect on MMP-3 gene expression was examined by pretreatment of quercetin, fisetin or ferulic acid - similar flavonoidal and related compounds with luteolin - in addition to luteolin (Fig. 1). As shown in Fig. 2, luteolin inhibited IL-1β-induced MMP-3 gene expression. However, quercetin, fisetin, or ferulic acid did not affect MMP-3 gene expression.

Effect of luteolin, quercetin, fisetin or ferulic acid on proliferation of rabbit chondrocytes (cytotoxicity assay)

As can be seen in Fig. 3, luteolin, quercetin, fisetin or ferulic acid showed no significant cytotoxicity at the concentrations of 1, 10, 50, and 100 μM. The numbers of cells in luteolin-treated cultures were 100 ± 0%, 97 ± 9%, 98 ± 13%, 105 ± 7%, and 89 ± 14% for control, 1, 10, 50, and 100 μM luteolin, respectively. The numbers of cells in fisetin-treated cultures were 100 ± 13%, 86 ± 19%, 90 ± 16%, 94 ± 17%, and 92 ± 8% for control, 1, 10, 50, and 100 μM fisetin, respectively. The numbers of cells in quercetin-treated cultures were 100 ± 8%, 109 ± 10%, 85 ± 18%, 88 ± 12%, and 103 ± 11% for control, 1, 10, 50, and 100 μM quercetin, respectively. The numbers of cells in ferulic acid-treated cultures were 100 ± 11%, 83 ± 19%, 104 ± 8%, 106 ± 11%, and 85 ± 13% for control, 1, 10, 50, and 100 μM ferulic acid, respectively.
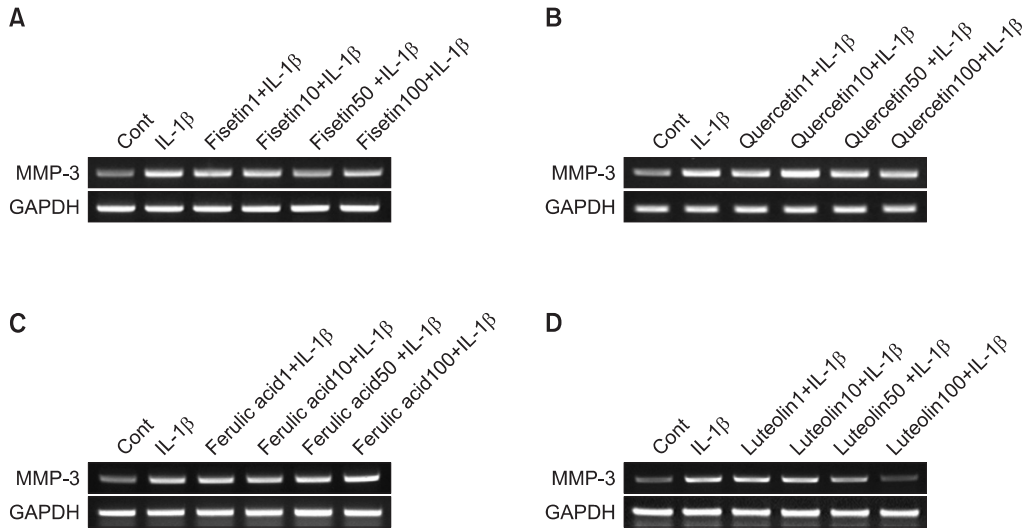
A

B

C

D

Fig. 2. Effect of luteolin, quercetin, fisetin, or ferulic acid on MMP-3 gene expression in rabbit chondrocytes. Primary cultured rabbit articular chondrocytes were pretreated with varying concentrations (1, 10, 50, and 100 μM) of luteolin, quercetin, fisetin or ferulic acid for 2 h and then stimulated with IL-1β (10 ng/mL) for 24 h. MMP-3 gene expression level was measured by RT-PCR. Three independent experiments were performed and representative data were shown (cont: control, concentration unit is μM).
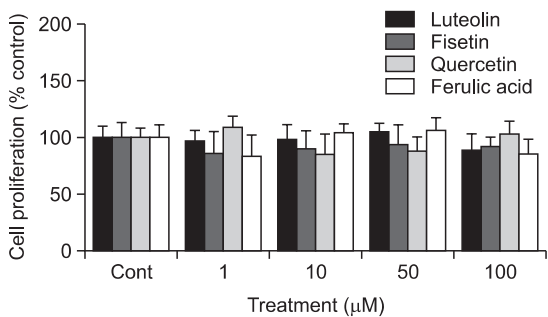

Fig. 3. Effect of luteolin, quercetin, fisetin, or ferulic acid on proliferation of rabbit chondrocytes. Chondrocytes were incubated for 72 h in the presence of varying concentrations of luteolin, quercetin, fisetin or ferulic acid. Cell viability was determined using SRB assay as described in Materials and methods. Each bar represents a mean ± S.E.M. of three independent experiments in comparison with that of the control set at 100%.

Fig. 4. Effect of luteolin on gene expression of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, or type II collagen in rabbit chondrocytes. Primary cultured rabbit articular chondrocytes were pretreated with varying concentrations (1, 10, 50, and 100 μM) of luteolin for 2 h and then stimulated with IL-1β (10 ng/mL) for 24 h. The gene expression level of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5 or collagen type II was measured by RT-PCR. Three independent experiments were performed and representative data were shown (cont: control, concentration unit is μM).

Effect of luteolin on gene expression of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, or type II collagen in rabbit chondrocytes

As shown in Fig. 4, luteolin also inhibited IL-1β-induced gene expression of MMP-1, MMP-13, ADAMTS-4, or ADAMTS-5, the other degradative enzymes related to the destruction of articular cartilage. Furthermore, luteolin showed the possible chondroprotective effect by abolishing IL-1β-induced deficits in type II collagen gene expression, in rabbit chondrocytes.

Effect of luteolin on IL-1β-induced secretion of MMP-3 from rabbit articular chondrocytes

If luteolin can affect the MMP-3 gene expression at the transcriptional level, it should be investigated whether luteolin affects IL-1β-induced secretion of MMP-3 proteins from rabbit articular chondrocytes. As can be seen in Fig. 5, stimulation with IL-1β (10 ng/mL) increased secretion level of MMP-3
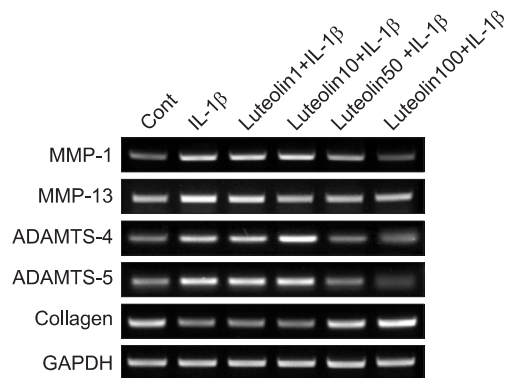
from chondrocytes. Luteolin inhibited IL-1β-induced secretion of MMP-3. This result means that luteolin can control the steps of protein synthesis and secretion of MMP-3.

Effect of luteolin on MMP-3 activity in rabbit articular chondrocytes

After treatment with IL-1β for 24 h, culture supernatants from rabbit articular chondrocytes were analyzed for caseinase activity by casein zymography, to investigate the effect of luteolin on the activity of MMP-3, which is known to degrade proteoglycans, one of the two major matrix components of cartilage. As can be seen in Fig. 6, IL-1β increased the activity of MMP-3 in rabbit articular chondrocytes, and this effect was
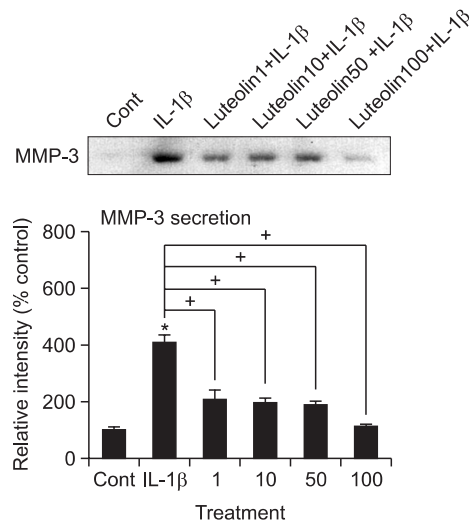
Fig. 5. Effect of luteolin on IL-1β-induced secretion of MMP-3 in rabbit articular chondrocytes. Primary cultured rabbit articular chondrocytes were pretreated with varying concentrations (1, 10, 50, and 100 μM) of luteolin for 2 h and then stimulated with IL-1β (10 ng/mL) for 24 h. Culture supernatants were collected for measurement of the levels of produced and secreted MMP-3 by western blot analysis. Three independent experiments were performed and representative data were shown (cont: control, concentration unit is μM).
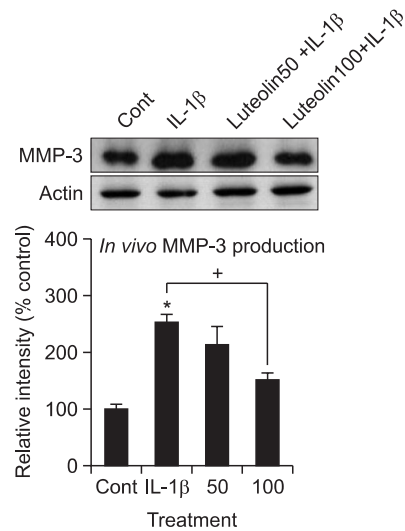

Fig. 6. Effect of luteolin on the enzyme activity of MMP-3 in rabbit articular chondrocytes. Primary cultured rabbit articular chondrocytes were pretreated with varying concentrations (1, 10, 50, and 100 μM) of luteolin for 2 h and then stimulated with IL-1β (10 ng/mL) for 24 h. Culture supernatants were collected for measurement of the enzyme activity of MMP-3 by casein zymography. Three independent experiments were performed and representative data were shown (cont: control, concentration unit is μM).

inhibited by pretreatment with luteolin.

Effect of luteolin on production of MMP-3 *in vivo*

To examine whether luteolin shows the potential effect *in vivo*, we checked the effect of intraarticular injection of luteolin

Fig. 7. Effect of luteolin on production of MMP-3 *in vivo*. The knee joint of rats were pretreated with 50 μM or 100 μM of luteolin for 3 h and stimulated with IL-1β (20 ng/30 μL) for 72 h, by intraarticular injection. Tissue lysates from articular cartilage homogenates were collected for measurement of the level of produced MMP-3 *in vivo*, by western blot analysis. Equal protein loading was evaluated by β-actin levels, and representative data were shown (cont: control, concentration unit is μM).
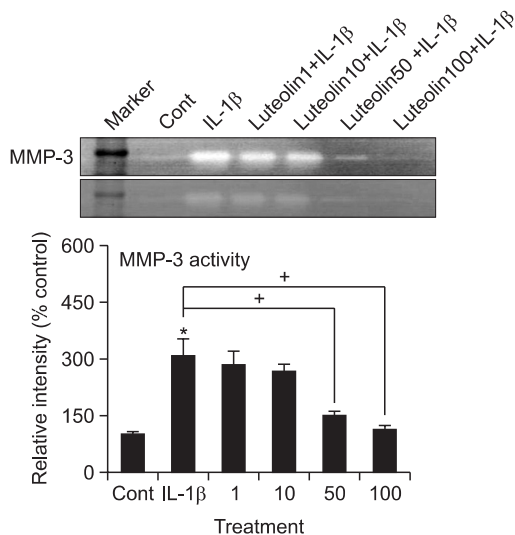
into the knee joint of rats on *in vivo* IL-1β-stimulated production of MMP-3 from articular cartilage tissues. As can be seen in Fig. 7, stimulation with IL-1β (20 ng/ 30 μL) increased the level of MMP-3 production from articular cartilage tissues. However, luteolin inhibited IL-1β-induced production level of MMP-3 *in vivo*, at the highest concentration tested.

DISCUSSION

Trial to develop a useful pharmacological tool for regulating the broken equilibrium between synthesis and degradation of articular cartilage during the progression of osteoarthritis can be a promising approach to the effective control of osteoarthritis. As aforementioned in introduction, luteolin, a flavonoidal compound derived from diverse medicinal plants showing anti-inflammatory and anti-oxidative activities, has shown anti-inflammatory, anti-oxidative, anti-carcinogenic and anti-arthritic effects in experimental models of rheumatoid arthritis and infectious arthritis (Manju and Nalini, 2007; Hou *et al.*, 2009; Lee and Kim, 2010; Choi and Lee, 2010; Lee and Han, 2011; Moncada-Pazos *et al.*, 2011; Sun *et al.*, 2012; Jung *et al.*, 2012; Impellizzeri *et al.*, 2013).

However, to the best of our knowledge, there have been no reports on the effects of luteolin on gene expression, secretion, and activity of MMP-3, which is an articular cartilage-degradative enzyme that decomposes proteoglycans, in primary cultured rabbit articular chondrocytes and production of MMP-3 in the rat knee.

Although osteoarthritis can be defined as a non-inflammatory disease in general, its development and progression is attributed to low-grade inflammation in intraarticular site, as well as to various inflammatory cytokines in articular tissues and fluids that are produced by chondrocytes and/or interact

with chondrocytes (Bonnet and Walsh, 2005; Kobayashi *et al.*, 2005; Loeser, 2006; Goldring *et al.*, 2008). IL-1β, an inflammatory cytokine produced by the cells in articular tissues including chondrocytes, can increase the gene expression level of MMPs and stimulate the progression of osteoarthritis. IL-1β plays a pivotal role in the initiation and progression of destruction of articular cartilage by inhibiting synthesis of collagen and stimulating MMPs expressions (Aida *et al.*, 2005; Kobayashi *et al.*, 2005; Pantsulaia *et al.*, 2010).

Among multiple MMPs in articluar tissues, MMP-3 has been reported to play an important pathophysiological role in osteoarthritis, by degrading components of the extracellular matrix, such as proteoglycans. MMP-3 levels were increased more than MMP-1 levels in patients suffering from osteoarthritis in knee joints compared to the control group (Garnero *et al.*, 2000; Lijnen, 2002).

As can be seen in results, we found that luteolin inhibited IL-1β-induced MMP-3 gene expression. However, quercetin, fisetin, or ferulic acid-similar flavonoidal and/or related compounds with luteolin-did not affect MMP-3 gene expression (Fig. 1, 2). This result suggests that luteolin suppresses the gene expression of MMP-3 at the transcriptional level.

In addition to MMP-3, MMP-1 and MMP-13 have been reported to play important roles in the destruction of cartilage in osteoarthritis. MMP-1 is a commonly detected metalloproteinase in synovial fluid from patients suffering from osteoarthritis (Freemont *et al.*, 1997; Goupille *et al.*, 1998; Kanyama *et al.*, 2000; Yoshihara *et al.*, 2000; Neuhold *et al.*, 2001; Jo *et al.*, 2003; Little *et al.*, 2009). Another degradative enzyme, ADAMTS-4, was reported to be a major aggrecanase in cartilage of mouse and ADAMTS-5 was known to be important in cartilage matrix destruction during osteoarthritis (Stanton *et al.*, 2005; Echtermeyer *et al.*, 2009).

Therefore, we examined the effect of luteolin on gene expression of MMP-1, MMP-13, ADAMTS-4, or ADAMTS-5. As can be seen in Fig. 4, luteolin inhibited IL-1β-induced gene expression of MMP-1, MMP-13, ADAMTS-4 or ADAMTS-5. At the same time, luteolin restored the gene expression of type II collagen that had been inhibited by IL-1β, in rabbit chondrocytes. Thus, the chondroprotective effect of luteolin are supported by its regulation of the gene expression of diverse proteases involved in the destruction of articular cartilage in osteoarthritis, as well as by its promotion of the expression of type II collagen at the transcriptional level.

Next, if luteolin can affect the MMP-3 gene expression at the transcriptional level, it should be investigated whether luteolin affects IL-1β-induced secretion of MMP-3 protein from rabbit articular chondrocytes. As can be seen in Fig. 5, stimulation with IL-1β increased the secretion level of MMP-3 from chondrocytes. Luteolin inhibited IL-1β-induced secretion level of MMP-3. This result shows that luteolin can control the steps of protein synthesis and secretion of MMP-3.

To investigate the regulatory effect of luteolin on the enzyme activity of MMP-3 secreted, culture supernatants from rabbit articular chondrocytes stimulated with IL-1β for 24 h were analyzed for caseinase activity by casein zymography. As shown in Fig. 6, when rabbit articular chondrocytes were stimulated with IL-1β, the enzyme activity of MMP-3 was increased. This effect was inhibited by pretreatment with luteolin. This result suggests that luteolin can suppress the proteolytic activity of MMP-3, in osteoarthritic articular cartilage tissues.

Finally, we examined the effect of intraarticular injection of luteolin into the knee joint of rats on *in vivo* IL-1β-stimulated production of MMP-3 in articular cartilage tissue. As shown in Fig. 7, luteolin inhibited IL-1β-stimulated production level of MMP-3 in articular cartilage tissue. This result shows that, in addition to its *in vitro* effects, luteolin exerts chondroprotective effects *in vivo* when administered via intraarticular injection.

Taken together, luteolin showed the chondroprotective effect by regulating the gene expression, secretion and activity of MMP-3, directly acting on articular chondrocytes. It can be developed as a novel agent for controlling cartilage damage in osteoarthritis via intraarticular administration, through future studies.

REFERENCES

Aida, Y., Maeno, M., Suzuki, N., Shiratsuchi, H., Motohashi, M. and Matsumura, H. (2005) The effect of IL-1beta on the expression of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in human chondrocytes. *Life Sci.* **77**, 3210-3221.

Aigner, T. and McKenna, L. (2002) Molecular pathology and pathobiology of osteoarthritic cartilage. *Cell Mol. Life Sci.* **59**, 5-18.

Birkedal-Hansen, H., Moore, W. G., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A. and Engler, J. A. (1993) Matrix metalloproteinases: a review. *Crit. Rev. Oral Biol. Med.* **4**, 197-250.

Blom, A. B., van Lent, P. L., Libregts, S., Holthuysen, A. E., van der Kraan, P. M., van Rooijen, N. and van den Berg, W. B. (2007) Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase-3. *Arthritis Rheum.* **56**, 147-157.

Bonnet, C. S. and Walsh, D. A. (2005) Osteoarthritis, angiogenesis and inflammation. *Rheumatology (Oxford)* **44**, 7-16.

Burrage, P. S., Mix, K. S. and Brinckerhoff, C. E. (2006) Matrix metalloproteinases: role in arthritis. *Front. Biosci.* **11**, 529-543.

Choi, E. M. and Lee, Y. S. (2010) Luteolin suppresses IL-1beta-induced cytokines and MMPs production via p38 MAPK, JNK, NF-kappaB and AP-1 activation in human synovial sarcoma cell line, SW982. *Food Chem. Toxicol.* **48**, 2607-2611.

Dean, D. D., Martel-Pelletier, J., Pelletier, J. P., Howell, D. S. and Woessner, J. F. Jr. (1989) Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. *J. Clin. Invest.* **84**, 678-685.

Echtermeyer, F., Bertrand, J., Dreier, R., Meinecke, I., Neugebauer, K., Fuerst, M., Lee, Y. J., Song, Y. W., Herzog, C., Theilmeier, G. and Pap, T. (2009) Syndecan-4 regulates ADAMTS-5 activation and cartilage breakdown in osteoarthritis. *Nat. Med.* **15**, 1072-1076.

Freemont, A. J., Hampson, V., Tilman, R., Goupille, P., Taiwo, Y. and Hoyland, J. A. (1997) Gene expression of matrix metalloproteinases 1, 3, and 9 by chondrocytes in osteoarthritic human knee articular cartilage is zone and grade specific. *Ann. Rheum. Dis.* **56**, 542-549.

Garnero, P., Rousseau, J. C. and Delmas. P. D. (2000) Molecular basis and clinical use of biochemical markers of bone, cartilage, and synovium in joint diseases. *Arthritis Rheum.* **43**, 953-968.

Goldring, M. B., Otero, M., Tsuchimochi, K., Ijiri, K. and Li, Y. (2008) Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. *Ann. Rheum. Dis.* **67(Suppl 3)**, iii75-iii82.

Goupille, P., Jayson, M. I., Valat, J. P. and Freemont, A. J. (1998) Matrix metalloproteinases: the clue to intervertebral disc degeneration. *Spine (Phila Pa 1976)* **23**, 1612-1626.

Hou, Y., Wu, J., Huang, Q. and Guo, L. (2009) Luteolin inhibits proliferation and affects the function of stimulated rat synovial fibroblasts. *Cell Biol. Int.* **33**,135-147.

Hu, P. F., Chen, W. P., Tang, J. L., Bao, J. P. and Wu, L .D. (2011) Protective effects of berberine in an experimental rat osteoarthritis model. *Phytother. Res.* **25**, 878-885.

Impellizzeri, D., Esposito, E., Paola, R. Di., Ahmad, A., Campolo, M., Pel, i A., Morittu, V. M., Britti, D. and Cuzzocrea, S. (2013) Palmitoylethanolamide and luteolin ameliorate development of arthritis

caused by injection of collagen type II in mice. *Arthritis Res.Ther.* **15**, R192.

Jo, H., Park, J. S., Kim, E. M., Jung, M. Y., Lee, S. H., Seong, S. C., Park, S. C., Kim, H. J. and Lee, M. C. (2003) The in vitro effects of dehydroepiandrosterone on human osteoarthritic chondrocytes. *Osteoarthritis Cartilage* **11**, 585-594.

Jung, H. A., Jin, S. E., Min, B. S., Kim, B. W. and Choi, J. S. (2012) Anti-inflammatory activity of Korean thistle Cirsium maackii and its major flavonoid, luteolin 5-O-glucoside. *Food Chem. Toxicol.* **50**, 2171-2179.

Kanyama, M., Kuboki, T. Kojima, S., Fujisawa, T., Hattori, T., Takigawa, M. and Yamashita, A. (2000) Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids of patients with temporomandibular joint osteoarthritis. *J. Orofac. Pain* **14**, 20-30.

Kobayashi, M., Squires, G. R., Mousa, A., Tanzer, M., Zukor, D. J., Antoniou, J., Feige, U. and Poole, A. R. (2005) Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. *Arthritis Rheum.* **52**, 128-135.

Kullich, W., Fagerer, N. and Schwann, H. (2007) Effect of the NSAID nimesulide on the radical scavenger glutathione S-transferase in patients with osteoarthritis of the knee. *Curr. Med. Res. Opin.* **23**, 1981-1986.

Lee, J. H. and Han, Y. (2011) Antiarthritic effect of lonicerin on Candida albicans arthritis in mice. *Arch. Pharm. Res.* **34**, 853-859.

Lee, J. H. and Kim, G. H. (2010) Evaluation of antioxidant and inhibitory activities for different subclasses flavonoids on enzymes for rheumatoid arthritis. *J. Food Sci.* **75**, H212-H217.

Lijnen, H. R. (2002) Matrix metalloproteinases and cellular fibrinolytic activity. *Biochemistry (Mosc)* **67**, 92-98.

Lin, P. M., Chen, C. T. and Torzilli, P. A. (2004) Increased stromelysin-1 (MMP-3), proteoglycan degradation (3B3- and 7D4) and collagen damage in cyclically load-injured articular cartilage. *Osteoarthritis Cartilage* **12**, 485-496.

Little, C. B., Barai, A. Burkhardt, D., Smith, S. M., Fosang, A. J., Werb, Z., Shah, M. and Thompson, E. W. (2009) Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development. *Arthritis Rheum.* **60**, 3723-3733.

Loeser, R. F. (2006) Molecular mechanisms of cartilage destruction: mechanics, inflammatory mediators and aging collide. *Arthritis Rheum.* **54**, 1357-1360.

Manju, V. and Nalini, N. (2007) Protective role of luteolin in 1,2-dimethylhydrazine induced experimental colon carcinogenesis. *Cell Biochem. Funct.* **25**, 189-194.

Mankin, H. J. (1982) The response for articular cartilage to mechanical injury. *J. Bone Joint Surg. Am.* **64**, 460-466.

Moncada-Pazos, A., Obaya, A. J., Viloria, C. G., López-Otín, C. and Cal, S. (2011) The nutraceutical flavonoid luteolin inhibits AD-AMTS-4 and ADAMTS-5 aggrecanase activities. *J. Mol. Med (Berl).* **89**, 611-619.

Moon, P. D., Jeong, H. S., Chun, C. S. and Kim, H. M. (2011) Bae-kjeolyusin-tang and its active component berberine block the release of collagen and proteoglycan from IL-1β-stimulated rabbit cartilage and down-regulate matrix metalloproteinases in rabbit chondrocytes. *Phytother. Res.* **25**, 844-850.

Neuhold, L. A., Killar, L. Zhao, W., Sung, M. L., Warner, L., Kulik, J., Turner, J., Wu, W., Billinghurst, C., Meijers, T., Poole, A. R., Babij, P. and DeGennaro, L. J. (2001) Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. *J. Clin. Invest.* **107**, 35-44.

Okada, Y., Shinmei, M., Tanaka, O., Naka, K., Kimura, A., Nakanishi, I., Bayliss, M.T., Iwata, K. and Nagase, H. (1992) Localization of matrix metalloproteinase 3 (stromelysin) in osteoarthritic cartilage and synovium. *Lab. Invest.* **66**, 680-690.

Pantsulaia, I., Kalichman, L. and Kobyliansky, E. (2010) Association between radiographic hand osteoarthritis and RANKL, OPG and inflammatory markers. *Osteoarthritis Cartilage* **18**, 1448-1453.

Shibakawa, A., Aoki, H., Masuko-Hongo, K., Kato, T., Tanaka, M., Nishioka, K. and Nakamura, H. (2003) Presence of pannus-like tissue on osteoarthritic cartilage and its histological character. *Osteoarthritis Cartilage* **11**, 133-140.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S. and Boyd, M. R. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **82**, 1107-1112.

Stanton, H., Rogerson, F. M. East, C. J., Golub, S. B., Lawlor, K. E., Meeker, C. T., Little, C. B., Last, K., Farmer, P. J., Campbell, I. K., Fourie, A. M. and Fosang, A. J. (2005) ADAMTS-5 is the major aggrecanase in mouse cartilage in vivo and in vitro. *Nature.* **434**, 648-652.

Sun, G. B., Sun, X., Wang, M., Ye, J. X., Si, J. Y., Xu, H. B., Meng, X. B., Qin, M., Sun, J., Wang, H. W. and Sun, X. B. (2012) Oxidative stress suppression by luteolin-induced heme oxygenase-1 expression. *Toxicol. Appl. Pharmacol.* **265**, 229-240.

Yoshihara, Y., Nakamura, H. bata, K., Yamada, H., Hayakawa, T., Fujikawa, K. and Okada, Y. (2000) Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. *Ann. Rheum. Dis.* **59**, 455-461.