Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Oleanolic acid (OA) regulates inflammation and cellular dedifferentiation of chondrocytes via MAPK signaling pathways

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Received June 26, 2016; **Accepted** March 25, 2017; **Published** March 31, 2017

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Doi: http://dx.doi.org/10.14715/cmb/2017.63.3.3

Abstract: Oleanolic acid (OA) is a bioactive triterpenoid in medicinal plants. It possesses various pharmacological properties, including analgesic, anti-inflammatory, and antitumor effects. The effects of OA in chondrocytes, however, are not well characterized. Here, we used rabbit articular chondrocytes as a cellular model to investigate the effects and regulatory mechanisms of OA on dedifferentiation and pro-inflammation. OA promoted dedifferentiation of chondrocytes by inhibiting type II collagen and pro-inflammatory activity by increasing cyclooxygenase-2 (COX-2) expression. Furthermore increased phosphorylation of p38 kinases and down-regulated phosphorylation of ERK was observed. Inhibition of p38 with SB203580 in OA-treated cells rescued the expression of type II collagen and suppressed the expression of COX-2. However, ERK inhibition with PD98059 accelerated the OA-induced inflammatory responses. These results suggest that OA induces loss of type II collagen expression via the p38 pathway and induces inflammation through the p38 and ERK pathways in rabbit articular chondrocytes.

Key words: Oleanolic acid; Chondrocytes; type II collagen; COX-2; MAPK pathway.

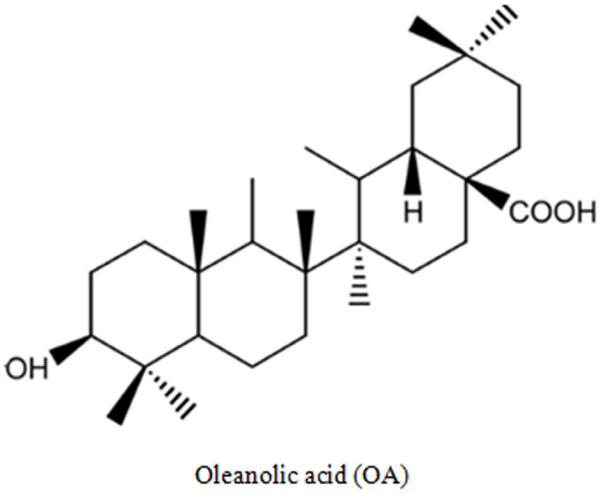
Introduction

During development, chondrocytes arise from mesenchymal cells (1). They are cartilage-specific cells that synthesize the cartilage-specific extracellular matrix (ECM) components and maintain its integrity (2). The composition of these components is maintained during biological processes, such as differentiation, cartilage development, and repair (1, 2). Cartilage is made up of ECM, a majority of which is composed of type II collagen, fibronectin, and sulfated proteoglycan macromolecules. Type II collagen is a phenotypic marker of chondorocytes, playing a critical role in the homeostasis of cartilage by regulating ECM synthesis, which is illustrated by the loss of the physicomechanical properties of the tissue leading to a variety of joint diseases such as osteoarthritis and rheumatoid arthritis. Chondrocyte dedifferentiation in vitro was defined by the loss of molecular markers that define a differentiated chondrocyte. As dedifferentiation progresses of chondrocytes, expression of ECM molecules as glycosaminoglycans (GAGs), type II collagen, and aggrecan is lost and increases in the expression of type I collagen (3, 4). The chondrocytes undergoes dedifferentiation by losing their normal phenotypic markers during the monolayer cell culture at high number of passages and through the development cartilage related pathologies. Progressive reduction of type II collagen expression is associated with osteoarthritis development in favor of nonspecific articular cartilage collagens (5).

Osteoarthritis is a degenerative disease associated with functional disability, stiffness, limited movement, and inflammation (6) and is characterized by destruction of chondrocytes. Non-steroidal anti-inflammatory drugs are used to treat osteoarthritis and to reduce its related symptoms (7).

Cyclooxygenase (COX) is the major enzyme that catalyzes the rate-limiting step in the conversion of arachidonic acid to prostaglandin E_2 (PGE_2), prostacyclin, and thromboxane A2 via oxygenation.The isoform COX-2 is mainly involved in the inflammatory response of various cell types, including endothelial cells, fibroblasts, and monocytes. Increased COX-2 expression has been linked with different pathological conditions, including osteoarthritis and rheumatoid arthritis (8). Mitogen-activated protein kinases (MAPKs) signaling cascades regulate diverse cellular functions, such as cell proliferation, growth, and apoptosis. p38 kinases, extracellular signal-regulated kinases (ERKs), and c-Jun NH2-terminal kinases (JNKs) constitute the three subclasses of MAPKs (9).

Various inflammatory mediators, including TNF-α, IL-β induce the expression of COX-2. Augmented COX-2 upregulate the E-prostanoid receptors (EPs) and increases the synthesis of prostaglandin E2 (PGE2). PEG2 induces the expression of MMPs, a disintegrin and MMPs with thrombospodin repeats-5 (ADAMTS-5) through EP4 receptor (overexpressed in arthritis). Moreover, PGE2 decrease the synthesis of proteoglycans and promote their release from cartilage. The expression of PGE2 and COX-2 is increased in osteoarthritic cartilage. Inflammatory mediators enhance the production of nitric oxide (NO) through MAPK by stimulating the expression of the inducible nitric oxide synthase (iNOS). Furthermore, NO increase MMP activity, induce chondrocyte apoptosis and inhibits pro-



Oleanolic acid (OA)

Figure 1. Chemical structure of oleanolic acid.

teoglycan synthesis. The COX-2, PGE2 and NO are associated with cartilage degeneration in arthritis via MAP kinases pathways (10).

Oleanolic acid (3 β-hydroxyolean-12-en-28-oic acid; OA), a naturally occurring triterpenoid (Fig. 1) is exists in a free acidic form or combined form as a glycoside. OA is found extensively in food products such as vegetable oils and glossy privet fruit (11). Indeed, its name OA originates from a species of olive plant (*Olea europaea*). OA exhibits various biological properties, including anti-cancer and anti-inflammatory activities (12, 13). A recent report showed that OA has anti-inflammatory effects in human endothelial cells (14). Also, OA regulated the NF-*k*B signaling which exhibits high anti-inflammatory activity (15).

However, the effects of OA on differentiation and the inflammatory response has not been investigated in detail in normal chondrocytes, and the mechanisms by which OA acts are not clearly understood. The purpose of this study was to (i) determine if OA could regulate dedifferentiation and inflammation in chondrocytes, and (ii) elucidate the molecular mechanisms of OA-induced dedifferentiation and inflammation.

Materials and Methods

Reagents and culture media

OA was purchased from Cayman Chemicals (Ann Arbor, MI, USA). It was dissolved in sterile dimethylformamide (DMF, Sigma-Aldrich, St. Louis, MO, USA) and <1% DMF final concentration was used in the medium. Same concentration of DMF was maintained in each condition. All primary antibodies (Type II collagen, COX-2, Phospho-ERK, p38, and Actin) were purchased from Cell Signaling Technology (Beverly, MA, USA). SB203580 (SB) was from BIOMOL (Plymouth Meeting, PA, USA), and PD98059 (PD) was purchased from Calbiochem (Billerica, MA, USA). Collagenase type II and antibiotics (penicillin and streptomycin) were obtained from Sigma-Aldrich. Fetal-bovine serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Carlsbad, CA, USA).

Isolation and culture of rabbit articular chondrocytes

Articular cartilage was obtained from 2-weeks-old New Zealand white rabbits (Koatech, Pyeongtaek, Korea), and cartilage fragments were digested using collagenase (381 units/ml) in DMEM. Chondrocytes were collected by centrifugation, and 5×10^4 cells were plated in 35 mm culture dishes in the presence of DMEM medium (10% fetal bovine serum (v/v), 50 units/ml penicillin, and 50 µg/ml streptomycin) at 37°C in a humidified incubator [air and CO2 (95%:5%) for stabilization]. On day 5, cells attained 70% confluence and were treated with OA or other reagents. The study was carried out according to the guidelines approved by the ethics committee of Kongju National University, Republic of Korea.

Western blot analysis

For western blot analysis, whole cell lysate was prepared for protein extraction in radioimmunoprecipitation assay (RIPA) buffer containing 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl [pH 7.4], 1% nonidet-40 (NP-40), 150 mM NaCl, phosphatase (1 mM of Na_3VO_4 and NaF), and protease inhibitors (1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 10 µg/ml of leupeptin, aprotinin, and pepstatin A). Bicinchoninic acid assay (BCA) method was used to quantitate levels of crude cellular proteins by using a standard curve of bovine serum albumin. Equal amounts of protein were loaded on gels and size-fractionated by SDS-polyacrylamide gel electrophoresis (PAGE). Thereafter, proteins were transferred to nitrocellulose (NC) membranes and incubated for 1 h in blocking buffer (5% non-fat dry milk in Tris-buffered saline/tween-20). All the blots were probed with primary antibodies at 4°C for 14 h. The membranes were washed and incubated for 2 h with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich). Blots were washed with Tris-buffered saline/Tween-20, developed using EZ-Western detection kit (DaeilLab service, Seoul, Korea), and the bands were visualized under a Fuji LAS-4000 imager (Fuji Film Co., Tokyo, Japan). The western blot data were quantified by densitometric analyses using Image J (National Institutes of Health, Bethesda, MD, USA).

Proliferation assay

The methylthiazole tetrazolium (MTT) assay was performed to investigate the effect of OA on chondrocyte proliferation. As described above, rabbit chondrocytes were isolated, seeded (2×10^4 cells/well) in 96-well plate, and incubated at 37°C in CO2 incubator for 24 h before OA treatment. Then, 10 µl of reagent 1 (MTT, 10 mg/ml) was added to each well and kept in a CO_2 incubator for 4 h until purple formazan crystals developed. Subsequently, 100 µl of reagent 2 (MTT solubilization buffer, 10% SDS with 0.01N HCl and DMSO) was added. Cells were again incubated for 12 h, and optical density (OD) was recorded at 600 nm using a spectrophotometer. The experiment was performed in quadruplicate.

Immunofluorescence staining

Chondrocytes were plated at the density of 5×10^4 cells on 35 mm culture dishes containing coverslips and treated or untreated with 75 µg/ml of OA in the presence or absence of 20 µM SB203580 or PD98059 inhibitors for 24 h. After different reagents treatment, these cells were fixed with 3.5% paraformaldehyde in PBS

for 20 min at room temperature and were permeabilized in PBS containing 0.1% Triton X-100 for 15 min. The fixed cells were incubated 1 h with antibodies against type II collagen and COX-2, and then incubated with secondary antibodies for 1 h. The cells were washed with PBS and incubated for 10 min with DAPI (0.1 µg/mL, Molecular Probes, Invitrogen) at room temperature. Next, the cells were washed three times with PBS, and observed under a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

Statistical Analysis

All the experiments were performed in triplicate except mentioned, and results are expressed as mean ± standard error (SE). Values were calculated from the specified number of determinations. The data were subjected to an analysis of variance (ANOVA) using Tukey's test to analyze differences, and results were considered significant at the level of $*p< 0.05$ and $**p< 0.01$.

Results

Effect of OA on the growth of rabbit chondrocytes

To evaluate the effect of OA on the cell proliferation/number in rabbit chondrocytes, cell was examined using the MTT assay and microscopy. Cells were exposed to various concentrations (0, 25, 50, 75, and 100 µg/ml) of OA for 24 h. The results demonstrated no significant difference in cell number with concentrations of lower than 50 µg/ml OA. As the concentrations of higher than 75µg/ml, the cell number was reduced (Fig. 2A and B).

OA causes dedifferentiation by downregulating the expression of type II collagen in chondrocytes

Next, we determined OA promoted cellular dedifferentiation by regulating the expression of type II collagen (Fig. 3). Chondrocytes were treated with various concentrations of OA for 24 h and 75 µg/ml for different time periods. Type II collagen expression progressively decreased with higher concentrations of OA and decreased over time with 75 µg/ml OA (Fig. 3A and B). These results suggest that OA induced the dedifferentiation of chondrocytes.

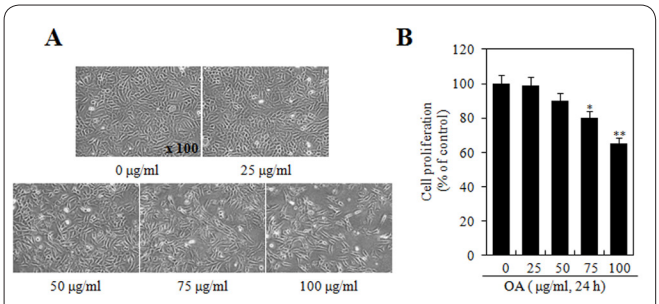


Figure 2. The effect of oleanolic acid (OA) on rabbit articular chondrocyte proliferation. Chondrocytes were treated with indicated concentrations of the OA for 24 h. (A) Cells were observed through a phase contrast microscope at the magnification of 100x. (B) Cell viability assay results, decrease in number of chondrocytes with increasing concentrations of OA. The experiment was performed in triplicate and presented as the means ± SE. The probability value of $*p< 0.05$ and $**p< 0.01$ was considered significant compared to the control.

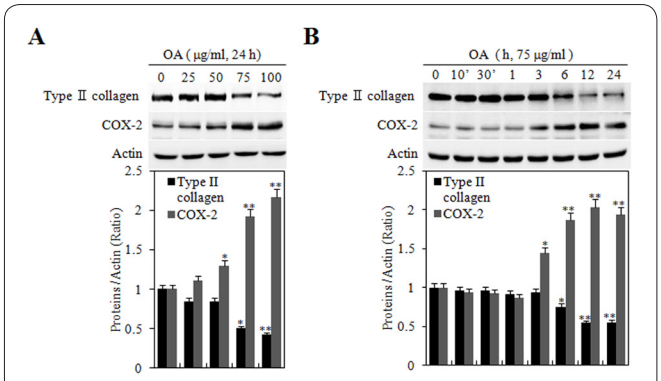


Figure 3. The effects of the oleanolic acid (OA) on type II collagen and cyclooxygenase 2 (COX-2) in chondrocytes. (A) Type II collagen and COX-2 expression with indicated concentration of OA for 24 h. (B) Chondrocytes were treated with 75 µg/ml of OA for different time periods. (A and B) Protein expression was determined by western blot analysis and actin was used as loading control. The relative amount of type II collagen and COX-2 were quantified by a densitometric analysis (ImageJ). The experiment was performed in triplicate and presented as the means ± SE. The probability value of $*p< 0.05$ and $**p< 0.01$ was considered significant compared to the control.

Pro-inflammatory response of OA through upregulation of COX-2 expression

Following exposure of chondrocytes to 75 µg/ml for the indicated time periods or 0, 25, 50, 50, and 100 µg/ml of OA for 24 h (Fig. 3), COX-2 expression was examined by western blot. OA treatment in chondrocytes dose- and time-dependently induced COX-2 expression (Fig. 3A and 3B). Thus, OA is capable of inducing COX-2 expression in rabbit articular chondrocytes.

OA induces dedifferentiation via p38 and inflammation through p38 and ERK-1/-2 pathways

MAPK signaling pathways regulate a variety of cellular activities, such as cytokines, inflammation, cell proliferation, differentiation, immunoregulation, and apoptosis (16, 17). Here, we evaluated the effects of OA on the activation of ERK and P38 kinase. Chondrocytes

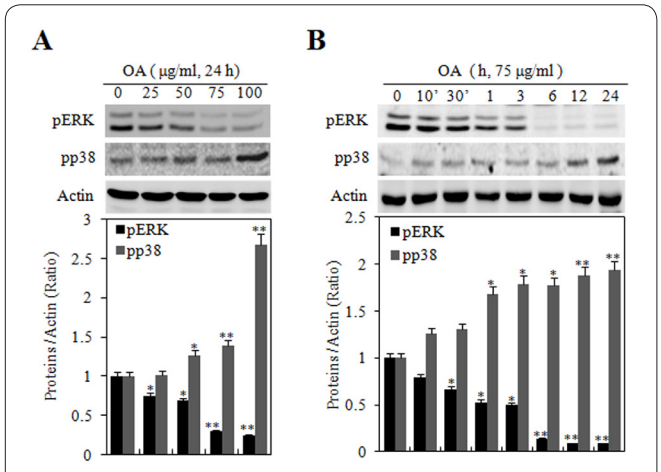


Figure 4. The effect of oleanolic acid (OA) on p38 and ERK expression in chondrocytes. Chondrocytes were untreated or treated (A) with the indicated concentrations of OA for 24 h, or (B) with 75 µg/ml OA for the indicated time periods. Expression of the ERK and p38 was determined by western blot analysis. Expression of actin was used as a loading control. The experiment was performed in triplicate and presented as the means ± SE. The probability value of $*p< 0.05$ and $**p< 0.01$ was considered significant compared to the control.

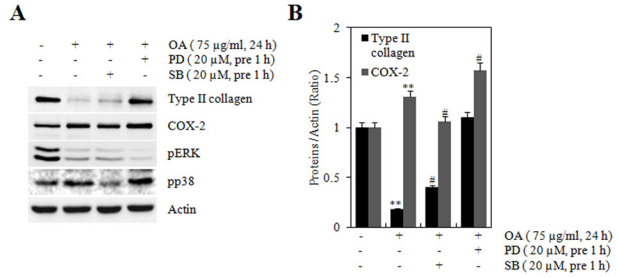


Figure 5. Oleanolic acid (OA) regulated expression of type II collagen and COX-2 rabbit articular chondrocytes. Chondrocytes were untreated or treated with 75 µg/ml of OA for 24 h in the absence or presence of the of 20µM concentrations of SB203580 or PD98059 for 24 h. Inhibitors were treated one hour before treating with OA. (A) OA downregulated the type II collagen and pERK expressions. While upregulated COX-2 and pp38 expressions. All the expressions were detected by western blot analysis using actin as loading control. (B) The relative amounts of type II collagen and COX-2 were quantified by a densitometric analysis (ImageJ). The experiment was performed in triplicate and presented as the means ± SE. The probability value of $^{**}p< 0.01$ was considered significant compared to the control and $^{\#}p< 0.05$ compared to the OA.

were treated with 75 µg/ml for the indicated time periods or 0~100 µg/ml of OA for 24 h, and western blot was performed to identify the protein expressions of pp38 and pERK. As shown in Figure 4, phosphorylation of ERK was decreased and phosphorylation of p38 was increased. Next, cells were pretreated with 20 µM PD98059 (PD) and SB203580 (SB) for 1 h and then treated with OA (Fig.5 and Fig.6). SB inhibited OA-induced dedifferentiation and expression of COX-2. Inhibition of ERK with PD results in increased COX-2 expression but did not regulated OA induced dedifferentiation. These findings indicate that OA-induces chondrocyte dedifferentiation through the p38 pathway and inflammation via ERK-1/-2 and p38 pathways.

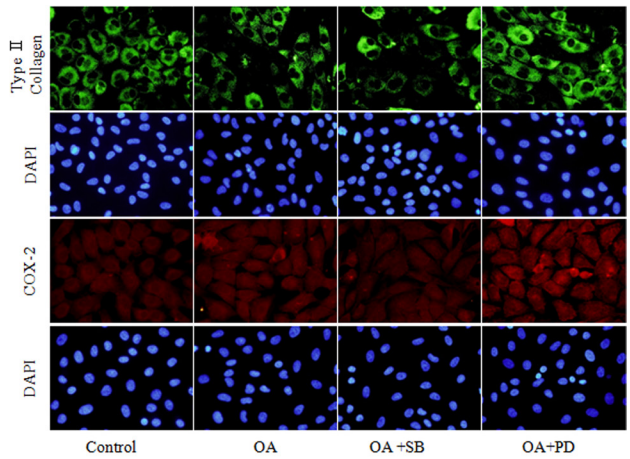


Figure 6. Fluorescent immunocytochemical analysis. Chondrocytes were untreated or treated with 75 µg/ml of OA for 24 h in the absence or presence of the indicated SB203580 or PD98059. Cell were exposed with monoclonal antibodies, coupled with secondary antibodies labelled with fluorescein (FITC-Green) for type II collagen or rhodamine (TRITC-Red) for COX-2 and nuclear counter staining was executed using DAPI (blue). Representative micrograph of chondrocytes of each treatment is shown.

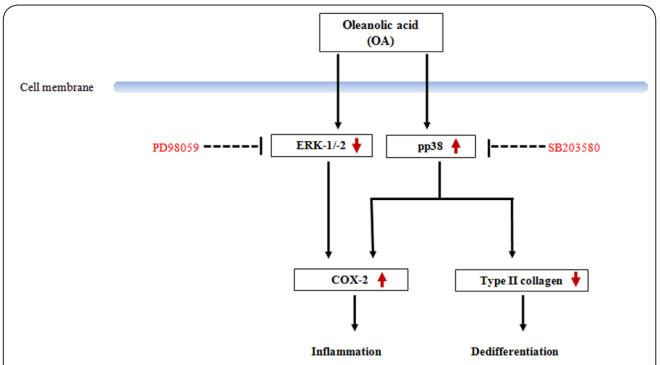


Figure 7. Schematic summary of oleanolic acid (OA)-induced dedifferentiation and inflammation of chondrocytes. The OA regulates dedifferentiation through the p38 pathway and modulates COX-2 expression through the p38 and ERK pathways.

Discussion

OA is a ubiquitous triterpenoid phytocompound with various beneficial effects. Currently, multiple pathways of OA are under investigation. Recently, OA was reported to inhibitory effect on tumor growth *in vivo* and induce apoptosis in various tumor cells such as Liver cancer (HepG2), Breast cancer (MDA-MB-231), Gastric cancer (BGD-823) and Osteosarcoma (15) (12). It increased Bax, caspase-3 and -9 expression and induced accumulation of reactive oxygen species (18). OA is also reported to induce cell cycle arrest by modulating ERK-p53 mediated cell cycle arrest and induced apoptosis in HCC cells via the mitochondrial pathway (15, 18). In the present study, it was found that OA inhibited the proliferation of rabbit articular chondrocytes (Fig. 2).

OA have novel modulatory effects on matrix metalloproteinases (MMPs) and type I collagen expression and to be a possible chemotherapeutic substance for chronic liver fibrosis. Furthermore, contemporary molecular biology techniques have helped to understand the unique pharmacological properties of triterpenoids (19). Triterpenoids regulate the induction of phase 2 enzymes, including NAD(P)H-quinone oxidoreductase and heme oxygenase 1, through the nuclear factor erythroid 2-like type 2 (Nrf2)-kelch-like ECH associating protein 1 (Keap1) signaling cascade (20). These compounds also have an important role in transforming growth factor beta/Smad signaling (19), but their effects vary depending on the experimental conditions. Investigation of such signaling pathways could be useful in understanding the molecular mechanisms underlying the beneficial effects of these compounds. These phytoconstituents possess various bioactivities, including antioxidative, hepatoprotective, anti-tumorigenic, anti-inflammatory, and osteoprotective effects, and have been shown to have low systemic toxicity. OAA (oleanolic acid acetate), a derivative of OA is known to have therapeutic effects, including atopic dermatitis and inflammatory bone loss *in vivo*. In addition, OAA significantly inhibited the type-II collagen specific IgG2a:IgG1 ratio in rheumatoid arthritis (21). However, there is a need to explore further the effects of OA in chondrocytes. In the present study, treatment of rabbit articular chondrocytes with OA was shown to induce the dedifferentiation by type II collagen expression decrease in a dose- and time-dependent manner (Fig. 3).

The anti-inflammatory effect of OA was first reported in the 1960s (22), it derivative 2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oic acid (CDDO) has been generated, which is more potent than the parent

compound in anti-inflammatory actions and more effective in inhibiting iNOS and COX-2 as a basis of its anti-inflammatory effects (19). *Choi et al.* demonstrated that OA improve inflammation and ulcers in arthritis (21). COX-2 has topical inflammatory activity by converting arachidonic acid to prostaglandin and is involved in different important functions, such as inflammation (23). COX-2 inhibitors have been shown to reduce inflammation and cartilage degradation in animal arthritis models (24). COX-2 also, paradoxically related to cancer cell growth. However, recent evidence also suggests that COX-2 may pro-apoptotic and involved in the apoptotic effect of apoptosis inducers (11). Wang *et al.* demonstrated that COX-2 activation is involved in OA-induced apoptosis and cell cycle arrest (11). Moreover, in previous studies reported that in human coronary SMC, OA is able to induce PGI_2 release through a mechanism involving COX-2 up-regulation via MAPK pathways (25). We hypothesized that the inflammatory effect OA in chondrocytes was mediated by COX-2. The effect of OA on inflammation was evaluated using western blot, and we found that OA up-regulated COX-2 (Fig. 3). By contrast, our data indicate that OA alone in chondrocytes showed pro-inflammatory effects.

MAPK signaling cascades also play a vital role in the transduction of extracellular signals by integrating, amplifying, and then eliciting specific cellular responses, such as differentiation, cell growth, and inflammation (26, 27). In order to explore ERK and p38 signaling cascades in OA induced chondrocyte dedifferentiation and inflammation, their respective phosphorylation patterns were assessed by western blot. Numerous studies have shown that the MAPK (p38 and JNK) pathways are involved in the regulation of inflammation, proliferation, and differentiation (28, 29). OA derivatives also demonstrated apoptosis in leukemia cells and lung cancer through MAPK signaling (30). Previously, we reported that p38, ERK, and Akt regulated differentiation of rabbit articular chondrocytes (31). In the present study, OA activated the p38 pathway and suppressed the ERK pathways, two pathways that belong to the MAPK signaling system (Fig. 4).

In the current study, OA inhibited chondrocyte proliferation, as measured by the MTT assay and phase contrast microscopy. Western blot results revealed that OA stimulation caused dedifferentiation and pro-inflammation in rabbit articular chondrocytes. Selective inhibition of p38 led to the revival of type-II collagen, indicating the involvement of p38 protein in the regulation of dedifferentiation. The pro-inflammatory effect was blocked by selective inhibition of p38 and promotion of COX-2 expression by inhibition of ERK, suggesting a pivotal role of the ERK and p38 cascade in regulating COX-2.

Collectively, these results suggest that MAPKs regulated the pro-inflammatory response through p38 and ERK and dedifferentiation via p38 in OA treated rabbit articular chondrocytes. However OA exhibited the inflammatory response and dedifferentiation effects, the current study provides the basic information in understanding the prospective molecular mechanism that would be useful in development of drug for joint related ailment such as arthritis.

Acknowledgments

The National Research Foundation of Korea (NRF, 2014-R1A1A3049653) and Korean Health Technology R&D project, Ministry of Health & Welfare, Republic of Korea (A120960) are greatly acknowledged to support this study.

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