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Simvastatin-dependent actin cytoskeleton rearrangement regulates differentiation via the extracellular signal-regulated kinase-1/2 and p38 kinase pathways in rabbit articular chondrocytes

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ABSTRACT

Alterations in cell morphology involve changes in the actin cytoskeleton and play crucial roles in determining chondrocyte phenotypes. Although the effects of simvastatin (SV) have been demonstrated in various cell types, the mechanisms and effects of SV on chondrocyte differentiation and actin cytoskeletal rearrangement are still unclear. Here, we investigated the roles of actin filament rearrangement on SV-induced differentiation of rabbit articular chondrocytes. Treatment with SV caused actin remodeling in comparison with that in untreated chondrocytes, as determined by immunofluorescence staining. Moreover, treatment with cytochalasin D (CD) and jasplakinolide (JAS), which modulate actin filament formation, resulted in reorganization of the actin cytoskeleton compared with that induced by SV in chondrocytes. In addition, CD synergistically enhanced the SV-induced increase in type II collagen expression, whereas JAS dramatically inhibited SV-induced differentiation. We also found that differentiation via SV-dependent actin cytoskeleton changes was regulated by the extracellular signal-regulated kinase (ERK)−1/2 and p38 kinase pathways. These results demonstrated that actin cytoskeletal rearrangement by SV regulated type II collagen expression and suggested that ERK-1/2 and p38 kinase pathways may play important roles in SV-induced type II collagen expression by altering actin cytoskeletal reorganization in rabbit articular chondrocytes.

1. Introduction

Osteoarthritis (OA), a common form of arthritis, has a negative impact on joint function and patient quality of life (Loeser, 2010). Effective treatments for OA have not yet been developed, despite the increasing need for interventions. The degeneration of cartilage in OA occurs via repair of damaged extracellular matrix (ECM) in chondrocytes and activation of enzymes produced by chondrocytes to decompose the matrix, inhibit matrix synthesis, and accelerate cartilage erosion (Sandell and Aigner, 2001). The ECM is composed of many tissue-specific macromolecules, including collagens (types II, IX, and XI), proteoglycans, and aggrecans. The synthesis and degradation of the ECM is completely controlled by chondrocytes (Reginato et al., 1994), which are derived from mesenchymal cells during embryonic development (DeLise et al., 2000) and have a reversible phenotype (Sandell and Aigner, 2001). When the differentiated chondrocytes are cultured in serial monolayer, they lose their properties and transformed into fibroblastic cells (Yu et al., 2018). The dedifferentiation chondrocytes can show the characteristics of the chondrocytes through the three-dimensional culture, which is called re-differentiation (Yu and Kim, 2015).

Statins are a group of drugs that act as inhibitors of hydroxy-methylglutaryl coenzyme A reductase, which is involved in mevalonate synthesis. The use of statins in clinical practice has increased owing to their efficacy in the prevention and treatment of hypercholesterolemia. Statins have also been shown to affect vascular wall composition (Treasure et al., 1995), bone metabolism (Uzzan et al., 2007), fracture healing (Pauly et al., 2009) and inflammation (Pauly et al., 2009; Uzzan et al., 2007).

Actin cytoskeleton is an important regulator of chondrocyte phenotype (Kim et al., 2003). Previous reports have indicated that inhibiting actin polymerization in chondrocytes induces re-expression of cartilage matrix genes (Newman and Watt, 1988) and reduces the expression of type I collagen (Hoshiba et al., 2008). Additionally, retinoic acid (RA) (Benya et al., 1988) treatment of chondrocytes or chondrocytes passaged monolayer culture (Parreno et al., 2017) results in dedifferentiation due to changes actin cytoskeletal architecture. A previous study by Kim et al. (2003) reported that reorganization of the

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actin cytoskeleton by CD modulates nitric oxide-induced dedifferentiation, apoptosis, and cyclooxygenase-2 expression via mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways. Yu et al. (2010, 2016a) showed that berberine induces dedifferentiation by actin cytoskeleton reorganization via the phosphoinositide 3-kinase/Akt and p38 kinase pathways and 2-deoxy-D-glucose regulates dedifferentiation through the beta-catenin pathway in rabbit articular chondrocytes.

Based on previous studies, statins control the proteins involved in actin cytoskeleton reorganization, and changes in actin cytoskeleton structure are closely related to the differentiation or dedifferentiation of chondrocytes, suggesting that SV may be closely involved in the differentiation of chondrocytes. However, SV-dependent actin cytoskeletal reorganization in chondrocytes has not been studied. Therefore, in this study, we investigated the mechanisms of action of SV in chondrocyte differentiation, with a focus on cytoskeletal architecture reorganization. Our findings suggested that SV-induced differentiation of chondrocytes was mediated by cytoskeletal architecture reorganization via inhibition of the ERK-1/2 and p38 kinase pathways.

2. Materials and methods

2.1. Isolation and monolayer culture of rabbit articular chondrocytes

Chondrocytes were obtained from arthrodial cartilage of New Zealand White rabbits (Koatech, Pyeoungtaek, Republic of Korea) as described previously (Yu et al., 2016b). All animal experimental procedures were in compliance with the guidelines of the Ethics Committee of Kongju National University. Cartilage tissues were finely cut into pieces (~1 mm^3), and then digested with 0.2% collagenase type II (Sigma-Aldrich, St. Louis, MO, USA) in TESCA buffer in Dulbecco's modified Eagle's medium (DMEM) for 7 h at 37 °C with 5% CO_2. After incubation, the suspension was transferred to a new polypropylene tube and centrifuged at 200 g for 5 min, finally discard supernatant which contained cell debris and tissue. The individual cells were re-suspended in DMEM supplemented with 10% (v/v) fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin and plated on culture dishes at a density of 2×10^4 cells/dish. After 3 days in culture, the cells were treated with SV (Sigma-Aldrich). SV was hydrolyzed for activate from lactone prodrug form in ethanolic NaOH (15% [v/v] ethanol and 0.25% [w/v] NaOH) at 60 °C for 1 h and then filter sterilized (Nyilasi et al., 2010). Stock concentration of SV was 50 mM and stored at − 20 °C. To induce dedifferentiation, passage 0 chondrocytes were serially subcultured up to passage 2 by repeatedly plating 1.7×10^4 cells/dish. The medium was changed every two days after seeding and after three days, the chondrocytes were treated with either 50 μM or graded concentrations (0 μM, 10 μM, 30 μM, 50 μM) of SV for predetermined times (0 min, 10 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h) or 24 h, respectively. CD (Sigma-Aldrich) and JAS (Molecular Probes, OR, USA), which were used to induce reorganization of the actin cytoskeleton were added simultaneously with SV.

2.2. Western blot analysis

Whole-cell lysates were prepared as described previously (Yu et al., 2016b). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (Millipore Corp., Billerica, MA, USA). The nitrocellulose membrane was blocked with 5% nonfat dry milk in Tris-buffered saline Tween 20 (0.1% Tween, pH 8.0, TBST) and incubated with antibodies against type II collagen or extracellular signal-regulated protein kinase. The bands were developed using a peroxidase-conjugated secondary antibody, followed by ECL reagents (Dogen, Seoul, Republic of Korea). The following antibodies were used: goat anti-collagen type II monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; SC-7764; 0.2 μg/ml; RRID: AB_2260775); mouse anti-GAPDH (Santa Cruz Biotechnology; SC-166545; 0.2 μg/ml; RRID: AB_2107299); rabbit anti-phospho-p38 MAP kinase (Cell Signaling Technology, Danvers, MA, USA; #9211; 0.2 μg/ml; RRID: AB_331640); rabbit anti-phospho-ERK (Cell Signaling Technology; #9101; 0.2 μg/ml; RRID: AB_2315113); anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA; A0545; 80 ng/ml; RRID: AB_257896); anti-goat IgG (Chemicon International, Billerica, MA, USA; AP106P; 40 ng/ml; RRID: AB_92411); and anti-mouse IgG (Enzo Life Sciences International, Farmingdale, NY, USA; ADI-SAB-100; 80 ng/ml; AB_11001642). All of the experiments reported in this study were performed three times and the results were reproducible.

2.3. Immunofluorescence staining

Chondrocytes cultured on coverslips were fixed with 3.5% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. The cells were permeabilized and blocked with 0.1% Triton X-100% and 5% fetal calf serum in PBS for 30 min. The fixed cells were washed and incubated for 1 h with antibody against type II collagen (MAB8887; 0.2 μg/ml, Merck Millipore, Milano, Italy). The cells were washed, incubated with fluorescein-conjugated secondary antibodies for 30 min, and observed under a fluorescence microscope. For double labeling of F-actin, cells were subsequently stained for 30 min at room temperature with rhodamine-conjugated phalloidin (Sigma-Aldrich) that bound with high affinity to F-actin. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Burlington, ON, Canada). The cells were washed three times with PBS and observed under a BX51 fluorescence microscope (Olympus, Tokyo, Japan).

2.4. Alcian blue staining

Cells were fixed with 3.5% paraformaldehyde for 15 min and stained with 0.1% Alcian blue in 3% acetic acid (pH 2.5) overnight. Chondrocytes were washed 3 times with phosphate-buffered saline (PBS), and 6 M guanidine HCl was added for 6 h. Production of sulfated proteoglycan was measured at 595 nm using ELISA reader. Rabbit joint cartilage explants were fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C, washed with PBS, dehydrated with graded ethanol, embedded in paraffin, and sectioned at 4 mm thickness. The sections were stained by standard procedures using Alcian blue.

2.5. Immunohistochemistry

Rabbit joint cartilage explants were fixed in phosphate-buffered saline containing 4% paraformaldehyde for overnight at 4 °C, dehydrated with increasing concentrations (30%, 50%, 70%, 85%, 95%, 100%) of ethanol, and embedded in paraffin wax (Fisher Scientific. Fairlawn, NJ, USA). Each specimen was cut into 4 μm slices as described previously (Yu et al., 2016b), and the dewaxed paraffin sections were incubated with an antibody against type II collagen (Merck Millipore) for overnight at 4 °C and visualized with a kit purchased from DAKO Co. (Dako Cytomation, Copenhagen, Denmark). The sulfated-proteoglycan was stained with Alcian blue (Sigma-Aldrich) and the sections were counterstained with hematoxylin or nuclear fast red.

2.6. Data analysis and statistics

All data were expressed as the mean ± the standard deviation (S.D.). Comparisons between two groups were made using one-way analysis of variance, and all pairwise comparisons between groups were conducted using the Turkey post hoc test; p values less than ≤ 0.05 were considered significant. The results shown were representative of at least three separate experiments.