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Research article

Simvastatin induces differentiation of rabbit articular chondrocytes *via* the ERK-1/2 and p38 kinase pathways

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ABSTRACT

Statins are competitive inhibitors of hydroxy-methyl-glutaryl Coenzyme A (HMG-CoA) reductase, a key enzyme involved in the conversion of HMG-CoA to the cholesterol precursor mevalonate. Some statins, such as simvastatin (simvastatin), have been shown to have anti-cancer and anti-inflammatory effects, reducing cartilage degradation in osteoarthritic rabbits in vivo. However, the regulatory mechanisms undergirding simvastatin mediated chondrocyte differentiation have not been well elucidated. Thus, we investigated the action and mechanism of simvastatin on differentiation of rabbit articular chondrocytes through western blot analyses, RT-PCR, and immunohistochemical (IHC) and immunofluorescence (IF) staining. Simvastatin treatment was found to induce type II collagen expression and sulfated-proteoglycan synthesis in a dose- and time-dependent manner. Indeed, RT-PCR revealed increased expression of type II collagen on treatment with simvastatin. Both IHC and IF staining indicated differentiation of chondrocytes. Simvastatin treatment reduced activation of ERK-1/2 and stimulated activation of p38 kinase. Inhibition of ERK-1/2 with PD98059 enhanced simvastatin induced differentiation, whereas inhibition of p38 kinase with SB203580 inhibited simvastatin induced differentiation. Simvastatin treatment also inhibits loss of type II collagen in serial monolayer culture. Collectively, our results indicate that ERK-1/2 and p38 kinase regulate simvastatin-induced differentiation of chondrocytes in opposing manners. Thus, these findings suggest that simvastatin may be a potential therapeutic drug for osteoarthritis.

1. Introduction

Osteoarthritis (OA), a common form of arthritis, has a major impact on joint function and patient quality of life [1]. OA is not a single disease entity, but instead represents a disease group with different underlying mechanisms and pathophysiologies affecting joint cartilage. Articular cartilage is a uniquely designed biomaterial that forms the smooth, gliding surface of the diarthrodial joints. Chondrocytes are derived from mesenchymal cells in which the differentiated phenotype is reversible [2]. The phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix (ECM) molecules, including type II collagen and proteoglycans such as aggrecan [3–5]. Chondrocytes are the only resident cell type found in cartilage responsible for synthesis and turnover of the ECM [6]. Cartilage ECM is primarily composed of large quantities of collagens and proteoglycans (PGs), which give cartilage its tensile strength and rigidity, enabling it to resist stress. Collagen type II is the main isoform, while types VI, IX, X, and XI are found in small amounts in articular cartilage. Collagen type II is found predominantly in cartilage. SOX-9 is a transcription factor that plays a critical role in the specific activation of type II collagen during chondrocyte differentiation [7,8]. High levels of SOX-9 protein correlate with type II collagen synthesis, while dedifferentiated as well as hypertrophic chondrocytes loss SOX-9.

PGs are large molecules containing a protein core with glycosaminoglycan branches [9]. Destruction of articular cartilage ECM is the hallmark pathology of OA. Moreover, an imbalance of anabolic and catabolic activity is thought to be an essential feature of OA cartilage degeneration.

Statins are competitive inhibitors of hydroxy-methyl-glutaryl Coenzyme A (HMG-CoA) reductase, and their use has been pervasive in clinical practice for the prevention and treatment of coronary heart disease [10]. The effects of statins also have been

Abbreviations: HMG-CoA, hydroxy-methyl-glutaryl Coenzyme A; ECM, extracellular matrix; IHC, immunohistochemichemistry; IF, immunofluorescence; RT-PCR, reverse transcription polymerase chain reaction; OA, osteoarthritis; ECM, extracellular matrix; PGs, proteoglycans; BMP-2, bone morphogenetic protein-2; MAPKs, mitogen activated protein kinases; ERK-1/2, extracellular signal-regulated kinase-1/2; SB, SB203580; PD, PD98059; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase

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demonstrated on vascular wall composition [11], fracture healing [12], and bone metabolism [13]. Statins have been shown to have anti-inflammatory effects unrelated to their lipid-lowering abilities [14,15]. Previous reports have indicated that statins led to expression of bone morphogenetic protein-2 (BMP-2) and type II collagen [16] mRNA, and reduced cartilage degradation in OA rabbits *in vivo* [17]. A number of studies aimed at investigating the effects of statins on chondrocytes have been recently performed. One statins class, mevastatin, increases mRNA expression of aggrecan and type II collagen as well as prostaglandins synthesis by fetal rat chondrocytes on day 2. However, longer (10 days) culture with mevastatin decreased the expression of these mRNAs. Similarly, atorvastatin in OA chondrocytes inhibits IL-1β production and MMP-13 mRNA expression levels, and increased type II collagen and aggrecan mRNA expression. These findings suggest that statins may have potential chondroprotective effects mostly by reducing cartilage degradation [18,19].

Mitogen activated protein kinases (MAPKs) are among the most widespread and heavily studied signaling pathways in eukaryotic cells. MAPKs are responsible for the conversion of a variety of extracellular stimuli into specific cellular responses that range from positive to negative roles in cell differentiation, apoptosis, inflammation, and cell proliferation [20–22]. During chondrogenesis of chick mesenchymal cells, the phosphorylation of p38 is increased and the phosphorylation of ERK is decreased [23]. In agreement with this, prevention of p38 kinase activity inhibits chondrogenesis, whereas a blockage of ERK activity enhances chondrogenesis [23]. These data thus support opposing roles of p38 and ERK in chondrogenesis, with p38 being necessary for chondrogenesis, whereas ERK signaling represses chondrogenesis. MAPK signaling is also likely involved in the transduction of mechanical signals in cartilage development. Shear stress has been shown to modulate MAPK signaling in articular chondrocytes. Increasing evidence indicates that p38 and ERK pathways are associated with de-differentiation and differentiation of chondrocytes [24,25].

Simvastatin, a member of the statin drug class, is known to have anti-cancer and anti-inflammatory effects. However, the underlying molecular mechanisms of simvastatin on differentiation of chondrocytes have not been well established. Thus, the aim of this study is to establish the action and mechanism of simvastatin on differentiation of rabbit articular chondrocytes. In addition, we also sought to investigate how the ERK-1/2 and p38 kinase pathways play key roles in these actions.

2. Material and methods

2.1. Materials

Simvastatin was purchased from Sigma-Aldrich (Saint Louis, MO, USA), and SB203580 (SB) was obtained from Biomol (Plymouth Meeting, PA, USA). PD98059 (PD) purchased from Calbiochem (San Diego, CA, USA). Goat anti-collagen type II polyclonal antibody for western blot analysis (Cat# SC-7764, RRID: AB_2260775) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and mouse anti-collagen type II monoclonal antibody (Cat# MAB8887, RRID: AB_2260779) for immunofluorescence staining was obtained from Chemicon International (Temecula, CA, USA). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Cat# SC-166545, RRID: AB_2107299) was purchased from Santa Cruz Biotechnology Inc. The rabbit *anti*-phospho-p38 (Cat# 9211S, RRID: AB_331640) and rabbit *anti*-phospho-ERK (Cat# 9101, RRID: AB_2315113) polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The rabbit anti-p38 (Cat# sc-535, RRID: AB_632138) and rabbit anti-ERK2 (Cat# sc-154, RRID: AB_2141292) polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. Anti-rabbit IgG antibody (Cat# A0545, RRID: AB_257896) was obtained from Sigma-Aldrich and anti-goat IgG antibody (Cat# AP106P, RRID: AB_92411) was purchased from Chemicon International. Anti-mouse IgG antibody (Cat# ADI-SAB-100, RRID: AB_11001642) was obtained from Enzo Life Sciences International Inc (Plymouth Meeting, PA, USA).

2.2. Cell viability assay

Chondrocytes (1×104 cells/well) were seeded in 96-well culture plates and incubated overnight for attachment. After incubation for 3 days, various concentrations (0, 10, 30, and 50 μM) of simvastatin were added. The medium was removed at the indicated times and 10 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium (MTT) solution (Sigma-Aldrich), dissolved in the culture medium at a final concentration of 5 mg/mL, were added to each well and the plates incubated for 4 h at 37 °C. After completing the incubation, 100 μL of solubilization buffer (10% sodium dodecyl sulfate with 0.01 N HCl) were added to dissolve the MTT tetrazolium crystals, and the cells were incubated overnight at 37 °C. Finally, the absorbance of each well was analyzed by a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 595 nm.

2.3. Isolation and monolayer culture of rabbit articular chondrocytes

Rabbit knee articular chondrocytes (Lateral condyle of femur-articular surface, medial condyle of femur, Lateral condyle of tibia, medial condyle of tibia) were isolated from New Zealand White Rabbits (2-weeks-old, Koatech, Pyeongtaek, Republic of Korea). The study was approved by the Ethics Committee at the Kong-ju National University. Cartilage was harvested from an area at a distance from the femur and calf and it were digested with 0.2% collagenase type II in TESCA buffer (50 mM TES, 0.36 mM Calcium chloride, pH 7.4) for 7 h in a 37 °C, 5% CO_2 incubator. Chondrocytes were seeded at a density of 2×10^4 cells/dish in a 35 mm dish at 37 °C, 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin (50 unit/mL), and streptomycin (50 μg/mL). The medium was changed every 2 days after seeding. After 3 days, the cells were treated with simvastatin (Sigma-Aldrich). PD98059 (Calbiochem), a selective inhibitor of ERK-1/2, or SB203580, a specific inhibitor of p38 kinase, (Biomol) was added 3 h prior to simvastatin. In some experiments, passage (P) 0 cells were cultured to P4 by plating cells at a density of 2×10^4 cells/dish. The differentiation status of articular chondrocytes was determined by examining the expression of type II collagen by western blot analysis, or by determining the production of sulfated proteoglycan by Alcian blue staining.

2.4. Western blot analysis

Whole cell lysates were prepared and subjected to SDS-PAGE. Proteins were extracted using a radio immunoprecipitation assay (RIPA) lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors 10 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL aprotinin, 1 mM AEBSF [4-(2-Aminoethyl) benzene sulfonyl fluoride hydrochloride] and phosphatase inhibitors (1 Mm NaF, 1 mM Na_3VO_4) on ice for 1 h. Cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4 °C, and then the supernatant was collected. The protein concentration of the whole cell lysates was determined using a bicinchoninic acid (BCA) assay and protein samples were boiled for 5 min in 1 × SDS sample buffer (125 mM Tris-HCl, pH 6.8, 7.5% glycerol, 2% SDS, and 0.02% bromophenol