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Full paper

Salinomycin causes dedifferentiation via the extracellular signal-regulated kinase (ERK) pathway in rabbit articular chondrocytes

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ABSTRACT

Salinomycin (SAL), a monocarboxylic polyether antibiotic isolated from *Streptomyces albus*, modulates various cellular responses, including proliferation, apoptosis, and inflammation. However, the effect of SAL on the dedifferentiation of chondrocytes remains unclear. Thus, we investigated the effects and regulatory mechanisms of SAL on the dedifferentiation of rabbit articular chondrocytes. Our results indicate that SAL-induced a loss of type II collagen and decreased sulfated proteoglycan levels in a dose- and time-dependent manner, as assessed by western blot analysis and alcian blue staining. Consistent with dedifferentiation, we found that type II collagen expression was decreased and type I collagen and SOX-9 expression was increased using RT-PCR. Immunohistochemical and immunofluorescence staining also indicated dedifferentiation of chondrocytes. SAL treatment activated the mitogen-activated protein (MAP) kinase signaling pathway. Among the MAP kinases, extracellular signal-regulated kinase (ERK) was phosphorylated and translocated into the nucleus from the cytosol following SAL treatment. Inhibition of ERK with PD98059 (PD) rescued the SAL-induced decrease in type II collagen, increase in type I collagen, and reduction in sulfated proteoglycan. Our findings suggest that SAL induces dedifferentiation via the ERK pathway in rabbit articular chondrocytes.

1. Introduction

Osteoarthritis (OA) is one of the most common chronic diseases and a major cause of disability among the elderly (1). With an aging worldwide population, the incidence of OA is rapidly increasing, and it is anticipated that OA will become the fourth leading cause of disability in the coming decades (2). However, despite the growing number of OA patients, the causes of this disorder have not been fully elucidated. OA is characterized by progressive degradation of articular cartilage and insufficient synthesis of cartilage-specific ECM because of a loss of chondrocyte phenotype (dedifferentiation), and chondrocyte apoptosis (3). Differentiated chondrocytes, which are the only cells found in normal mature cartilage, synthesize sufficient amounts of cartilage-specific extracellular matrix (ECM) to maintain matrix integrity (1). Further, differentiated articular chondrocytes are distinguished by their ability to synthesize cartilage-specific ECM molecules, type II collagen, and sulfated-proteoglycan. Dedifferentiation of these chondrocytes results in loss of type II collagen expression and an increase in type I and type III collagen. Importantly, modulation of matrix metalloproteinases (MMPs) and increased numbers of apoptotic cells are correlated with the degradation of cartilage matrix (4–6). Thus, understanding the mechanisms by which ECM synthesis is lost during OA may be useful for developing novel therapeutics.

Interestingly, previous work has shown that pro-inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, play a crucial role in biochemical alterations of cartilage and chondrocytes (7). Nitric oxide (NO) produced by IL-1β-stimulated chondrocytes causes dedifferentiation through the activation and expression of MMPs, thereby inhibiting proteoglycan synthesis and type II collagen expression. It was further shown that NO-induced chondrocyte dedifferentiation was regulated by mitogen-activated protein (MAP) kinases such as extracellular signal-regulated protein (ERK) and p38 kinase (8,9).

Various stimuli can activate the Raf/MEK/ERK signaling pathways, including mitogen and growth factor stimulation (10). This

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pathway is highly specific, as Raf can only activate MEK, and MEK can only activate ERK (11). ERK is primarily localized in the cytoplasm of resting cells, and translocates to the nucleus following acute stimulation (12,13). Thus, it is possible that extracellular stimulation of the Raf/MEK/ERK could contribute to OA through chondrocyte dedifferentiation.

Salinomycin (SAL) is a potassium ionophore isolated from *Streptomyces albus* that was originally used an antibiotic (14). In addition to other natural ionophores, it is used commercially as a coccidiostat in poultry and to promote growth in cattle. Recently, it was shown that SAL can regulate apoptosis, proliferation, and differentiation in diverse cell types (15,16). Not surprisingly, SAL, like other ionophores, has shown a broad spectrum of bioactivity in a variety of cancers. However, its effects on chondrocytes remain unknown. In this study, we evaluated whether SAL could regulate dedifferentiation in chondrocytes. Our findings indicate that SAL promotes the dedifferentiation of chondrocytes through the activation of ERK kinase.

2. Materials and methods

2.1. Isolation and monolayer culture of rabbit articular chondrocytes

Rabbit knee articular chondrocytes were isolated from New Zealand White Rabbits (2-weeks-old, KOATECH, Pyeoungtaek, Republic of Korea). The study was approved by the Ethics Committee at the Kong-ju National University. Cartilage slices were digested with 0.2% collagenase type II for 7 h in a 37 °C CO_2 incubator. Primary cell cultures were seeded at a density of 2×10^4 cells/dish in a 6-well plate at 37 °C, 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum, penicillin (50 unit/mL), and streptomycin (50 μg/mL). The medium was replaced every 2 days after seeding. After 3 days, the cell cultures were treated with salinomycin (SAL; Sigma, Saint Louis, MO, USA). PD98059 (Calbiochem, San Diego, CA, USA), which was used to inhibit MEK1/2, was added 1 h prior to SAL. In some experiments, passage (P) 0 cells were cultured to P2 by plating cells at a density of 2×10^4 cells/cm^2. The differentiation status of articular chondrocytes was determined by examining the expression of type II collagen by western blot analysis, or by determining the expression of type I collagen by RT-PCR.

2.2. Western blot analysis

Whole cell lysates and chondrocyte nuclear extracts were prepared and subjected to SDS-PAGE. Proteins were extracted using a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors and phosphatase inhibitors on ice for 30 min. Cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4 °C. The protein concentration of the whole cell lysates was determined using a bicinchoninic acid (BCA) assay. 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 blue) containing 1% mercaptoethanol. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline for 1 h at room temperature and washed three times with Tween-Tris-buffered saline. The following antibodies were employed to detect proteins: anti-collagen type II (Santa Cruz, California, CA, USA; 1:1000 dilution), anti-pERK (Cell Signaling Technology, Beverly, MA, USA; 1:1000 dilution), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Santa Cruz, California, CA, USA; 1:3000 dilution). The membrane was incubated with primary antibodies overnight at 4 °C. The blots were developed using a peroxidase-conjugated secondary antibody (1: 3000 dilution) for 2 h at room temperature. The enhanced chemiluminescence reagent was used to identify reactive bands. Finally, the bands were quantified using the LAS4000 (Fuji Film, Tokyo, Japan).

2.3. Reverse transcriptase (RT) – PCR

Cells were plated onto 35-mm culture dishes at a density of 2×10^4. After 3 days, SAL was added to the culture medium and incubated for 24 h. Cultures were washed with cold PBS, and the cells were harvested by scraping with a rubber policeman. Cells cultured without SAL were used as a negative control. Lysates were microcentrifuged at 13,000 rpm for 10 min at 4 °C, and the cell pellets were collected for mRNA extraction. Total RNA was isolated with TRIZOL reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (0.5 μg) was used for cDNA synthesis with the Maxime RT-PCR PreMix Kit (Intron, Seongnam, Republic of Korea). The following primers and conditions were used for PCR in rabbit articular chondrocytes: for type II collagen (370-bp product), 5′-GACCCCATGCAGTACATGCG-3' (sense) and 5′-AGCCGCCATTGATGGTCTCC-3' (antisense) with an annealing temperature of 52 °C; for type I collagen (441-bp product), 5′-GGCTTTCCTGGAGAGAAAGG-3' (sense) and 5′-ATAGAACCAGCAGGGCCAGG-3' (antisense) with an annealing temperature of 60 °C; for SOX-9 (386-bp product), 5′-GCGCGTGCAGCACAAGAAGGACCACCCGGATTACAAGTAC-3' (sense) and 5′-CGAAGGTCTCGATGTTGGAGATGACGTCGCTGCTCAGCTC-3' (antisense) with an annealing temperature of 60 °C; for GAPDH (299-bp product), 5′-TCACCATCTTCCAGGAGCGA-3' (sense) and 5′-CACAATGCCGAAGTGGTCGT-3' (antisense) with an annealing temperature of 50 °C. The primers were obtained from Genotech (Daejeon, Republic of Korea). PCR products were resolved on a 1% agarose gel and visualized with UV light after ethidium bromide staining.

2.4. Immunofluorescence staining

Cells were fixed with 3.5% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS. The cells were then blocked with 5% skim milk to prevent non-specific reactions. The fixed cells were washed and incubated for 2 h with antibodies against type II collagen (1:100) and pERK. The cells were washed and incubated with secondary antibodies for 1 h, followed by washing with PBS. The fluorescence images were recorded with a BX51 fluorescence microscope (Olympus, Tokyo, Japan).

2.5. Immunohistochemical staining

Rabbit articular cartilage specimens were fixed in 4% formaldehyde, embedded in paraffin, and cross-sectioned. For immunohistological analysis of type II collagen, sections were deparaffinized in xylene and rehydrated in graded alcohol. After washing with PBS, the sections were first incubated with 3% hydrogen peroxide for 15 min. The sections were immersed in 0.1 M sodium citrate buffer (pH 7.2) and heated at 90 °C in a water bath for 40 min. Next, sections were blocked with 10% fetal bovine serum for 10 min and incubated for 1 h at room temperature with a mouse anti-type II collagen antibody (1:50 dilution) as the primary antibody. The primary antibody was detected using the avidin–biotin conjugate method according to the instructions provided in the DAKO kit (Dako Cytomation, Copenhagen, Denmark). Peroxidase activity was detected using the DAKO DAB kit (Dako Cytomation). After the sections were rinsed with PBS, the nuclei were counterstained with