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2-Deoxy-D-glucose Regulates Dedifferentiation but not Cyclooxygenase-2 Expression through Reorganization of Actin Cytoskeletal Architecture in Rabbit Articular Chondrocytes

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Actin cytoskeletal architecture is believed to be a crucially important modulator of chondrocyte phenotype. 2DG (2-Dexoy-D-glucose) induces reorganization of actin cytoskeletal architecture in chondrocytes. In this study, we have investigated the effects of 2DG on dedifferentiation and inflammation via reorganization of cytoskeletal architecture in rabbit articular chondrocytes, with a focus on p38 kinase pathway. Treatment of 2DG alone reduced type II collagen and COX-2 expression in chondrocytes. But, 2DG reduced type II collagen was recovered by CD, disruptor of actin cytoskeletal architecture, whereas did not affect on COX-2 expression and production of PGE_2 compared with 2DG alone treated cells. Treatment of 2DG with JAS, inducer of cytoskeletal architecture polymerization, accelerated reduction of type II collagen expression and synthesis of proteoglycan but did not affect on COX-2 expression and production of PGE_2. Also, 2DG stimulated activation of p38 kinase. This result showed that 2DG regulates type II collagen but not cyclooxygenase-2 expression through reorganization of cytoskeletal architecture via p38 kinase pathway in rabbit articular chondrocytes.

Key Words: Actin, Cytoskeleton, Dedifferentiation

INTRODUCTION

Cartilage is the smooth, glistening white tissue that covers the surface of all the diarthrodial joints (Archer et al., 1994). Chondrocytes of articular cartilage are differentiated from mesenchymal cells during embryonic development (Sandell and Adler., 1999; DeLise, 2000). It is composed of compact extracellular matrix (ECM) including collagen and proteoglycan. The phenotype of differentiated chondrocyte is distinguished by type II collagen expression and synthesis of proteoglycan such as aggrecan. Normal chondrocytes are required for sufficient amounts of cartilage-specific extracellular matrix. Destruction of extracellular matrix such as type II collagen and proteoglycan lead to arthritis by structural and biochemical changes in chondrocytes (Sandell and Aigner, 2001). Therefore, Synthesis and maintenance of extracellular matrix (ECM) including collagen and proteoglycan are important for function of articular chondrocytes (Poole, 1999; Sandell and Adler, 2001).

Cyclooxygenase (COX) is a key enzyme required for the conversion of arachidonic acid to prostaglandin. Two types of COX, COX-1 and COX-2, have been identified. Under various conditions, COX-1 is produced constitutively and constantly in many types of cells whereas COX-2 is produced inducibly and transiently by response to various stimuli (Wu, 1995; Dubois et al., 1998; Smith et al., 2000). In arthritis, COX-2 is expressed in chondrocytes and cynoviocytes (Smith et al., 1996).

Cells were composed of cytoskeleton such as actin filaments, intermediate filaments, and microtubules (Chang and Goldman, 2004). The cytoskeleton was involved in maintaining of cell shape and structure, and gives it support along with their responsibility with movement around the cells. The modulation of phenotype is associated with significant

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change in actin cytoskeletal architecture (Kim et al., 2003). In previous reported that various cellular responses such as differentiation, proliferation, apoptosis, and inflammation were regulated by changes in cell morphology. So, actin cytoskeletal architecture is believed to be a crucially important modulator of cellular responses (Benya et al., 1988; Brown, 1988).

2-Deoxy-D-gucose (2 DG) is a synthetic analogue of glucose, is known as an inhibitor of glycosylation and glycolysis in a variety of cells (Woodward and Hudson, 1954; Wick, 1957). Up until now, it was studied that 2DG regulates cell responses such as proliferation and apoptosis with focus on cancer cells (Halicka et al., 1995; Zhang et al., 2006; Coleman et al., 2008). But, the effects of 2DG are not well understood in normal cells including chondrocytes.

In this study, we have investigated the role and modulator mechanism of 2DG on differentiation and inflammation in rabbit articular chondrocytes, focusing on the role of p38 pathway. Here, we found that dedifferentiation by 2DG were regulated by reorganization of cytoskeletal architecture through activation of p38 kinase but not inflammation in rabbit articular chondrocytes.

MATERIALS AND METHODS

1. Culture of rabbit articular chondrocytes and experimental conditions

Rabbit articular chondrocytes were released from joint cartilage of 2-week-old New Zealand White rabbits by enzymatic digestion. To summarize, cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 units/ml of solid, sigma) and individual cells were suspended in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg) supplemented with 10% (v/v) bovine calf serum, 50 μg/ml streptomycin, and 50 units/ml penicillin and were then plated on culture dishes at a density of 5×10^4 cells/cm^2. The medium changed every 2 days after seeding. The cells from day 3 cultures were treated with the indicated pharmacological reagents. We used 2-deoxy-D-glucose (Sigma, Missouri USA), Cytochalasin D (sigma) and Jasplakinolide (molecular probes). The medium changed with free-glucose medium (DMEM, Gibco-BRL, Gaithersburg) before treating of the indicated pharmacological reagents

2. Western blot analysis

For Western blot analysis, whole cell lysates were prepared by extracting protein 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. Protein were separated by SDS-poly acrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose sheet was blocked with 5% non-fat milk in Tri-buffered saline, and proteins were detected using the following antibodies: human anti-goat type II collagen (Santacruz, CA, USA), murine anti-COX-2 (Cayman Chemical, MI, USA), human anti-rabbit p42/44 (Cell Signaling, MA) for 2 h, washed three times with TBST, incubated with secondary antibodies conjugated with horseradish peroxidase (Sigma).

3. Alcian blue staining

The cells were fixed with 95% methanol at -20℃ for 2 min and stained with the 0.1% Alcian blue in 0.1 M HCl for overnight. The chondrocytes were washed three times with PBS buffer and then added the 6 M guanidine HCl for 6 h. Production of sulfated proteoglycan was measured at 620 nm by Enzyme-Linked Immunosorbent Assay (ELISA).

4. PGE_2 assay

PGE_2 production was determined by measuring the levels of cellular and secreted PGE_2 using a PGE_2 assay kit (Assay Designs, MI, USA). Briefly, chondrocytes were seeded in standard 96-well microtier plates at a density of 2×10^4 cells/well. Following addition of the various pharmacological reagents, total cell lysates were used to quantity the amount of PGE_2, according to the manufacturer's protocol. PGE_2 levels were calculated against a standard curve of PGE_2.

5. Immunofluorescence staining

Rabbit chondrocytes were fixed in 3.5% paraformaldehyde in PBS for 15 min at room temperature, washed three times with PBS. The cells were permeabilized with 0.1% Triton X-100 in PBS. The fixed cells were washed

three times with PBS and incubated with rhodamine-conjugated phalloidin for 1 h, and observed under a fluorescence microscope.

RESULTS

1. Actin cytoskeletal architecture is regulated by 2DG in rabbit articular chondrocytes

We initially determined the effects of 2DG on the changes of actin cytoskeletal architecture, as determined by immunofluorescence staining for F-actin conjugated rhodamine. As expected, Treatment of 2DG caused reorganization of actin cytoskeletal architecture to round shape of cells. Also, CD and JAS induced the changes of actin cytoskeletal architecture in the different types of pattern, respectly. Interestingly, cells of 2DG treated with CD or JAS were showed different patterns of actin cytoskeleton compared with 2DG only treated cell. The above result indicates that 2DG induces reorganization of cytoskeletal architecture.

2. Dedifferentiation but not COX-2 expression is modulated by 2DG through reorganization of actin cytoskeleton in chondrocytes

The structural changes of actin cytoskeleton are known to regulate cellular response, and our previous study indicated that actin cytoskeleton acts as a modulator of dedifferentiation and inflammation. Also, 2DG leads to expression of COX-2 protein with a molecular weight of 66~70 kDa as compared with the generally expressed 72~74 kDa protein by inhibiting glycosylation. To examine the effects of 2DG on differentiation and inflammation, we detected the expression of type II collagen and COX-2 in chondrocytes. Treatment of 2DG reduced type II collagen, a maker for dedifferentiation of chondrocytes, and induced COX-2 expression as determined by Western blot analysis. Also, 2DG reduced type II collagen was inhibited by CD but did not effects on COX-2 expression whereas 2DG reduced type II collagen was accelerated by JAS but did not affect on COX-2 expression. These results indicate that 2DG induces dedifferentiation through reorganization of cytoskeletal architecture but did not effect on COX-2 expression. COX-2 expression by 2DG was regulated by the other mechanisms not reorganization of actin cytoskeltal architecture.

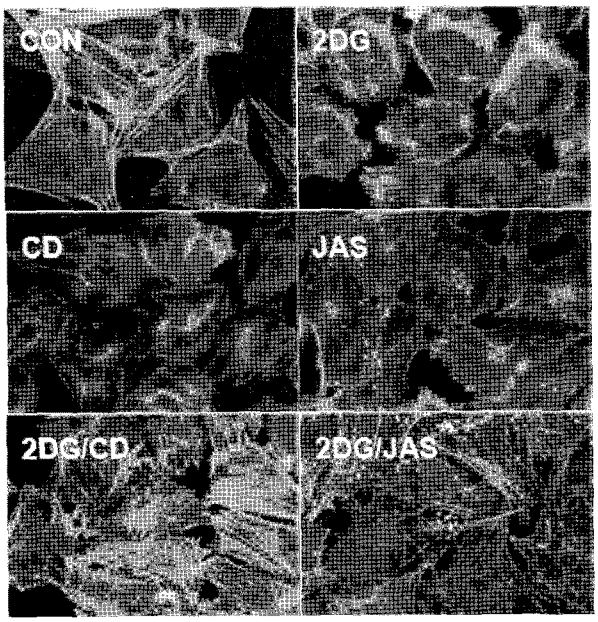

Fig. 1. Actin cytoskeletal architecture is regulated by 2DG in rabbit articular chondrocytes. Rabbit articular chondrocytes were treated with 5 mM 2DG, 0.1 μM CD, and 50 nM JAS or treated with 5 mM 2DG in the presence of CD or JAS for 24 h. Chondrocytes were stained for F-actin with rhodamine-conjugated phalloidin and analyzed by immunofluorescence microscopy. The data represent the results of a typical experiment conducted at least three times with similar results.

3. Type II collagen expression but not COX-2 expression is regulated by 2DG via reorganization of cytoskeletal architecture in articular chondrocytes

The effect of 2DG on sulfated proteoglycan synthesis and PGE_2 production was investigated using Alcian blue staining and PGE_2 assay. 2DG only reduced sulfated proteoglycan synthesis and PGE_2 production in articular chondrocytes. As expected, however, sulfated proteoglycan synthesis was regulated by 2DG with CD or JAS, but CD and JAS did not effect on PGE_2 production by 2DG. These data indicate that 2DG not only causes sulfated proteoglycan synthesis but also stimulates PGE_2 production. But, PGE_2 production by 2DG was regulated by the other mechanisms not reorganization of actin cytoskeltal architecture.

4. Type II collagen expression is regulated by 2DG through p38 pathway in association with reorganization of cytoskeleton in chondrocytes

To investigate the signaling pathway stimulating dedifferentiation of 2DG treated cells, we initially examined changes in the activity of ERK-1/2 and p38 kinase. These two kinase subtypes were selected for study, based on results from previous results indicating that the proteins regulate chondrocyte differentiation. When chondrocytes were treated with 2DG, p38 activity was increased, as determined by Western blot analysis. The p38 kinase activity was increased as determined by phosphorylation status of the protein. But, level of ERK1/2 phosphorylation was not changed by 2DG (data not shown). This result indicates that activity of p38 kinase is required for dedifferentiation by 2DG.

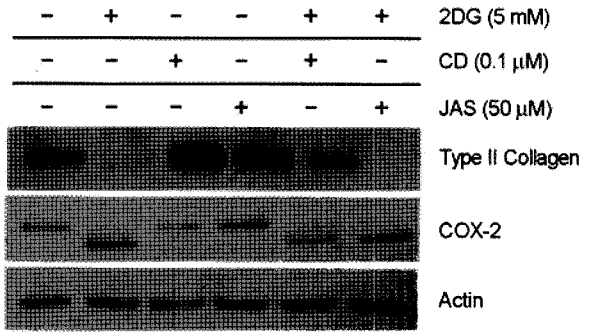

Fig. 2. Type II collagen expression but not COX-2 expression is regulated by 2DG via reorganization of cytoskeletal architecture in articular chondrocytes. Rabbit articular chondrocytes were treated with 5 mM 2DG, 0.1 μM CD, and 50 nM JAS or treated with 5 mM 2DG in the presence of CD or JAS for 24 h. Expression of type II collagen and COX-2 was determined by Western blot analysis. Actin was detected as loading control. The data shown represent a typical result from at least four independent experiments.

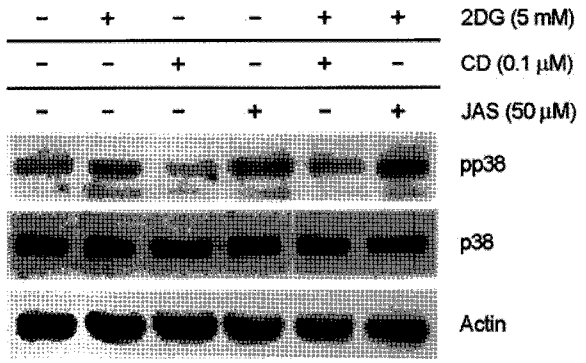

Fig. 4. Type II collagen expression but not COX-2 is regulated by 2DG through p38 pathway in associate with reorganization of cytoskeleton in chondrocytes. Rabbit articular chondrocytes were treated with 5 mM 2DG, 0.1 μM CD, and 50 nM JAS or treated with 5 mM 2DG in the presence of CD or JAS for 24 h. Protein levels of p38 and pp38 were detected by Western blot analysis. Actin was detected as loading control. The data represent the results of a typical experiment conducted at least three times.

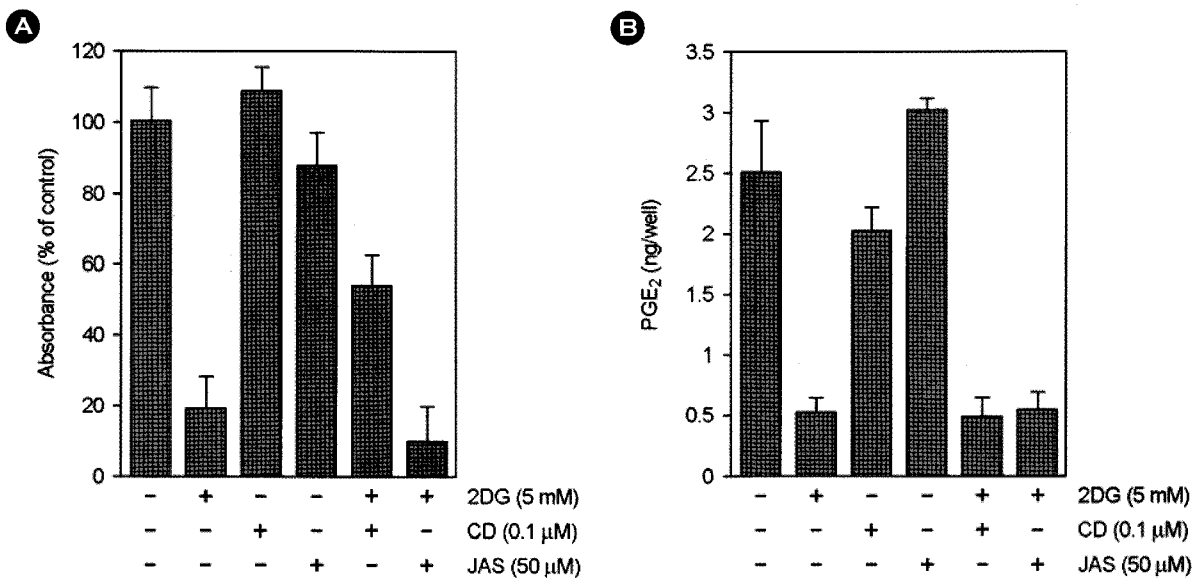

Fig. 3. Type II collagen expression but not COX-2 expression is regulated by 2DG via reorganization of cytoskeletal architecture in articular chondrocytes. Rabbit articular chondrocytes were treated with 5 mM 2DG, 0.1 μM CD, and 50 nM JAS or treated with 5 mM 2DG in the presence of CD or JAS for 24 h. The accumulation of sulfated proteoglycan was determined by Alcain blue staining **(A)**. Levels of cellular and secreted PGE_2 were determined by using a PGE_2 assay kit. The data represent the average values with standard deviation (n=4).

DISCUSSION

Actin cytoskeletal architecture is believed to be a crucially important regulator of chondrocyte phenotype (Kim et al., 2003). 2DG is a stable glucose analogue that is widely used as a competitive inhibitor of glucose (Woodward and Hudson, 1954; Wick, 1957). Interestingly, we have shown that 2DG induced reorganization of cytoskeletal architecture in chondrocytes. Our previous studies in articular chondrocytes demonstrated that actin cytoskeletal architecture by using CD and JAS regulated defferentiation and inflammation of articular chondrocytes were modulated by extracellular signal regulated protein kinase-1/2 (ERK-1/2) and p38 kinase (Kim et al., 2003).

The results of the present study demonstrate that 2DG regulates dedifferentiation by reorganization of cytoskeletal architecture in articular chondrocytes, focusing on p38 kinase signaling. 2DG treated cells with CD reduced dedifferentiation whereas with JAS accelerated dedifferentiation. In contrast, 2DG with CD or JAS treated chondrocytes did not effects upon inflammation response such as COX-2 expression and production of PGE_2 compared with 2DG alone treated cells as shown by Western blot analysis, Alcian blue staining and PGE_2 assay in Fig. 2 and 3. Also, we demonstrated that activation of p38 kinase by 2DG treatment is required for dedifferentiation via reorganization of cytoskeletal architecture. Therefore further studies are necessary to explain collectively signaling mechanism by using inhibitor of p38 kinase. In summary, our results suggest that 2DG causes dedifferentiation through reorganization of cytoskeletal architecture but not cyclooxygenase-2 expression in rabbit articular chondrocytes.

Acknowledgements

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