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Impaired glycolytic metabolism causes chondrocyte hypertrophy-like changes *via* promotion of phospho-Smad1/5/8 translocation into nucleus

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SUMMARY

Objective: Hypertrophy-like changes are often observed in chondrocytes during the development of osteoarthritis (OA). These changes play a crucial part in the OA-associated cartilage degradation and osteophyte formation. However, the pathogenesis leading to such changes is still unknown. In this study, we investigated the mechanism by which these hypertrophy-like changes are induced from the viewpoint of impaired glycolytic metabolism.

Methods: The effect of sodium fluoride (NaF) on glycolytic metabolism of cultured chondrocytes was confirmed by measurement of intracellular adenosine triphosphate (ATP) production. Translocation of phosphorylated Smad1/5/8 to the nucleus was evaluated by subcellular fractionation and Western blotting. Chondrocyte hypertrophy-like changes were investigated by real-time RT-PCR and Western blot analysis of differentiation markers.

Results: ATP production was dose-dependently decreased by NaF in the human chondrocytic cell line HCS-2/8. In addition, both chondrocyte proliferation and differentiation were inhibited, whereas cell death was promoted by treatment with NaF. Interestingly, combinational treatment with NaF and lactate enhanced translocation of phospho-Smad1/5/8 to the nucleus, as well as gene expression of *ALP*, *VEGF*, *COL10a1*, and matrix metalloproteinase13 (*MMP13*), which were the markers of late mature and hypertrophic chondrocytes. Furthermore, the production of type X collagen and activation of MMP9 were also promoted under the same conditions.

Conclusions: These findings suggest that decreased ATP production by NaF promotes hypertrophy-like changes *via* activation of phospho-Smad1/5/8 in the presence of lactate. Novel metabolic aspects of OA pathogenesis are indicated herein.

Introduction

Osteoarthritis (OA) is one of the most prevalent joint diseases and is characterized by an age-dependent, slowly progressive degeneration of articular cartilage[1]. In OA, distinct stages can be recognized during disease progression in terms of the degeneration of cartilage[1]. At the initiation stage, the behavior of the chondrocytes becomes altered due to aging or excess mechanical stress, and the cells acquire a hypertrophy-like phenotype, producing type X collagen and matrix metalloproteinase13 (MMP13)[1,2]. Next, during the progression stage, the release and activation of MMP13 lead to degradation of the extracellular matrix (ECM) surrounding the chondrocytes; and the fragmented ECM molecules, forming an altered biomechanical environment, influence these chondrocytes such that the hypertrophy-like changes become accelerated[1,2]. Thereafter, at the late and end stages, chondrocyte clusters, which are formed in an unsuccessful attempt to repair the cartilage tissue, appear in severely damaged cartilage and peri-articular formation of new bone (osteophytosis) occurs[1,2]. Eventually, the cartilage tissue degenerates, thus exposing the underlying bone[1,2]. Under such conditions, this disease clinically leads to loss of joint function[3]. The incidence of OA is thought to be associated with many risk factors, such as metabolic disease, aging, obesity, and mechanical stress[1,2]. However, the pathogenic mechanism, in

Abbreviations: OA, osteoarthritis; NaF, sodium fluoride; MMPs, matrix metalloproteinases; ECM, extracellular matrix; MCTs, monocarboxylate transporters; ATP, adenosine triphosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; GAG, glycosaminoglycan; BMP, bone morphogenetic protein; ACAN, aggrecan.

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particular, the mechanism governing regulation of these hypertrophy-like changes at the initiation stage in OA, is still unclear.

To estimate possible factors involved in the hypertrophy-like changes, we focused on two experimental models[4]. One of the models is an arthritis model induced by the intra-articular injection of monoiodoacetate (MIA), which inhibits the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), one of the glycolysis enzymes[5]. In this model, widespread chondrocytes are depleted throughout the cartilage tissue, cartilage ECM is also lost in the central part of the articular cartilage, and, furthermore, chondrocytes form clusters at the surface of articular cartilage[4]. The other one is the K/BxN mouse model, in which a spontaneously erosive arthritis is manifested[6,7]. The phenotype is characterized by many features common to rheumatoid arthritis (RA), including leukocyte invasion, synoviocyte proliferation, and erosion of cartilage and bone[6,7]. Recently, it was reported that the arthritis in the K/BxN mouse is caused by autoantibody against glucose-6-phosphate isomerase (GPI), which is another of the glycolysis enzymes[7]. Since these two animal models commonly exhibit severe cartilage destruction, this cartilage destruction may be caused by inhibition of the glycolysis enzymes. Therefore, we hypothesized that a part of cartilage destruction might be triggered by dysregulation of glucose metabolism in chondrocytes. Indeed, it is well known that cartilage is an avascular tissue and that chondrocytes generate energy in the form of adenosine triphosphate (ATP), mostly dependent on anaerobic metabolism of glucose[8]. Therefore, if this metabolism is impaired, intracellular ATP production is profoundly decreased; and the chondrocytes are forced to degenerate, following the pathway to hypertrophy-like changes.

Bone morphogenetic protein (BMP) signaling has been already known to be required for onset of chondrocyte hypertrophy[9]. Since Smad1/5/8 signaling, is a canonical pathway of BMP signaling, involvement of this signaling pathway in the hypertrophy-like changes due to impaired glycolysis is suspected. To examine this hypothesis, we used an *in vitro* culture system employing primary rat chondrocytes isolated from epiphyseal cartilage[10] and two useful immortalized chondrocytic cells lines, human chondrocytic HCS-2/8 cells[11–13] and rat chondrocytic RCS cells[14]. In the present study, we clarified that intracellular ATP production decreased by a few specific inhibitors of glycolysis caused chondrocyte hypertrophy-like changes *via* activation of Smad1/5/8 by lactate, which is an end product of glycolysis.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), α-modification of Eagle's medium (αMEM), and fetal bovine serum (FBS) were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan), ICN Biomedicals (Aurora, OH), and Nichirei Bioscience Inc. (Tokyo, Japan), respectively. Plastic dishes and multi-well plates were obtained from Greiner Bio-One (Frickenhausen, Germany). Sodium fluoride (NaF) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium lactate, iodoacetate sodium salt, anti-β-actin, anti-proliferating cell nuclear antigen (PCNA), and anti-type X collagen antibodies were obtained from Sigma (St. Louis, MO). Anti-monocarboxylate transporter (MCT)2, anti-MMP13, and anti-SOX9 antibodies were from Millipore (Temecula, CA). Anti-MMP9 was purchased from Triple Point Biologics, Inc. (Forest Grove, OR); and anti-nuclear factor (NF)-κB p65 and anti-MCT1, from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Smad1/5/8 from Cell Signaling Technology (Beverly, MA) and anti-lamin B1 from Life Technologies (Grand Island, NY) were also employed.

Cell culture

HCS-2/8[11–13] cells were inoculated at a density of 4×10^4 cells/cm^2 into culture dishes containing DMEM supplemented with 10% FBS and incubated at 37°C under 5% CO_2. RCS cells[14] were cultured in monolayer, pellet and collagen gel. For monolayer, RCS cells were seeded at a density of 2.5×10^4 cells/cm^2 in DMEM containing 10% FBS and incubated at 37°C under 5% CO_2. For pellet culture, 5×10^6 cells per pellet were centrifuged and then cultured at 37°C under 5% CO_2. For collagen gel 3-dimensional (3-D) culture, the cells were embedded at a density of 2×10^5 cells/dish into collagen scaffold (Cellmatrix; Nitta Gelatin Inc. Osaka, Japan) and cultured under normoxia or hypoxia (5% O_2) by using multigas incubator (BIO-LABO, Tokyo, Japan) at 37°C. Rat chondrocytes were isolated from the epiphyseal cartilage of 5-day-old Wistar rats as described previously[10]. The isolated chondrocytes were seeded at a density of 1×10^4 cells/cm^2 and cultured at 37°C under 5% CO_2 in αMEM containing 10% FBS. The Animal Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences approved all of the procedures.

Cell proliferation assay

HCS-2/8 cells were inoculated into a 96-well multi-plate at a density of 3×10^4 cells/well; and the next day the medium was replaced with serum-free medium containing NaF at various concentrations (1, 5 and 10 mM). Then the cells were cultured for 16 h. The effect of NaF on cell proliferation was determined by performing a cell proliferation enzyme-linked immunosorbent assay (ELISA), 5-bromo-2′-deoxyuridine (BrdU) colorimetric (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol[15].

Detection of intracellular ATP

For measurement of intracellular ATP, an ATP bioluminescence assay kit was obtained and used according to manufacturer's (Roche Applied Science) recommendations. Briefly, after HCS-2/8 cells had been treated with NaF in the absence or presence of sodium lactate for 16 h, they were harvested. The cells were then boiled for 2 min for the preparation of cell lysates, and 50 μl of samples/standards was transferred to a white plate. Then, the luciferase reagent was added, and the emitted light was measured with a luminometer (Fluoroskan Ascent FL; Thermo Labsystems, Franklin, MA).

Detection of cytotoxicity

For detection and quantification of cell death by NaF, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of rat chondrocytes and measurement of the lactate dehydrogenase (LDH) activity in conditioned media of HCS-2/8 cells by using an *in situ* cell death detection kit and cytotoxicity detection kit (Roche Applied Science), respectively.

Luciferase assay

The firefly luciferase reporter construct containing the Smad binding element (SBE) and hypoxia response element (HRE) at -1006/-954 of VEGF promoter[16] and herpes simplex virus thymidine kinase promoter-*Renilla* luciferase reporter plasmid (pRL-TK, internal control: Promega, Madison, WI) were used. HCS-2/8 cells were transfected with 0.9 μg of reporter plasmid in combination with 0.1 μg of pRL-TK using Fugene6 reagent (Roche, Basel, Switzerland). The dual luciferase system (Promega) was used as described previously[16].