Glucosamine Regulates Differentiation of a Chondrogenic Cell Line,
ATDC5

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Osteoarthritis (OA) is a slowly progressing chronic joint disease. Glucosamine (GlcN) is a saccharide that is widely used to relieve symptoms associated with OA. However, the mechanism of the effects of GlcN on articular cartilage remains unclear. We studied the effects of GlcN and its analogues, including chitin derivatives included in health supplements containing GlcN, on a chondrogenic cell line, ATDC5. We examined the effects of these saccharides on the proliferation and differentiation of ATDC5 cells. Glucosamine analogues, such as N-acetyl glucosamine and chitobiose, did not affect the proliferation or differentiation of ATDC5 cells. While GlcN did not affect the proliferation of ATDC5 cells, it inhibited their differentiation. Next, we examined whether GlcN affects mineralization and glycosaminoglycan (GAG) production by ATDC5 cells. Mineralization was markedly inhibited by addition of GlcN to the cell culture medium. Moreover, GlcN induced the formation of sulfated GAG in ATDC5. We also analyzed the mRNA levels in ATDC5 cells. GlcN reduced the mRNA levels of Smad2, Smad4 and MGP. GlcN might inhibit expression of MGP mRNA and induce the production of chondroitin sulfate in ATDC5 cells. The mechanism by which GlcN inhibits mineralization may be by regulating the expression of mRNA for the Smad2 and Smad4 chondrogenic master genes.

Key words glucosamine; ATDC5; chondrocyte; glycosaminoglycan; Smad

The elasticity and compression resistance of cartilage are maintained by chondrocytes and the cartilage extracellular matrix. Osteoarthritis (OA) is caused by interactions among various factors such as aging, irregular dietary habits, disease, medication and mechanical stress. Osteoarthritic cartilage leads to reduction in the cartilage extracellular matrix and ectopic mineralization of cartilage.1—3 Glucosamine (GlcN) has been administered in recent years to reduce the pain associated with OA.4 GlcN is one of the basic constituents of complex saccharides such as hyaluronan and chondroitin sulfate. Hyaluronan is a straight-chain macromolecule mucopolysaccharide in which N-acetyl glucosamine and glucuronic acid are linked.5 Chondroitin sulfate consists of N-acetyl galactosamine and glucuronic acid and is present in cartilage as side chains of aggregan.6 Alleviation of OA symptoms as a result of GlcN intake has been reported at the clinical level.7,8 Much of the detailed mechanism underlying this alleviation of OA symptoms as a result of GlcN intake, however, remains unknown. Although chitin hydrolyzates are used in health supplements containing GlcN, the effectiveness of the GlcN analogues derived from chitin, such as N-acetyl glucosamine and chitobiose, has not been studied.

Endochondral ossification is the morphosis of bone in which chondrocytes differentiate from mesenchymal cells and then are replaced by bone as a result of subsequent proliferation, maturation and hypertrophy.9 In this process, some tissues, such as articular cartilage and costicartilage, remain as cartilage without being replaced by bone. Much of the detailed mechanism underlying this cartilage retention is unknown. Various transcription factors mediate the proliferation and differentiation of chondrocytes by regulating production of the extracellular matrix during chondrogenesis and osteogenesis.10,11 In the stage prior to mineralization of cartilage, the level of alkaline phosphatase (ALP) activity was elevated.12 On the other hand, mature chondrocytes produce glycosaminoglycans (GAGs) such as hyaluronan and chondroitin sulfate and are responsible for maintaining the properties of cartilage.13 Type II collagen (Col2) is a well-known component of the cartilage extracellular matrix. In the course of chondrogenesis, Col2 is present in the extracellular matrix in differentiating and mature chondrocytes. However, Col2 was reported to disappear during the course of osteogenesis, while chondrocytes were reported to disappear during in the mineralization phase.14 Matrix gla protein (MGP) is another cartilage extracellular matrix protein. MGP has been detected in the vascular endothelial cells of arteriosclerotic lesions, so MGP is thought to be closely associated with mineralization.15,16 Bone gla protein (BGP) is a bone-specific matrix protein. BGP increases as chondrogenesis and osteogenesis progress and plays an important role in maintenance of the mineralized bone matrix.17

Bone morphogenetic proteins (BMPs) and transforming growth factor β (TGF-β) are well-known growth factors that regulate the proliferation and differentiation of chondrocytes.18 After binding to their receptors located in the cell membrane, BMPs and TGF-β phosphorylate the Smad family and regulate the expression of cartilage-specific genes.19 Smad2, a downstream effector of a TGF-β receptor activation, a factor for bone formation, and is known to be a transcription factor that promotes differentiation of chondrocyte.19,20 In addition, Smad2 is known to regulate expression of cartilage-specific genes present downstream of Sox9 and Runx2 by forming heterodimers with those transcription factors.21,22 Smad4 forms heterodimers with phosphorylated Smad2 and other Smad subtypes and is known to regulate differentiation of cartilage through interaction with various transcription factors.19,23 Sox9 is a transcription factor belonging to the Sox family and is reported a master gene of chondrogenic differentiation that regulates induction of mesenchymal cell differentiation into chondrocytes and inhibition of differentiation into hypertrophic chondrocytes.24,25 Runx1 is known to be a transcription factor that is expressed

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in hypertrophic chondrocytes and is necessary for endochondral ossification. Runx2 is a bone-specific transcription factor that is expressed in hypertrophic chondrocytes and is essential for replacement of cartilage by osteocytes.

Previously we showed that calcium and magnesium regulated matrix mineralization positively and negatively, respectively, in a chondrogenic cell line, ATDC5. GAGs are also important components of cartilage. In this study, using ATDC5, we examined the effects of GlcN and its analogues on mineralization, differentiation and proliferation of chondrocytes as well as expression of cartilage-tissue-specific genes. We also examined the mechanism by which GlcN affects cartilage metabolisms.

**MATERIALS AND METHODS**

**Cell and Culture Conditions** A chondrogenic cell line, ATDC5, was used. ATDC5 was purchased from the RIKEN Cell Bank. The cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 (DMEM/F12) medium (Invitrogen, Japan) supplemented with 5% fetal bovine serum (Invitrogen, Japan) and penicillin (50 IU/ml)—streptomycin (50 μg/ml). Cells were subcultured every second day by using trypsin/EDTA (Sigma-Aldrich, Japan) and maintained at 37 °C in a humidified atmosphere of 5% CO₂/95% air.

**GlcN and Its Analogues** GlcN and its analogues in this study were kind gifts from Yaizu Suisankagaku Industry (Shizuoka, Japan). Glucose (Glc), glucosamine hydrochloride (GlcN), N-acetyl glucosamine (GlcNAc), chitosan dimer (GlcN₂), chitobiose (GlcNAc₂), lactosamine (Gal-GlcN), and N-acetyl lactosamine (Gal-GlcNAc) were each added to the culture medium to a final concentration of 1 mg/ml in order to examine the effects of GlcN and its analogues on the differentiation of ATDC5.

**Normal culture medium (N)** was used as a control.

**Alkaline Phosphatase Staining** The ALP staining solution contained 0.05 M AMP Buffer (pH 9.8), 10 mM naphthol AS-BI phosphate (Sigma-Aldrich, Japan) and 1 mM Fast Red (Sigma-Aldrich, Japan). ATDC5 cells were cultured in 24-well plates at approximately 2.5 × 10⁴ cells per well. After culturing for 1 d, GlcN and its analogues were added and the cells were cultured for 3 d. During culturing, the culture medium was replaced every 3 d. Then the cells were post-fixed in 20% formalin, rinsed twice with water and then allowed to react with AR staining solution at room temperature for 10 min; they were then rinsed by water and mineralization was assessed. After culturing for 1 d, GlcN and its analogues were added and the cells were cultured for 35 d, the culture medium was replaced every 3 d. After culturing, the cells were post-fixed in 20% formalin, rinsed twice with water and then allowed to react with AR staining solution at room temperature for 10 min; the signals were normalized relative to the control culture.

**Alizarin Red Staining** Alizarin red (staining) is a method for examining sites of calcium deposition. For AR staining, a 1% AR solution (pH 6.38) was prepared from Alizarin Red S-Certified (Sigma-Aldrich, Japan) and 28% aqueous ammonia. ATDC5 cells were cultured in 24-well plates at approximately 2.5 × 10⁴ cells per well. After culturing for 1 d, GlcN and its analogues were added and the cells were cultured for 35 d, the culture medium was replaced every 3 d. Then the cells were post-fixed in 20% formalin, rinsed twice with water and then allowed to react with AR staining solution at room temperature for 10 min; the signals were normalized relative to the control culture.

**RNA Extraction and RT-PCR** ATDC5 cells were cultured in 6-cm dishes at approximately 5 × 10⁵ cells per dish. After culturing for 1 d, GlcN and its analogues were added and the cells were cultured for 3 d. RNA was extracted using TRIZOL Reagent according to the manufacturer’s instructions (Invitrogen, Japan). After dissolution in DPC-treated water, the extracted RNA was quantified using a spectrophotometer. cDNA was prepared from 1 μg of total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Japan). Amplification was performed in 10 μl reaction mixture and using EX Taq (Takara Bio, Japan). The primer sequences used for each PCR are outlined below. Initial denaturation was performed at 94 °C for 30 s, annealing temperatures ranged from 58—64 °C for 30 s, and extension was performed at 72 °C for 3 min to conclude the reaction. Primers for the Smad2 were 5'-CCCCACTCTATCCAGAAAAAC-3' (upstream) and 5'-GGAAACCTGCATTTGGT-GTT-3' (downstream); for Smad4, 5'-GCCTTACTACCCAC-CTGAAAGGA-3' (upstream) and 5'-GGATTTACTGTCAGCCAC-CCAGC-3' (downstream); for Sox9, 5'-ATGTCGAGGAAC-TCTGGGTGGTTCGCCCTGT-3' (upstream) and 5'-ATTCC-TCCTCCCGGATGAGTTGGTGCAC-3' (downstream); for Runx1, 5'-ACTTCTTCTGGTCTCCGCTA-3' (upstream) and 5'-GTTACGAGAATTCAACGACC-3' (downstream); for Runx2, 5'-ATGGTGGAGATCCGACGACC-3' (downstream) and 5'-CCATCTCTCTCCAGATGTT-3'; for Runx1, 5'-ACTTCTTCTGGTCTCCGCTA-3' (upstream) and 5'-GTTACGAGAATTCAACGACC-3' (downstream); for Runx2, 5'-ATGGTGGAGATCCGACGACC-3' (downstream) and 5'-CCATCTCTCTCCAGATGTT-3'; for Runx1, 5'-ACTTCTTCTGGTCTCCGCTA-3' (upstream) and 5'-GTTACGAGAATTCAACGACC-3' (downstream); for Runx2, 5'-ATGGTGGAGATCCGACGACC-3' (downstream) and 5'-CCATCTCTCTCCAGATGTT-3'; for Runx1, 5'-ACTTCTTCTGGTCTCCGCTA-3' (upstream) and 5'-GTTACGAGAATTCAACGACC-3' (downstream); for BGP, 5'-ATGACCTCTGACCCAGACACACCAT-3' (upstream) and 5'-CCCTGCAAGTAGTTACACAGACACCAT-3' (upstream); for BGP, 5'-ATGACCTCTGACCCAGACACACCAT-3' (upstream) and 5'-CCCTGCAAGTAGTTACACAGACACCAT-3' (upstream); for BGP, 5'-ATGACCTCTGACCCAGACACACCAT-3' (upstream) and 5'-CCCTGCAAGTAGTTACACAGACACCAT-3' (upstream).
CAT-3 (downstream). The PCR products were transferred to a 1.5% agarose gel and visualized with ethidium bromide. All gels were digitally imaged using a flat head scanner and Adobe Photoshop Elements software. Within each series, all adjustments were made in parallel to all gels used for comparison. The band intensities of these digital images were determined using ImageJ software for gels from at least 3 different experiments. The signals were normalized to those of GAPDH transcripts.

RESULTS

Effects of GlcN and Its Analogues on Differentiation and Proliferation of ATDC5 Cells First, we investigated whether GlcN and its analogues affect the ALP activity of chondrocytes. The chondrogenic cell line, ATDC5 was cultured in culture medium to which GlcN or one of its analogues (Glc, GlcNAc, (GlcN)₂, (GlcNAc)₂, Gal-Glc, Gal-GlcN and Gal-GlcNAc) had been added. The ALP activity of the cells determined by ALP staining, and the differentiation of the cells was examined. The results showed that only GlcN inhibited ALP activity (Figs. 1A, B). The GlcN analogues did not affect the ALP activity of the ATDC5 cells. As a result of quantifying the staining using ImageJ image analysis software, comparison of GlcN to Glc, the negative control, indicated that the ALP activity was decreased to about 1/3 of its original level by GlcN. GlcN under the conditions of the current experiments, neither GlcN nor its analogues affected ATDC5 cell proliferation according to an MTT assay (data not shown).

Effects of GlcN on Mineralization of ATDC5 Cells The effects of GlcN on mineralization of ATDC5 cells were studied next. ATDC5 cells were cultured for 35 d in the normal medium and also in the presence of Glc or GlcN. After culturing, the extent of ATDC5 mineralization was determined by AR staining of the cells. The results showed that GlcN markedly inhibited mineralization in comparison to Glc as a negative control (Fig. 2). These results indicated that GlcN, by inhibiting the differentiation of ATDC5 cells, also inhibits their mineralization.

Effects of GlcN on GAG Production of ATDC5 Cells Chondrocytes produce GAGs which are involved in maintenance of cartilage function. Because we found that GlcN inhibited the ALP activity and mineralization of ATDC5 cells, this experiment investigated the effects of GlcN on GAG production by the cells. ATDC5 cells were cultured for 35 d in the normal medium and in the presence of Glc and GlcN, and the distribution of GAGs in the extracellular matrix of the cells was compared by AB staining. Total GAG production was also compared by AB staining (pH 2.5). As a result, the staining area for total GAG in the ATDC5 cells cultured with GlcN was smaller than that in the cells cultured in the normal culture medium or with Glc (Fig. 3A). These results for total GAG staining were similar to those for ALP activity and mineralization. Next, the production of sulfated GAGs was compared by AB staining (pH 1.0). In contrast to the staining for total GAG, the staining area for sulfated GAGs was larger when the cells were cultured with GlcN than with Glc (Fig. 3B). These results indicate that GlcN may induce
sulfated GAG production in ATDC5.

Effects of GlcN on mRNA Levels for Chondrocyte Matrix Genes in ATDC5

GlcN did not completely inhibit ATDC5 differentiation but appeared to induce production of sulfated GAGs. This experiment examined the effects of GlcN on the expression of genes for extracellular matrix proteins, which are also major components of the extracellular matrix of cartilage. The total RNA of the cell cultures was extracted, and the effects of Glc and GlcN on the mRNA levels for chondrocyte matrix proteins, such as Col2, MGP and BGP, were examined by RT-PCR. Exposure to Glc and GlcN did not result in any marked differences in mRNA levels for Col2 and BGP. However, GlcN decreased the level of MGP mRNA to about 2/3 of that with Glc (Fig. 4). Thus, GlcN decreased the level of MGP mRNA, indicating that it affects not only GAG production but also the extracellular matrix of cartilage and regulates ATDC5 differentiation.

Effects of GlcN on mRNA Levels for Chondrogenic Transcription Factors in ATDC5

Because we found that GlcN affected the levels of mRNA for MGP in ATDC5, this experiment investigated whether GlcN affects the levels of mRNA for cartilage-specific transcription factors in ATDC5. The total RNA was extracted from cell cultures, and the effects of GlcN on the levels of mRNA for cartilage-specific transcription factors were determined by RT-PCR. GlcN decreased the levels of Smad2 and Smad4 mRNA to about 2/3 of that with Glc. The levels of mRNA for Runx2, and Sox9 were not affected by GlcN (Fig. 4). These results indicate that GlcN regulates the mRNA levels of cartilage transcription factors such as Smad2 and Smad4.

DISCUSSION

GlcN is currently used as a health supplement to reduce the pain of OA. GlcN sold as a health supplement is often manufactured by hydrolysis of chitin from the shells of shrimp or crabs. In addition to GlcN, hydrolysis of chitin produces compounds like GlcNAc, (GlcN)2 and (GlcNAc)2. A recent study found that not only GlcN but also GlcNAc expresses the chondroprotective activity. Moreover, an in vivo study found that oligosaccharides such as chitosan dimer were detected in the serum study after oral administration of chitosan oligomer and chondroitin sulfate. However, the effects of GlcN analogues on OA are insufficient. Thus, we studied the effects of GlcN and its analogues on chondrocytes by using the ATDC5 cell line.

Our data found that, in comparison to GlcN analogues, GlcN reduced the ALP activity of ATDC5 cells. This suggests that GlcN may work by regulating the differentiation of chondrocytes. The GlcN analogues did not regulate chondrocyte differentiation. Addition of GlcN or its analogues did not affect the proliferation of ATDC5 cells. Thus, GlcN at the concentration used in this study was not cytotoxic to ATDC5 cells.

GlcN is metabolized to GAGs such as hyaluronan and chondroitin sulfate via N-acetyl glucosamine and N-acetyl galactosamine in the extracellular matrix of cartilage. GlcN is believed to reduce the pain of OA since it serves as a source of GAGs. In animal experiments, intake of GlcN has been reported to increase the amount of GAGs in the extracellular matrix of cartilage. A recent study, however,
ported that GlcN could not be detected in the synovial fluid after it had been orally administered. In addition, GlcN reaching the synovium has been reported to have a short half-life. These reports suggested that GlcN may not just be a source of GAGs but may also affect the cartilage because of other factors. In our study, GlcN may, as a source of GAGs, directly serve as a source of the extracellular matrix of cartilage as well as possibly regulating differentiation of chondrocytes as a signal molecule.

The main components of the extracellular matrix of cartilage are hyaluronan and aggrecan. These molecules play important roles in the proper functioning of articular cartilage because they help form a hydrated gel that facilitates smooth movement of the articular cartilage. Hyaluronan is a high-molecular-weight polysaccharides and does not contain a sulfate residue. Aggrecan is the major proteoglycan in the articular cartilage and consists of a core protein and side chains such as 4- and/or 6-chondroitin sulfate and keratan sulfate and consists of a core protein and side chains such as 4- and/or 6-chondroitin sulfate and keratan sulfate. Aggrecan is reported to play a role in prevention of such as 4- and/or 6-chondroitin sulfate and keratan sulfate and consists of a core protein and side chains such as 4- and/or 6-chondroitin sulfate and keratan sulfate. In our study, GlcN may, as a source of GAGs but may also affect the cartilage because of other factors. In our study, GlcN may, as a source of GAGs but may also affect the cartilage because of other factors.

In this study, we distinguished chondroitin sulfate and keratan sulfate, as sulfated GAGs, from the total GAG, which includes hyaluronan. The total GAG staining area and sulfated GAG staining area were estimated by alcan blue staining at pH 2.5 or 1.0, respectively. As a result, only the staining area of sulfated GAGs was increased, suggesting that GlcN might induce the production of sulfated GAGs. For this staining study, we assumed that inhibition of mineralization by GlcN was related to increased formation of sulfated GAGs such as chondroitin sulfate and/or keratan sulfate. In addition, sulfated GAGs may be crucial to inhibition of ectopic mineralization of the extracellular matrix of cartilage. These results suggest that aggrecan might be involved in the differentiation of chondrocytes, and might play a role in maintaining the elasticity of articular cartilage. Biochemical studies will be necessary to clarify the production of each GAG and/or estimate the contents of each GAG in response to GlcN addition to ATDC5 cell cultures.

Next, we examined the mRNA levels of cartilage-specific genes to identify those involved in inhibition of mineralization and induction of the sulfated-GAG staining area. As a result, the level of MGP mRNA in ATDC5 cells was found to be reduced by GlcN. MGP is an extracellular mineral-binding protein that plays important regulatory roles in the mineralization of cartilage and skeletal tissues. GlcN might have inhibited mineralization by mediating regulation of the MGP mRNA levels. Moreover, GlcN decreased the levels of mRNA for Smad2 and Smad4 in the ATDC5 cells. Smad2 is a well-known transcription factor downstream of TGF-β. Recent studies showed the possibility that Smad2 binds to Sox9 and Runx2 and regulates the process of tissue formation from cartilage to bone. Our data suggested that the effects of GlcN on mineralization and sulfated GAG induction might relate to its reduction of the Smad2 and Smad4 mRNA levels in ATDC5. Also, Smad2 and/or Smad4 might regulate chondrocyte differentiation, the amount of sulfated GAGs and mineralization via the mRNA level of MGP. It will be necessary to clarify the mechanisms by which GlcN regulates these genes by molecular biological methods such as promoter assay and northern blotting.

Our findings suggest that GlcN may inhibit mineralization of chondrocytes by acting as a signal molecule. As the mechanism, GlcN might regulate the levels of MGP and the amount of chondroitin sulfate in the cartilage matrix by regulating mRNA expression of the Smad2 and Smad4chondrogenic transcription factors. Moreover, in this study the GlcN analogues did not show these same effects as GlcN on the ATDC5 cell line.

The current experiments were carried out in vitro study using the ATDC5 cell line, so the safety of GlcN in humans was not addressed. Future studies should investigate the safety of GlcN in an in vivo animal experimental system and GlcN’s effectiveness in humans.

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