Effects of glucosamine derivatives and uronic acids on the production of glycosaminoglycans by human synovial cells and chondrocytes

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Abstract. Glucosamine (GlcN) has been widely used to treat osteoarthritis (OA) in humans. However, its chondroprotective action on the joint is poorly understood. In this study, to elucidate the chondroprotective action of GlcN, we examined the effects of GlcN-derivatives (GlcN and N-acetyl-D-glucosamine) and uronic acids (D-glucuronic acid and D-galacturonic acid) (0.1-1 mM) on the production of glycosaminoglycans (GAG), such as hyaluronic acid (HA), keratan sulfate and sulfated GAG by human synovial cells and chondrocytes. The results indicate that among GlcN-derivatives and uronic acids, GlcN but not N-acetyl-D-glucosamine, D-glucuronic acid and D-galacturonic acid induce the production of HA by synovial cells and chondrocytes at >0.25 and >0.1 mM ($p<0.05$), respectively, and the production levels are much higher (>10-fold) in synovial cells compared to chondrocytes. In contrast, neither N-acetyl-D-glucosamine, D-glucuronic acid nor D-galacturonic acid affected the production of keratan sulfate and sulfated GAG by these cells. Moreover, the experiments with ^{3}H-labeled GlcN indicated that GlcN can be incorporated and utilized for the production of GAG (including HA) by synovial cells and chondrocytes. In addition, GlcN (1 mM) up-regulates the expression of HA-synthesizing enzymes (hyaluronan synthases) in synovial cells and chondrocytes. Together these observations indicate that GlcN may exhibit chondroprotective action on joint diseases such as OA by modulating the expression of HA-synthesizing enzymes and inducing the production of HA (a major component of GAG contained in synovial fluid) especially by synovial cells.

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Key words: glucosamine, uronic acid, hyaluronic acid, glycosaminoglycan, synovial cells, chondrocytes

Introduction

Osteoarthritis (OA) is the most common joint disorder with an immense socioeconomic impact. OA is characterized by qualitative and quantitative changes in the architecture and composition of all the joint structures (1,2). An altered imbalance among the biosynthesis and degradation of matrix components leads to a progressive destruction of the tissue (3). Currently, several treatments are available for OA ranging from conservative treatments to surgical extremes. Conservative measures involve lifestyle modifications, physical therapy and pharmacological treatment with non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular injection of hyaluronic acid (HA) (4). Treatments for early OA are primarily based on symptomatic relief, whereas irreversible joint disability in advanced OA usually requires surgical intervention to relieve pain and improve joint function (5). However, current treatments are mostly targeting the OA symptoms and not addressing the destructed structure of articular cartilage in OA.

Glucosamine (GlcN), a naturally occurring amino monosaccharide, is present in the connective and cartilage tissues as a component of glycosaminoglycans (GAG). GlcN contributes to the maintainance of the strength, flexibility and elasticity of these tissues. Thus, GlcN has been widely used to treat OA for more than two decades in humans (6-9). Several short- and long-term clinical trials of OA have shown the significant symptom-modifying effects of GlcN (10-12). Moreover, the updated Osteoarthritis Research Society International (OARSI) recommendations for the management of hip and knee OA, have recently suggested that GlcN has symptom-relieving and structure-modifying effects in the OA knee (13). In this context, we previously revealed that GlcN can suppress the cytokine-induced activation of synovial cells (such as nitric oxide-, PGE_2- and IL-8-production), thereby possibly exhibiting anti-inflammatory actions in arthritis (14). However, the mechanism for the chondroprotective action of GlcN on the joint is not fully understood.

In the joints, synovial cells produce HA, a component of synovial fluid GAG, whereas chondrocytes produce chondroitin

sulfate, keratan sulfate (KS) and HA as components of cartilage matrix GAG (15-19). Thus, it is reasonable to speculate that GlcN may affect GAG production by synovial cells and chondrocytes. In this study, to elucidate the chondroprotective action of GlcN, we examined the effects of D-GlcN on the production of GAG, such as HA, KS and sulfated GAG (sGAG) by human synovial cells and chondrocytes, and compared them with those of the GlcN-derivative, N-acetyl-D-glucosamine (GlcNAc), and of the uronic acids, D-glucuronic acid (GlcU) and D-galacturonic acid (GalU) on GAG production.

Materials and methods

Reagents. D-Glucosamine hydrochloride (GlcN), N-acetyl-D-glucosamine (GlcNAc), and D-galacturonic acid (GalU) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). GlcU was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan).

Cells. Human synovial MH7A cells (20) were purchased from RIKEN Cell Bank (RCB1512, Tsukuba, Japan). MH7A cells were maintained in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS), penicillin and streptomycin at 37˚C in 5% CO_2. Human chondrocytes (SW1353) were purchased from ATCC (HTB94, USA). SW1353 cells were maintained in Leibovitz's L-15 medium (Gibco/Invitrogen, CA, USA) containing 10% FBS, penicillin and streptomycin at 37˚C under atmosphere.

Quantification of GAG. MH7A cells ($1x10^5$ cells/well) and SW1353 cells ($1.5x10^5$ cells/well) were seeded in 12-well plates overnight. The cells were incubated with 0.1-1 mM GlcN, GlcNAc, GlcU or GalU for 24 h, and then the culture supernatants were recovered for the measurements of GAG. Furthermore, the cells were washed twice with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and incubated with trypsin solution (0.1% trypsin, 0.02% EDTA in PBS) at 37˚C for a few minutes. The detached cells were collected into 1.5 ml tubes and then centrifuged (600 x g, 4˚C, 5 min). The supernatants were discarded, and the cells were lysed in 200 μl of lysis buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40). The cell lysates were sonicated and placed on ice for 5 min. After centrifugation (8000 x g, 4˚C, 5 min), the supernatants were used for the measurements of GAG. Concentrations of HA, KS and sGAG in the culture supernatants and cell lysates were measured using a hyaluronan assay kit, a keratan sulfate ELISA kit and a sulfated glycosaminoglycan quantitation kit (Seikagaku Biobusiness Corporation, Tokyo, Japan), respectively, according to the manufacturer's protocols. The detection limits of HA, KS and sGAG were 12.5, 2.5 and 250 ng/ml, respectively.

Incorporation of GlcN into glycosaminoglycans. MH7A cells ($2x10^5$ cells/well) and SW1353 cells ($3x10^5$ cells/well) were seeded in 6-well plates overnight. The cells were incubated with 0.06-1 mM GlcN containing 1/10,000 D-[6-^{3}H(N)]-glucosamine hydrochloride (740 GBq/mmol, Perkin-Elmer Japan, Kanagawa, Japan) for 24 h, and the culture supernatants were recovered. Furthermore, the cells were washed twice with ice-cold PBS and lysed in 2 ml lysis buffer (50 mM Tris-HCl pH 7.6, 3M guanidine hydrochloride) for 24 h at 4˚C. The culture supernatants and cell lysates were added to 222 μl of 10% solution of hexadecylpyridinium chloride (Nacalai Tesque) and incubated for 24 h at room temperature. Thereafter, the precipitates (GAG-hexadecylpyridinium complex) were collected by filtration with glass filters (GF/F, Whatman Japan KK/GE Healthcare Bio-Sciences KK, Tokyo, Japan). After washing twice with PBS, the filters were put into vials. Then, 10 ml of liquid scintillation cocktail (Aquasol-2, Perkin-Elmer, Japan) were added to each vial, and the ^{3}H-radioactivity was measured by a scintillation counter (LS 6500 Scintillation Counting System, Beckman Coulter, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). MH7A cells ($3x10^5$ cells/well) and SW1353 cells ($4.5x10^5$ cells/well) were seeded in 6-cm dishes overnight. The cells were incubated with 1 mM GlcN, GlcNAc, GlcU or GalU for 24 h, and then recovered by a cell scraper (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). According to the manufacturer's protocol, total RNA was purified using an RNeasy plus mini kit (Qiagen, CA, USA) and QIAshredder (Qiagen) to remove contaminated DNA, and stored at -80˚C. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a ReverTra Plus RT-PCR kit (Toyobo, Osaka, Japan), and PCR amplification was performed with KOD plus Taq polymerase in a thermal cycler (Eppendorf AG, Hamburg, Germany) for hyaluronan synthases (HAS) 1-3, hyaluronidases (HYAL) 1-3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), according to the manufacturer's protocol. In brief, cDNA was synthesized by reverse transcription of total RNA (500 ng) using ReverTra Ace reverse transcriptase and oligo(dt)20. To discriminate mRNA-derived PCR products from genomic DNA-derived products, intron-spanning PCR primers were used. The primers, annealing temperature and the number of PCR cycles are shown in Table I. PCR products were resolved by 2% agarose gel electrophoresis and stained with ethidium bromide. In our preliminary experiments, we tried to semi-quantitatively detect mRNA by using different numbers of PCR cycles. The results revealed that the amounts of RT-PCR products increased dependently on the cycle number. Thus, we decided to measure the mRNA levels by RT-PCR with the numbers of PCR cycles indicated in Table I. The detected bands were quantified using Multi Gauge (Version 3.0, FujiFilm, Tokyo, Japan).

Statistical analysis. Data are shown as the means ± SD, and were analyzed for significant differences by a Student's t-test in Excel. Differences were considered statistically significant at $p<0.05$.

Results

Effects of GlcN-derivatives and uronic acids on the production of GAG by human synovial cells and chondrocytes. GAG released from human synovial cells and chondrocytes were measured after treatment with GlcN, GlcNAc, GlcU and GalU using the culture supernatants. GlcN significantly increased

Table I. Gene specific PCR primers, annealing temperature (˚C) and number of PCR cycles.

Gene	Forward primer	Reverse primer	Annealing temperature (˚C)	PCR cycles for MH7A synovial cells	PCR cycles for SW1353 chondrocytes
HAS1	5'-gtctgtgactcggacacaagg-3'	5'-ggtagcataacccatcgtgagc-3'	55	32	32
HAS2	5'-gtttgtgattcagacactatgcttg-3'	5'-cagccattctcggaagtaggac-3'	55	27	37
HAS3	5'-gtgtgcgactctgacactgtg-3'	5'-cttagtgggggtctctgtgag-3'	59	33	32
HYAL1	5'-gaccagttccagggagctgcacgggccc-3'	5'-cagctgcccccctgggccgcactctccc-3'	61	32	32
HYAL2	5'-ggacctcatctctaccattgg-3'	5'-gctcaccactccagcccaag-3'	55	30	27
HYAL3	5'-gcggcccgtgcactgatggag-3'	5'-gagcaccacgccgggctgccc-3'	59	32	32
GAPDH	5'-accacagtccatgccatcac-3'	5'-tccaccaccctgttgctgta-3'	60	20	20

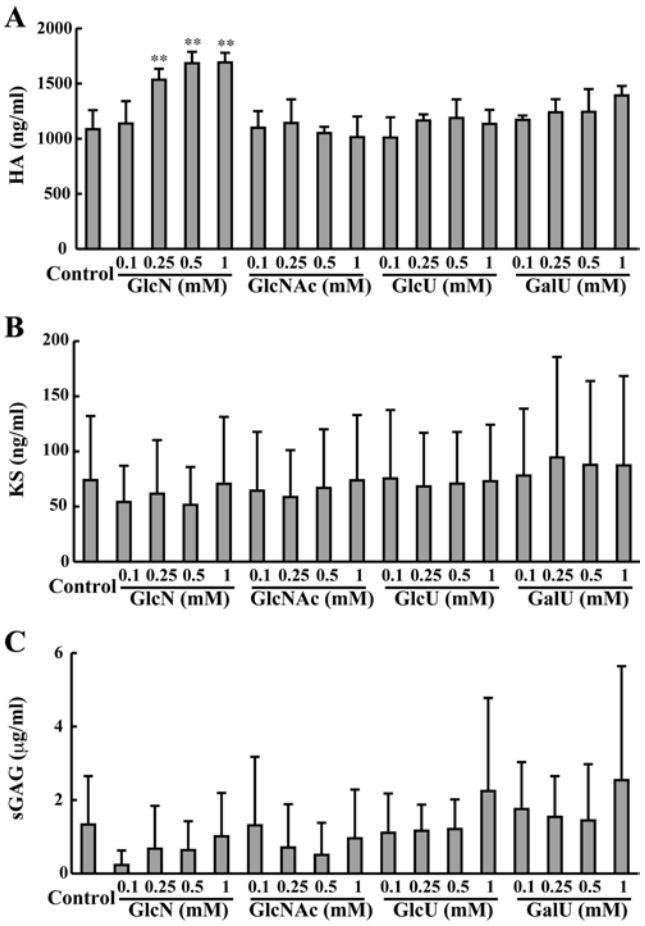

Figure 1. Effects of GlcN, GlcNAc, GlcU and GalU on the release of GAG from synovial cells. Human synovial cells (MH7A) were incubated without (control) or with 0.1-1 mM GlcN, GlcNAc, GlcU or GalU for 24 h, and extracellularly released HA (A), KS (B) and sGAG (C) were measured. Data are expressed as ng/ml or μg/ml, and represent the mean ± SD of three independent experiments. Values were compared between incubation without (control) and with GlcN, GlcNAc, GlcU or GalU. $^{**}p<0.01$.

the HA level in the culture supernatants from synovial cells ($p<0.01$) (Fig. 1A). In contrast, GlcNAc, GlcU and GalU did not essentially increase the extracellular level of HA (Fig. 1A). Of note, neither GlcN, GlcNAc, GlcU nor GalU affected the extracellular levels of KS or sGAG released from human synovial cells (Fig. 1B and C).

Similarly, GlcN at 0.1-1 mM significantly increased HA levels in the culture supernatants of chondrocytes ($p<0.05$) (Fig. 2A). In contrast, GlcNAc, GlcU and GalU did not increase the extracellular levels of HA (Fig. 2A). Similarly to our observations in synovial cells, neither GlcN, GlcNAc, GlcU nor GalU affected the extracellular levels of KS and sGAG released from human chondrocytes (Fig. 2B and C). Notably, HA levels were much higher (>10-fold) in the culture supernatants from synovial cells than from chondrocytes.

In recent years, the presence of intracellular HA has been proven in smooth muscle cells, fibroblasts and keratinocytes (21,22). Therefore, the effect of GlcN, GlcNAc, GlcU and GalU on the intracellular accumulation of GAG in human synovial cells and chondrocytes was determined. In synovial cells, only GlcN at 0.25-1 mM significantly increased the intracellular level of HA ($p<0.05$) (Fig. 3A). However, GlcN,

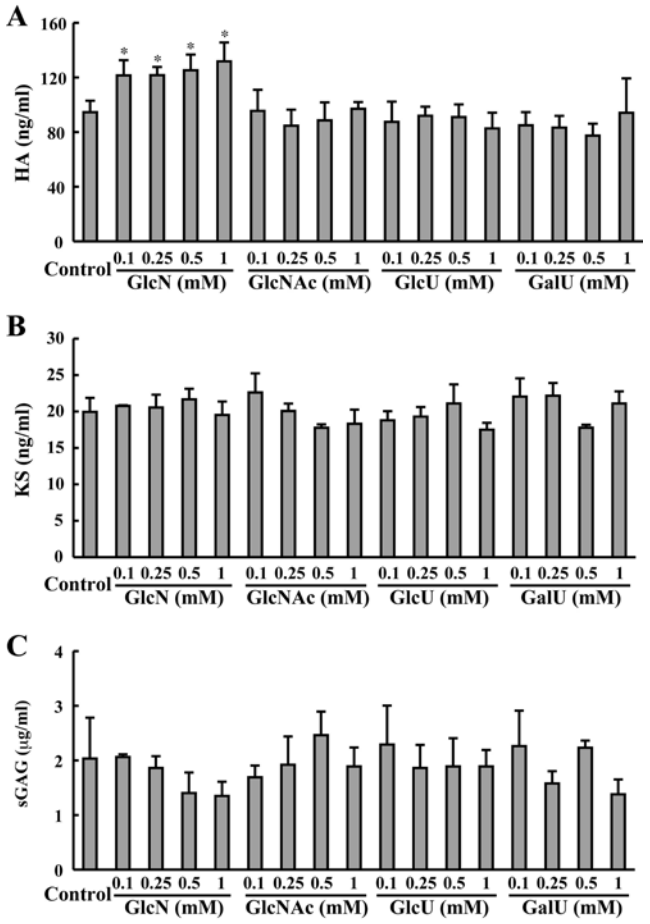

Figure 2. Effects of GlcN, GlcNAc, GlcU and GalU on the release of GAG from chondrocytes. Human chondrocytes (SW1353) were incubated without (control) or with 0.1-1 mM GlcN, GlcNAc, GlcU or GalU for 24 h, and extracellularly released HA (A), KS (B) and sGAG (C) were measured. Data are expressed as ng/ml or μg/ml, and represent the means ± SD of three independent experiments. Values were compared between incubation without (control) and with GlcN, GlcNAc, GlcU or GalU. $^{*}p<0.05$.

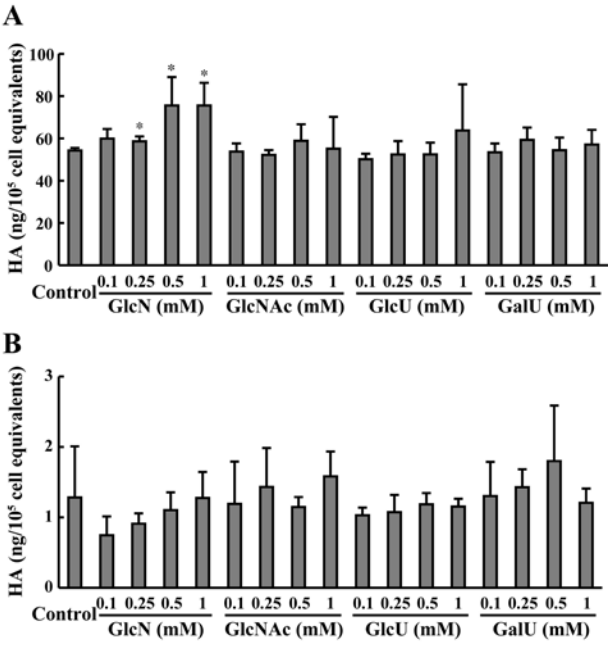

Figure 3. Effects of GlcN, GlcNAc, GlcU and GalU on the intracellular levels of HA in synovial cells and chondrocytes. Synovial cells (A) and chondrocytes (B) were incubated without (control) or with 0.1-1 mM GlcN, GlcNAc, GlcU or GalU for 24 h, and the intracellular levels of HA were measured. Data are expressed as ng/10^5 cell equivalents, and represent the means ± SD of three independent experiments. Values were compared between incubation without (control) and with GlcN, GlcNAc, GlcU or GalU. $^{*}p<0.05$.

GlcNAc, GlcU and GalU did not affect the intracellular level of HA in chondrocytes (Fig. 3B), although GlcN increased the extracellular release of HA by chondrocytes (Fig. 2). This may be due to the fact that the level of intracellular HA was below the detection limit (<12.5 ng/ml or <0.7 ng/10^5 cell equivalents) in chondrocytes. As expected, neither GlcN, GlcNAc, GlcU nor GalU affected the intracellular levels of KS and sGAG in synovial cells and chondrocytes (data not shown). Together these observations suggest that among amino GlcN-derivatives and the uronic acids examined, only GlcN is able to increase both the extracellular levels of HA released from synovial cells and chondrocytes, and the intracellular levels of HA in synovial cells. Moreover, the potential to produce HA is much higher (>10-fold) in synovial cells than chondrocytes.

Incorporation of GlcN into GAG. As mentioned above, GlcN increased the extracellular level of HA from synovial cells and chondrocytes and the intracellular level of HA in synovial cells. Thus, it is important to clarify whether the increase of HA in the culture supernatants and within the cells is due to the production of HA utilizing GlcN as a precursor. For this purpose, synovial cells and chondrocytes were incubated

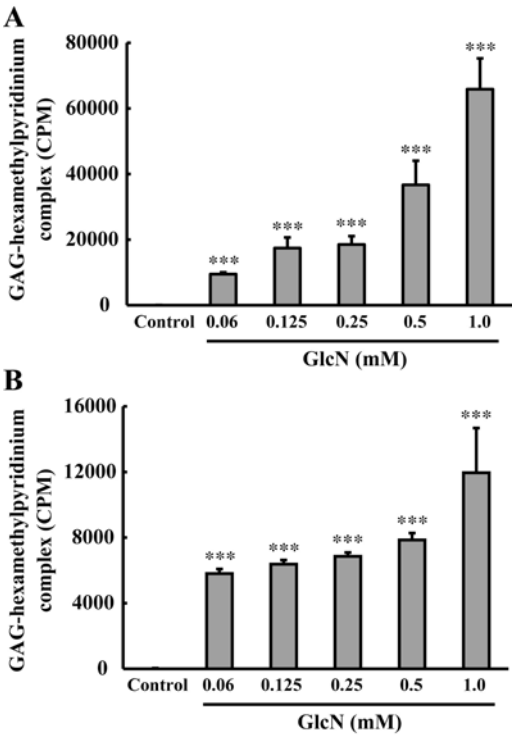

Figure 4. Incorporation of GlcN into GAG by synovial cells. Synovial cells were incubated without (control) or with 0.06-1 mM GlcN containing 1/10,000 of D-[6-^{3}H(N)]-glucosamine hydrochloride for 24 h and the incorporation of GlcN into GAG was determined by counting the radioactivities (CPM) of GAG-hexadecylpyridinium complex recovered from the culture supernatant (A) and cell lysates (B). Data are corrected by subtracting the background count of cells incubated only with ^{3}H-labeled GlcN. Data represent the means ± SD of three independent experiments. Values were compared between incubation without (control) and with GlcN. $^{***}p<0.001$.

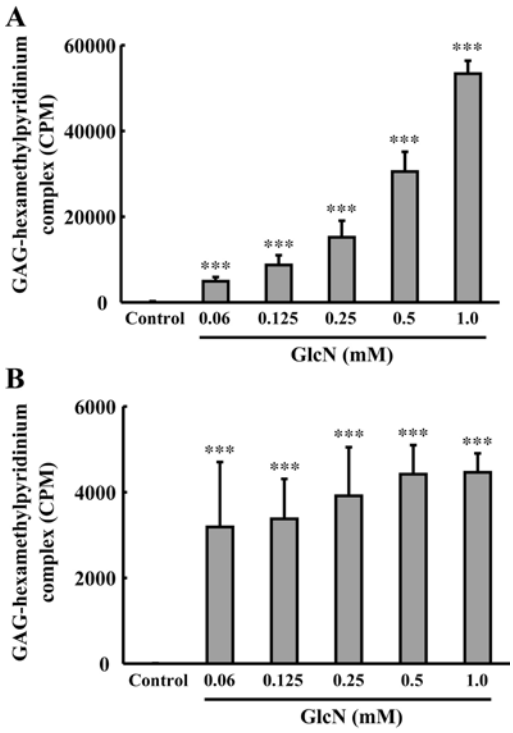

Figure 5. Incorporation of GlcN into GAG by chondrocytes. Chondrocytes were incubated without (control) or with 0.06-1 mM GlcN containing 1/10,000 of D-[6-^{3}H(N)]-glucosamine hydrochloride for 24 h, and the incorporation of GlcN into GAG was determined by counting the radioactivities (CPM) of GAG-hexadecylpyridinium complex recovered from the culture supernatant (A) and cell lysates (B). Data are corrected by subtracting the background count of cells incubated only with ^{3}H-labeled GlcN. Data represent the mean ± SD of three independent experiments. Values were compared between incubation without (control) and with GlcN. $^{***}p<0.001$.

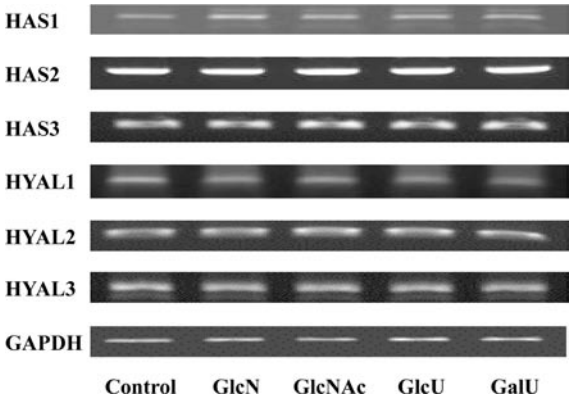

Figure 6. Effect of GlcN, GlcNAc, GlcU and GalU on the mRNA expression of HA-metabolizing enzymes in synovial cells. Synovial cells were incubated with 1 mM GlcN, GlcNAc, GlcU or GalU for 24 h. After the incubation, total RNA was purified, and the expression of mRNA for HAS1-3, HYAL1-3 and GAPDH was evaluated by RT-PCR. PCR products were resolved by 2% agarose gel electrophoresis, and stained with ethidium bromide. Data represent one of two independent experiments.

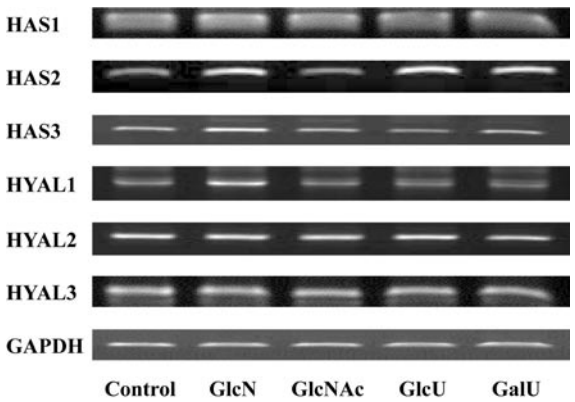

Figure 7. Effect of GlcN, GlcNAc, GlcU and GalU on the mRNA expression of HA-metabolizing enzymes in chondrocytes. Chondrocytes were incubated with 1 mM GlcN, GlcNAc, GlcU or GalU for 24 h. After the incubation, total RNA was purified, and the expression of mRNA for HAS1-3, HYAL1-3 and GAPDH was evaluated by RT-PCR. PCR products were resolved by 2% agarose gel electrophoresis, and stained with ethidium bromide. Data represent one of two independent experiments.

with GlcN containing ^{3}H-labeled GlcN, and the radioactivities in the GAG-hexadecylpyridinium complex containing the incorporated GlcN were measured. Importantly, the incorporation of ^{3}H-labeled GlcN into the extracellular GAG as well as the intracellular GAG in synovial cells was increased in a concentration-dependent manner (Fig. 4). Similarly, in chondrocytes, the incorporation of ^{3}H-labeled GlcN into the extracellular and intracellular GAG was increased in a concentration-dependent manner (Fig. 5). These observations indicate that GlcN is actually incorporated and utilized for the production of GAG (including HA) by synovial cells and chondrocytes.

Effects of GlcN-derivatives and uronic acids on the expression of HA-metabolizing enzymes. Finally, to reveal whether GlcN-derivatives (GlcN and GlcNAc) and uronic acids (GlcU and GalU) affect the expression of HA-metabolizing enzymes, such as HA-synthesizing enzymes (HAS) and HA-degrading enzymes (HYAL), synovial cells and chondrocytes were treated with GlcN, GlcNAc, GlcU and GalU, and then the expression levels of HAS1-3 and HYAL1-3 were measured by RT-PCR. In synovial cells, the expression of HAS1 was apparently increased by GlcN treatment, but not by GlcNAc, GlcU and GalU (Fig. 6). In contrast, the expression of HAS2, HAS3 and HYAL1-3 was not essentially affected by GlcN, GlcNAc, GlcU or GalU (Fig. 6). In chondrocytes, the expression of HAS2 was increased not only by GlcN but also GlcU and GalU, whereas the expression of HAS3 and HYAL1 was increased only by GlcN (Fig. 7).

Discussion

Synovial fluid secreted from synovial tissues is a HA-containing viscous liquid that plays a role as the lubricant and cushion for the joint movement (23). OA is characterized by qualitative and quantitative changes in the architecture and composition of the joint structures (1,2). In addition, the levels of HA and viscosity are decreased in the synovial fluid from OA patients (24). Thus, the intra-articular injection of HA is appreciated as a treatment of OA (25). Moreover, GlcN has also been used to treat OA in humans, because GlcN is present in the cartilage tissues as a component of GAG (6-9). However, the mechanism for the chondroprotective effects on the joint is poorly understood. In this study, in order to elucidate the chondroprotective action of GlcN, we examined the effects of

GlcN derivatives (GlcN and GlcNAc) and uronic acids (GlcU and GalU) on the production of GAG, such as HA, KS and sGAG by human synovial cells and chondrocytes.

The results indicate that among the GlcN derivatives and the uronic acids examined, only GlcN increased not only the extracellular levels of HA released from synovial cells and chondrocytes (Figs. 1 and 2), but also the intracellular levels of HA in synovial cells (Fig. 3). Moreover, the experiments with ^{3}H-labeled GlcN revealed that GlcN can be incorporated and utilized for the production of GAG (including HA) by synovial cells and chondrocytes (Figs. 4 and 5).

Synovial fluid is secreted from synovial tissues, and HA is a major component of GAG contained in the synovial fluid (24). Notably, the HA level in the culture supernatants from synovial cells was much higher (>10-fold) than that from chondrocytes (Figs. 1A and 2A), indicating that synovial cells play a more important role than chondrocytes in the production of HA in synovial fluid. In contrast, the incorporated level of GlcN into GAG was almost the same in synovial cells and chondrocytes. Furthermore, the sGAG levels (2 μg/ml and 300 ng/10^5 cell equivalents, data not shown) are much higher than the HA levels (100-1,000 ng/ml and 1-60 ng/10^5 cell equivalents) in the culture supernatants and in the cells, respectively. Based on these findings, it could be speculated that only a small portion of GlcN added to the culture system is utilized for the production of HA, especially by synovial cells, although GlcN is mostly utilized for the production of sGAG (including chondroitin sulfate, dermatan sulfate and KS) in both synovial cells and chondrocytes almost at the same level.

The concentration of HA in normal synovial fluid is 3.4-5 mg/ml, and is decreased to 2.5 mg/ml in OA (2,25). In this study, synovial cells extracellularly released 1000 ng/ml of HA in the absence of GlcN, and the HA concentration was increased up to approximately 2000 ng/ml in the presence of 1 mM GlcN (Fig. 1A). Thus, the concentrations of HA in conditional media (1-2 μg/ml) are much lower (<1/2000-fold) than those in synovial fluid (2.5-5 mg/ml). The difference in the concentrations of HA may be due to the fact that the number of synovial cells used in the *in vitro* assay is far less than that present in the synovial tissue *in vivo*.

RT-PCR analysis revealed that the expression of HAS1, one of HA-synthesizing enzymes, was apparently increased by GlcN in synovial cells (Fig. 6), suggesting that the up-regulated HAS1 may be involved in the GlcN-induced increase of HA production by synovial cells. In contrast, the expression of HAS2 was up-regulated by GlcN, GlcU and GalU, whereas the expression of HAS3 and HYAL1 was increased by GlcN in chondrocytes (Fig. 7), suggesting that the up-regulated HAS3 and possibly HAS2 may be involved in the GlcN-induced increase of HA production by chondrocytes. On the contrary, since GlcU and GalU did not increase the HA production by chondrocytes, the GlcU- and GalU-induced up-regulation of HAS2 is unlikely to play a role in the HA production. Interestingly, HYAL1, one of degrading enzymes, was also up-regulated by GlcN in chondrocytes. However, the up-regulated HYAL1 is not likely to play a role in the HA degradation, because the HA production was grossly increased by GlcN in chondrocytes despite the up-regulation of HYAL1.

In conclusion, the present study has revealed that among GlcN-derivatives and uronic acids, only GlcN induces the production of HA by synovial cells and chondrocytes, and the production level is much higher (>10-fold) in synovial cells compared with chondrocytes. Moreover, GlcN increases the expression of HA-synthesizing enzymes (HAS) in synovial cells and chondrocytes. Together these observations indicate that GlcN is likely to exhibit its chondroprotective actions on joint diseases, such as OA, by modulating the expression of HA-synthesizing enzymes and inducing the production of HA (a major component of GAG contained in the synovial fluid) especially by synovial cells. However, the *in vivo* effects of glucosamine on articular disorders should be further evaluated in the future.

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