ARTHRITIS & RHEUMATISM
Vol. 44, No. 5, May 2001, pp 1089–1095
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Preferential Incorporation of Glucosamine Into the Galactosamine Moieties of Chondroitin Sulfates in Articular Cartilage Explants

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Objective. **To determine the metabolic fate of glucosamine (GlcN) in intact articular cartilage tissue.**

Methods. **Intact articular cartilage explants were cultured for up to 13 days in Dulbecco's modified Eagle's medium supplemented with 1) 1-^{13}C–labeled GlcN, 2) 1-^{13}C–labeled glucose (Glc), or 3) no labeling. Every 3–4 days, samples were removed and frozen in liquid nitrogen for carbon-13 magnetic resonance spectroscopic (MRS) analysis. The metabolic products of the labeled precursors were determined from the MRS data based on resonance positions and comparison with known standards and published values.**

Results. **GlcN was taken up by the chondrocytes and incorporated selectively into the hexosamine, but not the hexuronic acid, components of the glycosaminoglycan chains of articular cartilage proteoglycan. The data also demonstrated that GlcN is the substrate of choice for the galactosamine moieties of the chondroitin sulfates, incorporating at levels 300% higher than with an equivalent amount of labeled Glc.**

Conclusion. **The results indicate that GlcN facilitates the production of proteoglycan components that are synthesized through the hexosamine biochemical pathway.**

Supported by NIH grants RR-02305, HL-07614, AR-45404, and AR-45242.

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Submitted for publication June 1, 2000; accepted in revised form January 4, 2001.

Osteoarthritis (OA) is one of the most prevalent musculoskeletal diseases in the US today. While the initiating event(s) is still largely unknown and certainly multifactorial, the hallmark of arthritis pathology is the net loss of articular cartilage matrix as a result of an imbalance of matrix biosynthesis, turnover, and degradation (1). Arthritis presents in patients as pain, which progresses with time and eventually leads to loss of joint function. This occurs in part because the chondrocytes can no longer maintain the correct articular cartilage phenotype and cannot sustain the proper balance of synthesis and degradation. While no cure exists for this debilitating disorder, new therapies include a variety of novel approaches including gene therapy (2), bioengineered matrices (3), and autologous cartilage grafts (4). Other approaches are aimed at modifying the disease process by affecting the expression of relevant matrix genes, such as those critical in the restoration of the cartilage matrix (i.e., proteoglycans) and those involved in the catabolic pathway, such as degradative enzymes involved in matrix degradation.

Considerable interest has been generated in the area of putative chondroprotective or disease-modifying biologic agents and compounds, including drugs affecting chondrocyte and synovial cell biosynthesis, nutraceuticals, and growth factors and their receptors. The extracellular matrix of articular cartilage is made up of a variety of collagens, proteoglycans, and water (5,6). The primary proteoglycan is aggrecan, which has a large core protein (250 kd) and contains predominantly chondroitin sulfate (CS) and keratan sulfate (KS) glycosaminoglycans (GAGs) of varying types and chain lengths. The repeating disaccharide unit in CS consists of an amino sugar, *N*-acetylgalactosamine (GalNAc), and a hexuronic acid, D-glucuronic acid (GlcUA), linked via a β-1,4 bond. The GalNAc moiety may be sulfated at the

C-4 carbon (CS4) or the C-6 carbon (CS6). In KS, the uronic acid is replaced with a galactose (Gal) linked to 2-acetoamidoglucosamine (GlcNAc) in a β-1,4 linkage. All of the disaccharide components ultimately are derived from glucose (Glc) (7).

The loss of proteoglycans from cartilage is an early and critical event in the process of the joint destruction that occurs in degenerative and inflammatory arthritis. Since there is no vasculature in normal adult articular cartilage, this specialized tissue is influenced by mediators and solutes that are diffusible from the vasculature of the synovial membrane, joint capsule, and subchondral bone marrow. One clearly beneficial outcome would be the restoration of a normal proteoglycan level and composition in articular cartilage. Substantial interest in both public and research areas has arisen over glucosamine (GlcN), a nutraceutical used clinically for years in much of Europe. There is increasing evidence that GlcN may affect both the symptoms of OA (i.e., pain) and the biosynthesis of proteoglycans. Clinical trials with GlcN show promise, but rigorous investigations of clinical efficacy and elucidation of the putative mechanism of action have proven difficult (8), although there are studies that have demonstrated that GlcN can alter the expression of proteoglycans and influence the aggrecanase activity responsible for proteoglycan degradation (9).

One potentially valuable tool for analyzing the metabolism of GlcN is carbon-13 magnetic resonance spectroscopy (^{13}C MRS). The characterization of GlcN in cartilage using a ^{13}C-labeled molecule would clearly demonstrate that there is a basis for the use of GlcN to affect proteoglycan biosynthesis. The ^{13}C isotope of carbon is a stable form of carbon that is normally present in very low amounts (~0.1%). However, it is possible to selectively enrich a specific carbon position on an organic molecule of interest (up to 99%), increasing the signal intensity of the labeled carbon position by 1,000%. Since the resonance positions of carbon atoms are highly sensitive to their molecular environments (10), this enrichment creates a uniquely identifiable carbon that can be used to trace a biosynthetic pathway. In fact, ^{13}C-labeled Glc has been used extensively to trace the pathway through glycolysis, the Krebs cycle, and alternate pathways in many metabolically active tissues, including neurons, myocytes, and hepatocytes (11–14), but, to date, ^{13}C-labeled compounds have not been used in studying cartilage metabolism or proteoglycan synthesis.

The recent interest in GlcN and its effect on OA has prompted us to investigate its metabolism in cartilage using ^{13}C-labeled compounds. The purpose of this work was to demonstrate the uptake and metabolism of GlcN by intact cartilage and its incorporation into cartilage proteoglycan. The characterization of the fate of the labeled GlcN in this model will definitively demonstrate that there is a basis for using GlcN to affect proteoglycan synthesis and determine if there is any preferential incorporation of the added GlcN into the hexosamine moieties of specific GAGs.

MATERIALS AND METHODS

Culture reagents. All materials for reagents and media were obtained from Gibco BRL (Grand Island, NY) unless otherwise noted. Sterile phosphate buffered saline (PBS) was prepared with PBS (Gibco #14190-136) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Pen/Strep; Gibco #15140-122), 0.25 μg/ml amphotericin B (Gibco #15295-017), and 2.4 gm/liter HEPES (Gibco #15630-080). Culture media were prepared with high-Glc Dulbecco's modified Eagle's medium (DMEM) (4.5 gm/liter Glc; Gibco #11965) supplemented with Pen/Strep, amphotericin B, 1× insulin–transferrin–selenium G (Gibco #41400-011), 2 m*M* glutamine (Gibco #25030-081), and 50 μg/ml ascorbic acid (Sigma, St. Louis, MO). Three different media were prepared with labeled carbohydrate supplements: 1) 1-^{13}C–labeled GlcN at 0.4 mg/ml (Isotec, Miamisburg, OH), 2) 1-^{13}C–labeled Glc at 0.4 mg/ml (Cambridge Isotopes, Andover, MA), and 3) control (no added labeling). Representative ^{13}C MRS spectra showing the resonance positions for the C-1–labeled carbons are plotted in Figure 1 for the substrates containing GlcN and Glc.

Explant cultures. Femoral joints from 1–2-year-old steers were obtained from a local slaughterhouse (Beirig Brothers, Vineland, NJ) within 6 hours after they were killed. After washing with 70% ethanol, cartilage plugs (mean ± SD weight 144.4 ± 17.8 mg) were removed from the joints with a sterile 7 mm–diameter cork borer. Samples were placed in sterile PBS and processed further under a sterile laminar flow hood. Residual bone and blood were scraped from the tidemark with a scalpel before the samples were washed 3 times with sterile PBS. The explants were transferred to a clean, sterile 6-well culture plate (1 sample per well). The samples were divided into 3 groups of 12 specimens each, depending on the culture medium. Four milliliters of the appropriate medium was added to each well. The samples were incubated at 37°C under 5% CO_2/95% air. Every 3–4 days, 3 samples from each group were removed and quickly frozen in liquid nitrogen to stop all biochemical processes. The samples were stored at 0°C while awaiting MRS. Media were refreshed for the remaining samples. All media were saved at 0°C for spectrophotometric analysis.

MRS measurements. All experiments were performed with a Bruker AM-500 spectrometer (Bruker Optics, Billerica, MA). Samples were coarsely chopped and placed in a 5 mm–diameter nuclear magnetic resonance tube with 0.5 ml PBS. Broadband, proton-decoupled ^{13}C MRS were obtained at 125.8 MHz using a composite pulse decoupling with a WALTZ-16 sequence and a 45° flip angle (90° flip angle 16 μs) with 32K points, sweep width 38 kHz, and a repetition delay of 400 ms. For the labeled samples, 25,000 acquisitions were

made, while 100,000 acquisitions were made for the unlabeled specimens. After data acquisition, the free-induction decays were line broadened with 20 Hz of exponential weighting, Fourier transformed to the frequency domain, and manually phased. Peaks were referenced relative to the carboxyl/carbonyl peak at 177.5 ppm. After data acquisition, the intensities were determined and normalized to the resonance from the C-6 carbon of GalNAc at 63.5 ppm.

Spectrophotometric assays of the bathing media. Proteoglycan concentrations in the media were measured spectrophotometrically as previously described (15), with some modifications. A solution of dimethylmethylene blue (DMMB) was prepared by mixing 8 mg of DMMB in 496.5 ml water containing 1 gm sodium formate, 2.5 ml ethanol, and 1 ml formic acid (pH 3.6, A_{535} 0.47). The assay was calibrated using standards containing 0–30 μl of 1 mg/ml CS C (Sigma) and a corresponding volume of DMEM to make a total volume of 50 μl in a 4.5-ml methacrylate cuvette. Two milliliters of the DMMB solution was added to each standard, and the percent transmittance was read immediately. Reagent blanks were used to recalibrate the spectrophotometer before each set of samples was read. Fifty microliters of the media samples was added to 2 ml of DMMB, and the transmittance was read. The quantity of proteoglycan in the samples was calculated using Beer's law and the standard curve. Glucose and lactate values were obtained with a commercially available immobilized-enzyme analyzer (Model 2300 STAT PLUS; YSI, Yellow Springs, OH).

RESULTS

Figure 2 shows representative ^{13}C spectra for 1) unlabeled control (natural abundance or 0.1% ^{13}C-labeled) cartilage, 2) cartilage tissue maintained in GlcN enriched to 99% ^{13}C at the C-1 carbon position, and 3) cartilage tissue maintained in Glc enriched to 99% ^{13}C at the C-1 carbon position. All samples were incubated in DMEM with the appropriate labeled supplement for 13 days prior to MRS analysis. The spectra are not drawn to scale because of variations in sample size and acquisition time.

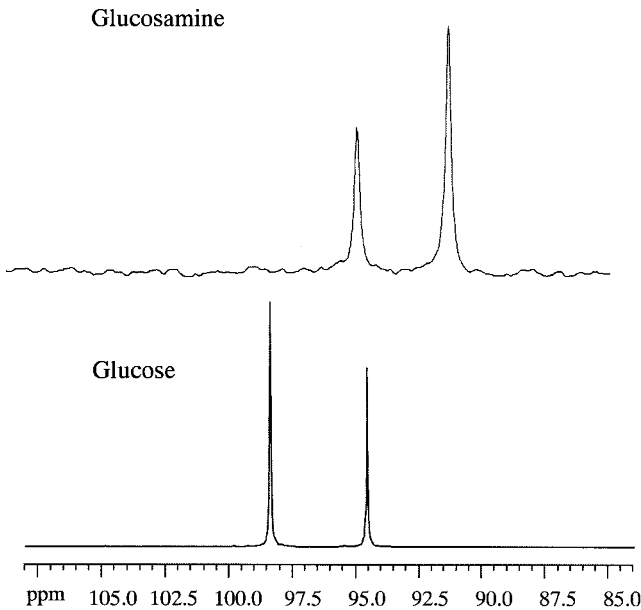

Figure 1. Representative ^{13}C magnetic resonance spectra of the labeled substrates, glucosamine, and glucose, in Dulbecco's modified Eagle's medium solution used for maintaining the cartilage samples.

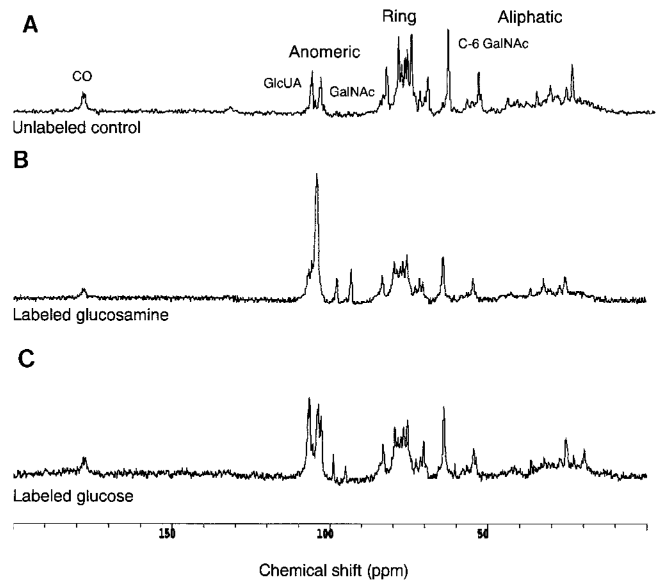

Figure 2. Representative ^{13}C magnetic resonance spectra (MRS) of cartilage samples maintained in culture for 13 days. The specimens were grown without ^{13}C labeling (**A**), with glucosamine (GlcN) labeled with ^{13}C at the C-1 position (**B**), and with glucose (Glc) labeled with ^{13}C at the C-1 position (**C**). The important resonance regions are labeled in the control spectrum. Relative increases in signal intensity are readily apparent in the anomeric (C-1) regions of the labeled GlcN and Glc specimens, indicating that the labeled carbohydrates are being metabolized by the chondrocytes for incorporation into cartilage proteoglycans. ^{13}C MRS spectra were acquired using a standard broadband proton-decoupled pulse sequence as described in Materials and Methods. CO = acetyl carbonyl; GlcUA = D-glucuronic acid; GalNAc = *N*-acetylgalactosamine.

The major resonance regions have been previously identified (16,17) but are repeated here for clarity of discussion. Aliphatic resonances appear in the region of 0–50 ppm. The C-3 carbon of lactate at 19.5 ppm is not apparent in the control spectrum but can be observed in the labeled Glc spectrum in Figure 2C. Resonances from the C-2–C-5 carbons of ring structures appear in the region of 50–90 ppm, including a sharply resolved peak at 63.5 ppm that corresponds to the C-6 carbon of GalNAc in cartilage. The outliers at 177.2 ppm and 177.8 ppm correspond to the carboxyl and acetyl

carbonyl (CO) resonances, respectively. The most important region of interest lies at ~90–120 ppm, where the anomeric (C-1) carbons resonate. No resonances from the Glc α and β anomers are visible in the control spectrum, but 2 broad, complex peaks in the region 100–110 ppm correspond to the expected resonance regions for the C-1 carbons of the GAG chains. The ^{13}C positions have been assigned for all of the carbons of the major GAG subunits in isolated, purified form (18–20).

Significant changes were visible in the spectra from the 2 labeled specimens, as shown in Figures 2B and C. Two new peaks became apparent in each spectrum, corresponding to the α and β anomers of unbound GlcN at 93 ppm and 97.5 ppm, respectively (Figure 2B), and the α and β anomers of unbound Glc at 95 ppm and 98.8 ppm, respectively (Figure 2C). Moreover, a prominent increase in signal intensity was evident in the region where the GAG species resonate. In the biosynthetic pathway for formation of GAG components, the integrity of the C-1 carbon position is maintained (7). That is, if the C-1 position has been labeled for Glc or GlcN, then products of the hexosamine pathway will be labeled at the C-1 position as well. The increases seen in these spectra indicate that the labeled precursors were being metabolized for production of cartilage proteoglycan. The increase in signal intensity over time for the specimens maintained in labeled GlcN is illustrated by the stacked plots shown in Figure 3. A spectrum from control, unlabeled cartilage at 6 days is also displayed for comparison.

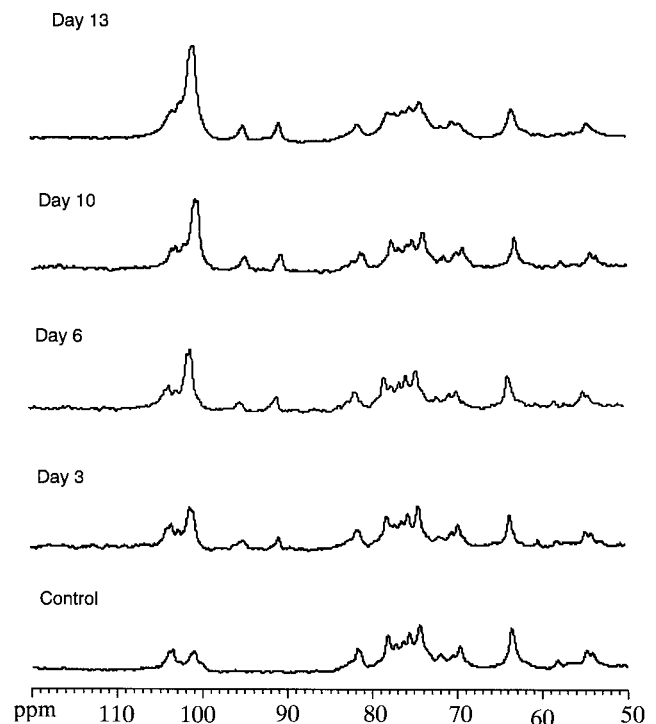

Figure 3. ^{13}C MRS spectra from specimens grown in labeled GlcN, illustrating the increase in signal intensity in the anomeric region over the course of the culture period, from day 3 through day 13. A spectrum from control, unlabeled cartilage is shown for comparison. See Figure 2 for definitions.

Expanded views of the anomeric region (90–120 ppm) are shown in Figure 4 for each of the spectra from Figure 2, along with solutions of CS standards (Sigma). The spectrum from cartilage supplemented with GlcN shown in Figure 4C reveals 5 overlapping resonances at 107.1, 106.6, 105.7, 104.3, and 103.7 ppm. Based on comparison with the CS6 solution shown in Figure 4A and on values from the literature (18–20), the resonances at 107.1 and 104.3 ppm were assigned to the GlcUA and GalNAc moieties, respectively, of CS6. From the CS4/CS6 mixture shown in Figure 4B, the resonances at 106.6 and 103.7 ppm were assigned to the corresponding GlcUA and GalNAc moieties of CS4. The final peak at 105.7 ppm arose from KS, whose components, GlcUA and GlcNAc, resonate too closely to be individually resolved (at 105.7 and 105.6 ppm, respectively).

In addition to the 5 peaks identified in the GlcN spectrum, both the Glc-labeled spectrum (Figure 4D) and control, unlabeled spectrum (Figure 4E) contained a peak at 102.8 ppm. This peak does not correspond to any peak positions for the major GAG species in cartilage. At this time, it is not known what molecular species corresponds to this resonance. However, this peak was conspicuously absent in the sample maintained with GlcN. In addition to CS and KS, several other forms of GAG are known to exist in cartilage in minor amounts, including hyaluronic acid, dermatan sulfate, and heparan sulfate. However, none of these GAG species is present in sufficient concentrations to be observable under these experimental conditions, especially when compared with the amounts of CS (55–90% total GAG) and KS (4–8% total GAG) (7,21).

The spectra from labeled GlcN and labeled Glc in Figures 4C and 4D clearly showed differences in enhancement profiles for the GAG components. The cartilage maintained in labeled GlcN showed a preferential and substantial increase in the aminated moieties (GalNAc), while the GlcUA components showed only a modest increase. In comparison, the Glc-labeled specimens showed an approximately equivalent increase for both subunits of CS. In order to better quantify the

effects of labeling, the intensities were determined for each of the peaks resonating in the GAG region. To normalize for intersample differences in volume and proteoglycan content, each of the peaks was normalized to the intensity from the C-6 carbon of GalNAc at 63.5 ppm. Graphs of normalized signal intensity as a function of culture time are shown in Figure 5.

DISCUSSION

The spectrum of unlabeled cartilage shown in Figure 2A is similar in appearance to previously pub-

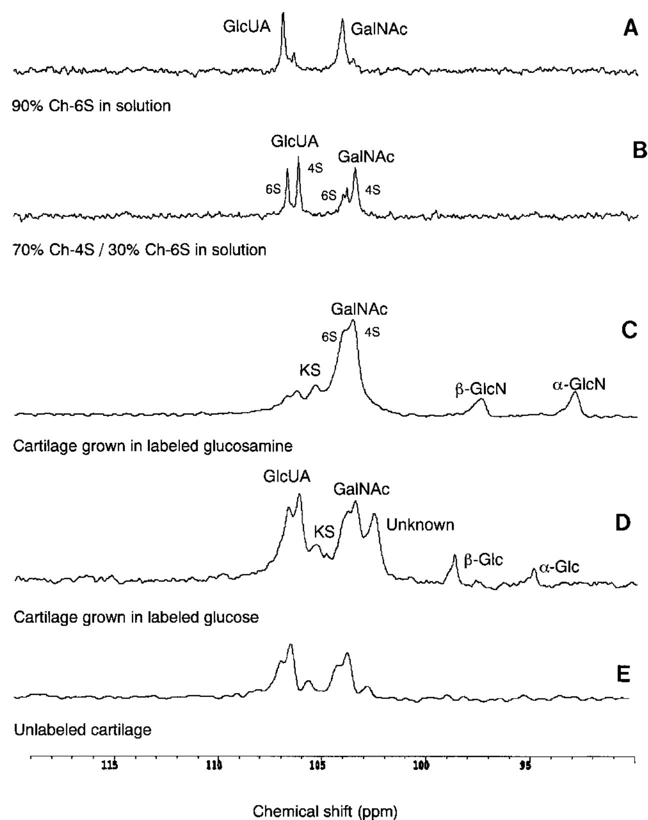

Figure 4. A–E, Expanded views of the anomeric region for spectra shown in Figure 2, along with spectra from chondroitin sulfate standards. Proteoglycan components metabolized from hexose and hexosamine substrates labeled with ^{13}C at the C-1 position appear in the anomeric region of ^{13}C MRS spectra. The chondroitin 6-sulfate (Ch-6S) and chondroitin 4-sulfate (Ch-4S) moieties can be individually resolved in the spectra from the cartilage explants. Based on comparisons with known standards of Ch-6S and Ch-4S and from values in the literature, the resonance peaks were assigned as follows: 107.1 ppm, GlcUA of Ch-6S; 106.6 ppm, GlcUA of Ch-4S; 105.7 ppm, GlcUA and 2-acetoamidoglucosamine (GlcNAc) of keratan sulfate (KS); 104.3 ppm, GalNAc of Ch-6S; 103.7 ppm, GalNAc of Ch-4S. See Figure 2 for other definitions.

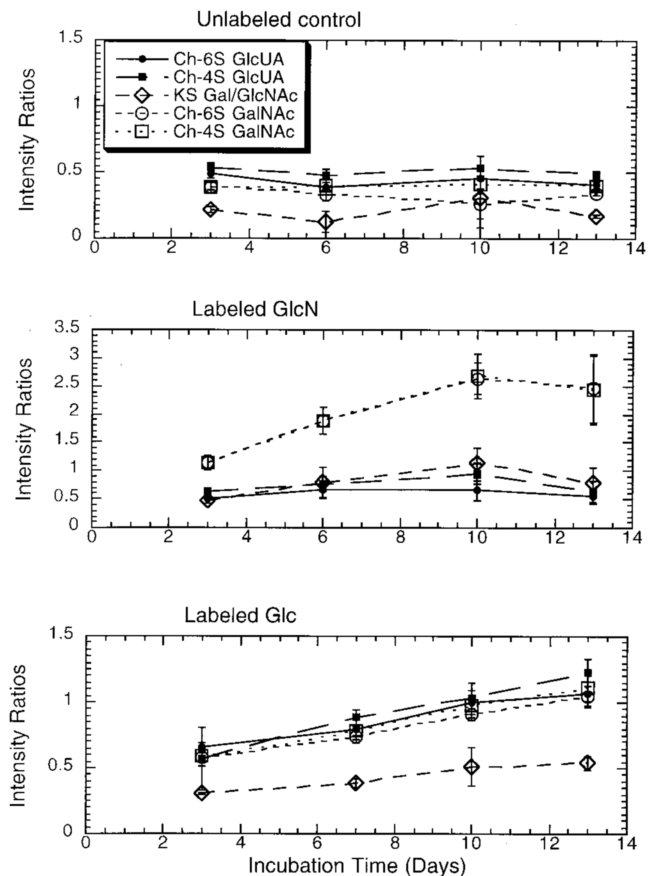

Figure 5. Intensity ratios for each of the peaks that resonates in the anomeric region of ^{13}C MRS spectra obtained on articular cartilage samples grown without ^{13}C labeling (top), samples grown in GlcN labeled at the C-1 position (middle), and samples grown in Glc labeled at the C-1 position (bottom). To normalize for intersample and experimental variability, all signal intensities were normalized to the C-6 peak of GalNAc at 63.5 ppm. See Figures 2 and 4 for definitions.

lished natural-abundance ^{13}C spectra of bovine nasal cartilage (16,22) obtained at lower magnetic field strengths and with lower spectral resolution. This spectrum reveals a complex pattern resulting from the numerous carbons present within the tissue. It is interesting to note that the ^{13}C spectrum of cartilage largely reflects the proteoglycan component. Previous research has indicated that collagen is restricted in motion and therefore is not observable with standard MRS techniques (21). On the other hand, the GAG chains have sufficient rotational mobility to render them effectively 100% visible on MRS (16,21). Therefore, under conditions of complete relaxation, where the nuclear spins are allowed to regain magnetic equilibrium between each excitation, the areas under the peaks are directly proportional to

the number of atoms at each carbon position in each molecule. For the experimental protocol used in this study, where the longitudinal relaxation time for all GAG components was 0.06 seconds (21) and the time between excitations was 0.4 seconds, this condition was met. Therefore, the changes in the various GAG components can be directly compared.

As expected, no increase in signal intensity was observed for any of the 5 resonances in the control, unlabeled cartilage, as shown in Figure 5A. This result demonstrates that the cartilage proteoglycan composition remained invariant and stable during the culture period. In contrast to the results with control cartilage, the GalNAc peaks in the labeled GlcN cartilage showed a substantial increase in relative signal intensity up to the tenth day of culture (Figure 5B). Moreover, the signal increase in GalNAc was comparable for both types of CS, producing enhancements of 641% by day 13 for CS6 and 518% for CS4 relative to the intensities from control cartilage. In comparison, these same resonances increased by only 215% and 180%, respectively, when cartilage was cultured with labeled Glc for 13 days (Figure 5C). Since the molar amount of labeled material was roughly equivalent in the 2 carbohydrate pools (6.7% for the GlcN medium and 8.1% for the Glc medium), these results indicate that GlcN was preferentially incorporated into the GalNAc products of the hexosamine pathway, at levels 300% higher than Glc.

In addition to the CS GalNAc resonances, the KS peak showed a larger enhancement with labeled GlcN than with Glc (289% versus 164%). Although it is not possible to differentiate the contributions from the 2 moieties because of the substantial overlap in peak intensities, it is probable that the larger signal observed with labeled GlcN reflects higher incorporation into the GlcNAc component, which is produced through the hexosamine pathway as well.

Unlike the aminated moieties of the GAG subunits, the GlcUA peak intensities showed modest increases when cartilage was cultured in labeled GlcN. The maximum enhancements for these resonances were 49% and 88% for CS6 and CS4, respectively. When cultured in labeled Glc, the corresponding increases were 145% and 143%. These results indicate that a thermodynamic or kinetic barrier exists in the biochemical pathway between GlcN and GlcUA.

To determine whether total proteoglycan content changed with GlcN supplementation, spectrophotometric assays were run on all tissue after papain digestion. As shown in Table 1, proteoglycan content was not statistically different for any of the cartilage populations.

Table 1. Metabolic characteristics of the cartilage samples as determined from spectrophotometric assays*

Sample	Labeled GlcN	Labeled Glc	Unlabeled control
Wet weight, mg	156 ± 14	158 ± 25	144 ± 21
PG in tissue, μg/mg tissue	28.6 ± 0.4	29.5 ± 0.5	31.0 ± 0.7
PG release, μg/mg tissue/day	0.6 ± 0.2†	0.8 ± 0.2	0.9 ± 0.4
Glc consumption, μmoles/mg tissue/day	0.70 ± 0.09	0.71 ± 0.13	0.65 ± 0.10
Lactate production, μmoles/mg tissue/day	0.99 ± 0.13	1.15 ± 0.19	0.91 ± 0.12

* Values are the mean ± SD. GlcN = glucosamine; Glc = glucose; PG = proteoglycan.
† $P < 0.05$ versus labeled Glc and unlabeled control.

In addition, GAG levels in the cartilage remained constant during the culture period (data not shown). Therefore, the increased signal observed with labeled Glc or GlcN reflects incorporation of newly formed GAG into a stable matrix. Under these circumstances, the maximum signal enhancement for any GAG component would be 1,000%, corresponding to complete replacement of the tissue. Therefore, the 518% and 641% increases observed for the GalNAc resonances with GlcN supplementation indicate that this aminated glucose derivative was incorporated into more than half of GAG during the course of the culture (13 days). In addition, as shown in Table 1, release of GAG into the medium was slightly depressed in the GlcN-supplemented cartilage compared with control and Glc-supplemented samples, suggesting that GlcN may also have an inhibitory role in proteoglycan breakdown, as has been postulated by others (9). However, the levels of proteoglycan released into the media represented a small fraction of the total proteoglycan in the cartilage for all of the sample groups.

GlcN uptake studies have previously been performed using radiolabeled isotopes such as sulfate-35 (23,24) or carbon-14 (25). Radiolabeling is a highly sensitive technique that can enable detection of as little as 10 ng of proteoglycan (26). However, radiolabeling cannot reveal with certainty the nature of the species that incorporates the radioactivity. Our results demonstrate, for the first time, that GlcN is preferentially incorporated into products of the hexosamine pathway in cartilage explants. Although these findings do not prove that GlcN provides therapeutic relief or chondroprotective benefits, these results clearly show that chon-

drocytes will metabolize GlcN and utilize it in newly synthesized proteoglycan when this carbohydrate is made available to them. Moreover, GlcN is the substrate of choice for the amine sugar moieties of GAG. Since the bioavailability of orally administered GlcN approaches 90% in humans and other animals (27,28), it is tenable to postulate that some portion of GlcN supplements will end up in the cartilage matrix. The issues that remain concern the relative proportion of GlcN that reaches the chondrocytes and the impact that newly synthesized GlcN-derived molecules have on articular cartilage matrix repair and function.

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