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J Rheumatol 2005;32;1775-1786

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# N-Acylation of Glucosamine Modulates Chondrocyte Growth, Proteoglycan Synthesis, and Gene Expression

DORIS E. TERRY, KAREN REES-MILTON, PATRICK SMITH, JOHN CARRAN, PARVIS PEZESHKI, CRAIG WOODS, PETER GREER, and TASSOS P. ANASTASSIADES

**ABSTRACT.** *Objective.* To examine the effects of glucosamine (GlcN) and some N-acylated (GlcNAcyl) derivatives on the proliferation and proteoglycan (PG) synthesis of bovine articular chondrocyte (BAC); and to expand these results to human articular chondrocytes (HAC) and study the modulation of gene regulation by these compounds.

*Methods.* The compounds tested were: glucose (Glc), GlcN.HCl, N-acetyl GlcN (GlcNAc), and N-butyryl GlcN, (GlcNBu). GlcNBu was synthesized from GlcN and butyric anhydride. For the chondrocyte cultures, both anchorage-dependent (AD) and an anchorage-independent (AI) system (alginate beads) were evaluated. Following the various additions, BAC were assessed for total cell number, DNA, or total PG synthesis at different times. Utilizing similar conditions, human cDNA microarrays were performed for the HAC after harvesting total RNA.

*Results.* For AD cultures, the addition of GlcN.HCl (0.1–5.0 mM) to BAC or HAC cultures inhibited cell proliferation and total PG synthesis in a dose-dependent manner. For AI cultures, the inhibitory effects of GlcN.HCl on cell proliferation were less prominent, and PG synthesis increased slightly more for the GlcNAcyl than the GlcN additions. In the AD system, the addition of GlcNAc did not result in the inhibitory effect of GlcN.HCl, while GlcNBu addition resulted in an increase in BAC proliferation and PG synthesis that could not be explained by the Bu moiety alone. For the HAC, additions of 0.1 mM GlcNBu resulted in upregulation of a large number of genes, with only a few downregulated, while GlcN addition resulted in no upregulation and one downregulated gene, for preset stringency criteria.

*Conclusion.* Addition of GlcNBu to BAC or HAC to AD cultures generally stimulated cell proliferation and PG synthesis, while addition of GlcN resulted in inhibition of these indicators. The inhibitory effects of the GlcN molecule appear to be related to the unsubstituted amino group. Additions of GlcNBu, but not GlcN, to HAC resulted in upregulation in the expression of a large number of genes. (J Rheumatol 2005;32:1775–86)

## Key Indexing Terms:

ARTICULAR CARTILAGE  
GLUCOSAMINE SULFATE

CHONDROCYTE  
ACYLATION

GLUCOSAMINE  
GENE REGULATION

Osteoarthritis (OA) in humans is the most prevalent form of arthritis and is considered the second leading disability in those over age 50 years<sup>1,2</sup>. One of the prominent alterations

that characterize osteoarthritic cartilage damage is a reduction of proteoglycan (PG) content, reflecting an imbalance between synthesis and release of PG. Both synthesis and release depend on the metabolic activity of cartilage cells<sup>3–5</sup>.

In humans, nonsteroidal antiinflammatory drugs (NSAID) are widely used for pharmacological management of OA, as well as rheumatoid arthritis (RA). However, the use of NSAID is limited by gastrointestinal (GI) toxicity, and the addition of some NSAID to bovine articular chondrocyte (BAC) culture systems is known to inhibit PG synthesis<sup>6,7</sup>. The net effect of selective cyclooxygenase 2 (COX-2) inhibitors on PG synthesis and on matrix homeostasis remains to be determined<sup>8</sup>. Another approach to treatment of OA is through protease inhibitors, but the clinical toxicity of serine and aspartate protease inhibitors for the treatment of OA has limited usage.

Glucosamine sulfate salt (GlcN.S), GlcN.HCl, and chondroitin sulfate and are receiving considerable attention for

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Supported by the Canadian Institutes of Health Research (MOP 43833), the National Science and Engineering Research Council of Canada (STP 246 039-01), and Farnam Companies, Inc., Phoenix, Arizona.

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Accepted for publication May 17, 2005.

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the treatment of joint pain in OA. For human use, the efficacy of GlcN for the treatment of OA is based on randomized clinical trials (RCT), utilizing mostly pain scores and functional assessments as primary endpoints<sup>9</sup>. Metaanalysis of RCT on GlcN for the symptomatic treatment of OA supports a positive effect of GlcN on pain for knee OA<sup>10</sup>. The majority of these RCT have been industrially sponsored. In North America, GlcN compounds, which are sold “over the counter” as nutritional supplements, are available in the form of GlcN.HCl, GlcN.S, and GlcNAc. The mechanism of action by which the GlcN compounds apparently ameliorate pain in OA is not understood.

Other over the counter supplements, such as chondroitin, collagen, and other so-called chondroprotective agents have gained popularity as a potential therapy for OA. Collectively, these alternative agents are sometimes included with other slow acting, disease modifying drugs, but they are chemically related to structural molecules of the cartilage matrix<sup>11</sup>.

The use of GlcN in OA as a “nutraceutical” is based on the presumption that ingested GlcN will reach the joints and serve as a substrate for the synthesis of articular cartilage glycosaminoglycans (GAG). GlcN-sulfate.S (GlcNS) is well absorbed in the gut and, unlike the NSAID, does not irritate the GI tract<sup>12</sup>. However, the serum concentrations of free GlcNS even after ingestion by humans of as much as 6 g of this compound were below the limits of detection available at the time<sup>13</sup>, making it difficult to confirm that sufficient GlcN would reach the articular cartilage to contribute significantly as a substrate for GAG synthesis. Recently, a sensitive method for detecting GlcN in rat plasma has been described, based on derivatization of GlcN and high performance liquid chromatography (HPLC), with a detection limit of 0.63  $\mu\text{g/ml}$  and a quantification limit of 1.25  $\mu\text{g/ml}$ <sup>14</sup>. Using this methodology, single-dose pharmacokinetics in the rat indicated that orally administered GlcN is rapidly absorbed and cleared and that the gut, rather than the liver, is responsible for the first-pass metabolism<sup>15</sup>. This method has yet to be utilized on human plasma. Further, it is not known to what extent the GlcN molecule is incorporated intact into the GAG or if it is previously deaminated to glucose. Overall, the pharmacokinetic evidence that GlcN acts as a “nutraceutical” by providing a more readily available substrate than glucose for GAG synthesis by HAC is not convincing.

GlcN has been shown to regulate the expression of several genes, including important growth factors such as transforming growth factor- $\beta$  (TGF- $\beta$ ), and the hexosamine pathway is required for glucose to modulate the transcription of these growth factors<sup>16-18</sup>.

The choice of the culture system used, specifically with respect to anchorage-dependent (AD) or anchorage-independent (AI) conditions, is an important consideration for new compound or drug evaluation in chondrocyte culture.

AD systems have advantages including, (1) uniform drug accessibility of the test compounds to all cells in the culture; (2) effects such as density-dependent inhibition of growth can be evaluated. In AI systems, concentrations of a drug within the cell aggregates (as in agarose, beads, droplets, etc.) are likely much less in the center of the aggregate than the periphery and such differences are generally not determined experimentally. Thus AI systems have disadvantages in terms of uniformity of drug delivery. However, AD systems obviously have disadvantages in terms of relative retention of the cartilage phenotype in culture (particularly with repeated subculture), which is an advantage of AI systems. Also, it should be noted that when repair does occur in damaged articular cartilage (as for example after proximal tibial osteotomy in OA), the repair is primarily by fibrocartilage, rather than fully differentiated hyaline cartilage<sup>19,20</sup>.

We investigated the role of N-acylation of GlcN on chondrocyte proliferation and GAG synthesis. The hexosamines in the GAG of the cartilage PG are N-acetylated, although the reason for N-acetylation of hexosamines in PG (or, more generally, in glycoproteins) is not understood. One of the more interesting N-acylated GlcN is N-butyryl GlcN (GlcNBu), which we had shown in preliminary studies to have stimulatory effects on BAC proliferation and total GAG synthesis, not seen with the parent molecule<sup>21,22</sup>.

We examined the effects of GlcN, GlcNAc, and GlcNBu over a wide range of concentrations on BAC proliferation and GAG synthesis experiments under AD and AI culture conditions. We extended the studies to HAC AD cultures, which have much lower rates of proliferation than the BAC. For the HAC cultures, we also carried out cDNA microarray studies, in order to determine differential gene expression between cells fed Glc, GlcN, or GlcNBu.

## MATERIALS AND METHODS

GlcN.HCl and GlcNAc were obtained from Sigma Chemical Company (St. Louis, MO, USA). Initially GlcNBu was chemically synthesized in 200 mg lots from GlcN.HCl and butyric anhydride in a bicarbonate system<sup>23</sup> and purified on a Bio-Gel P-2 chromatography column (Bio-Rad Laboratories, Hercules, CA, USA). The GlcNBu powder was reconstituted in water, applied on the water-pretreated column, and eluted with water at a flow rate of 1 ml/10 min. Column fractions corresponding to GlcNBu were pooled and lyophilized. Purity was verified by descending paper chromatography and HPLC.

We later used a synthesis best suited to producing larger amounts of material, based on a modification of the procedure<sup>24</sup>, utilizing the same starting materials and substituting NaOMe for bicarbonate. After synthesis, GlcNBu was purified by a Soxhlet extraction method from absolute ethanol. GlcNBu was dried under vacuum and analyzed by mass spectrometry, nuclear magnetic resonance, HPLC, and melting point.

*Bovine articular chondrocytes.* BAC were isolated from bovine articular cartilage (convex surfaces of the carpometacarpal joints of adult cows) by collagenase digestion, and subcultures were obtained and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS, 10%), Glc (1 mg/ml), sodium carbonate (3.7 g/l), penicillin-streptomycin (P/S) (2%), and phenol red as described from our laboratory<sup>25-27</sup>.

**AD cell culture.** Confluent subcultures were trypsinized, seeded at  $33 \times 10^3$  cells/well (6-well plates),  $5 \times 10^3$  cells/well (24-well plates) or  $10^3$  cell/well (96-well plates) in the above medium, and incubated overnight at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. Then the media were replaced with fresh glucose-free media containing the appropriate GlcN analog. The GlcN compounds used in this study include GlcN.HCl, N-acetyl GlcN (GlcNAc), and N-butyryl GlcN (GlcNBu).

**Cell proliferation assay for AD cultures.** After overnight incubation, the media were replaced with fresh media containing test materials of interest (see figure legends). At specified times, the cells were harvested by trypsinization with 0.25% trypsin in phosphate buffered saline (PBS, pH 7.4), and cell numbers were determined with a Coulter counter (Coulter Electronics, Hialeah, FL, USA).

**DNA synthesis for AD cultures.** To measure DNA synthesis at different timepoints, BAC (1000 cells/well) were plated in Corning Costar 96-well tissue culture plates. On the following day, the media were replaced with fresh DMEM containing the test compounds at the concentrations indicated. Three or 7 days later, the cells were pulse labeled with [ $^3$ H]-thymidine (0.3  $\mu$ Ci/well; ICN Biomedicals, Aurora, OH, USA) for 6 and 18 h in the same culture conditions. After labeling, the cells were washed 3 times with PBS, pH 7.4, and lysed in 90% hyamine hydroxide. The radioactivity incorporated into the cells was measured using high-throughput Microbeta liquid scintillation and luminescence counter (Wallac Trilux, Turku, Finland).

**AI alginate culture.** Articular chondrocytes were isolated from articular cartilage bovine ankle joints as described<sup>27</sup>. Primary AI cultures were set up of the isolated chondrocytes in alginate beads at a seeding density of  $0.2 \times 10^6$  cells/well as described<sup>28</sup>. The DMEM-F12, 10% FBS growth medium included 3 mM CaCl<sub>2</sub> for enhanced alginate bead stability in culture. Encapsulated chondrocytes were recovered from the alginate beads and enumerated as for the AD cultures at the end of each of 7, 14, and 21 day incubation periods. Cultures remaining after 7 and 14 days were re-fed with the same medium, after the original medium had been removed.

**Metabolic labeling for PG synthesis.** For radiolabeling studies in AD cultures, replated cells in 6-well or 24-well plates were seeded at high density, and the cells were confluent within 4 days. The media were replaced with fresh media containing test materials of interest and radiolabeled with 10  $\mu$ Ci/ml [ $^{35}$ S]-sulfate. After 4 days of incubation at 37°C, both media and cells were harvested. The AI cultures were radiolabeled in a similar fashion, except that [ $^{35}$ S]-sulfate labeling was for the 17–21 day period. The cells were counted as described, while the media were stored at –20°C until analyzed.

**Precipitation and quantification of newly synthesized proteoglycans.** For AD cultures, the rapid Alcian blue method for isolation and quantification of radiolabeled PG was employed as described<sup>29</sup>. Briefly, 20  $\mu$ l of media were spotted on cellulose acetate strips, air dried, and stained with 0.5% Alcian blue in destain solution (30 mM MgCl<sub>2</sub>, 0.1% CH<sub>3</sub>COOH, 10% ethanol) for 1 h. The strips were then destained, air dried, and cut in sections and the [ $^{35}$ S]-sulfate radioactivity was assessed with Microbeta liquid scintillation and luminescence counter. For qualitative analysis on sodium dodecyl sulfate polyacrylamide gel electrophoresis, total anionic glycoconjugates were isolated using the toluidine precipitation method<sup>29</sup> and were stored at –20°C.

For AI cultures, the radiolabeled PG were precipitated from the dissolved beads and 150  $\mu$ l of medium was precipitated with 8 volumes of absolute ethanol and the [ $^{35}$ S]-sulfate radioactivity in the washed precipitates was directly quantified (alginate partially interferes with Alcian blue precipitation method).

**Determination of dead cells.** Cells were seeded at  $5 \times 10^3$  cells/well overnight, and the media were replaced with fresh media containing the test material of interest. At the end of the culture period, the cells were harvested by trypsinization, washed with 1 ml of 2% serum in PBS, and centrifuged at 100  $\times$ g for 5 min. The cells were stained with propidium iodide

for 5 min and analyzed on the flow cytometer. Dead cells were found to vary between 3% and 6%, under the different experimental conditions, by this method<sup>30</sup>.

#### Human Articular Chondrocytes

**Cell culture and RNA isolation.** HAC were isolated from knee joint tissue resected during arthroplasty for OA. Visually healthy appearing cartilage from the margins of the resected joint was used. Cartilage pieces were sliced into 1 mm<sup>3</sup> slices and then treated overnight with collagenase (0.04%) to free HAC for culture. After the fourth passage, the media was changed to DMEM minimal media, without glucose, plus 10% fetal bovine serum and 2% P/S. After the cells acclimatized, they were trypsinized and counted by Coulter counter, and replated at a cell density of 5000 cells/cm<sup>2</sup> in 6-well cell culture plates (Corning, Corning, NY, USA). Medium was changed after 24 h and the experimental regimens started at that time. There were 8 replicate cultures for each condition described. Cultures were treated with 0.1 mM Glc, 0.1 mM GlcN, or 0.1 mM GlcNBu. These concentrations were based on previous cell proliferation experiments, where they did not have a major effect on proliferation, so that confluency was reached at about the same time in all the culture conditions. On the 20th day, total RNA was collected using a Purescript RNA isolation kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's instructions. Total RNA from each set of 8 cultures was pooled separately and quantified spectrophotometrically with 260 nm/280 nm ratios from 1.84 to 1.94. Seventy micrograms total RNA for each of the 3 conditions were sent to the Queen's University microarray facility (Kingston, ON, Canada) for analysis.

**Cell proliferation assay and quantification of newly synthesized PG.** Methods to quantify the rate of cell proliferation and total net PG synthesis for HAC cultures treated with 0.1 mM GlcN or GlcNBu, relative to the controls, were as described above for BAC.

**cDNA microarray and analysis.** Experimental cDNA probes (GlcN or GlcNBu treated replicate cultures) were labeled with Cy3, while control cDNA (Glc treated replicate cultures) was labeled with Cy5. Probes were hybridized to the array containing 19,000 human cDNA sequences, representing known genes and expressed sequence tags (EST). A second array was performed with experimental cDNA probes (GlcN or GlcNBu) labeled with Cy5, while control cDNA (Glc) was labeled with Cy3. The flip-fluor experiment was performed to ensure noninterference from the labeling tag. The arrays were obtained from the University Health Network Microarray Center (Toronto, ON, Canada) and printed on CMT-GAPS slides (Corning). The slides were scanned using the ScanArray 4000, and image analysis was performed with QuantArray 3.0. The data were normalized to ensure that median values for each dye label were the same for each gene, and log<sub>2</sub> ratios of Glc(–)/GlcN and Glc(–)/GlcNBu determined for both experiments.

Data analysis was as follows. Analysis of the microarray data involved several steps of sorting, conducted using Microsoft Excel sorting function to eliminate human bias toward particular genes. All genes that did not meet the specified criterion were removed from the data set. First, data were sorted into either GlcN vs control, or GlcNBu vs control (at Queen's University Microarray Center). These groupings were then subdivided into increased or decreased gene expression. The data sets were treated to independent analyses. The data sets were then sorted by level of regulation, with only genes exhibiting a significance level above 4-fold over the glucose-free cells being maintained for further sorting. Genes that survived the significance test were sorted by ascension number to group genes that appear at least twice on the list of significantly regulated genes. These data sets were then pooled with the corresponding data set from the flip-fluor experiment. The final data set consists of genes that appear twice on both "flipped" arrays with a level of regulation surpassing 4-fold.

## RESULTS

**BAC proliferation and PG synthesis under AD conditions.** Media supplemented with GlcN.HCl (1.25 mM) inhibited

chondrocyte proliferation, under AD conditions, after roughly 4 days of growth (Figure 1). In preliminary experiments, this concentration of GlcN.HCl (1.25 mM) was found to be the minimal concentration providing for close to maximal inhibition of BAC proliferation response (see also Figure 4A).

The rates of proliferation for all the treatments were similar up to Day 4, and thereafter the rate of proliferation of the cells treated with GlcN.HCl flattened out, so significantly lower final cell densities were achieved (i.e., by Day 6; Figure 1) in the GlcN treated cultures compared to the other conditions. The inhibition could not be accounted for by the HCl moiety of the GlcN.HCl, since supplementation with 1.25 mM HCl alone resulted in a slight stimulation of proliferation over controls. Supplementation with GlcNAc also resulted in a slight stimulation of proliferation over controls. No cell detachment or other morphological evidence of cell damage was observed in any of the conditions. Cell death assessed by propidium iodide staining in this and other experiments was 3%–6%, as indicated.

Since N-acetylation of GlcN appeared to abolish the antiproliferative effect of the parent molecule, we further studied the effect of N-acylation of GlcN in general on chondrocyte proliferation in the AD cultures. Proliferation studies with media containing the standard concentration of Glc, 0.5 mg/ml (2.77 mM), and supplemented with 1 mg/ml of GlcN.HCl (4.64 mM), GlcNAc (4.5 mM), or GlcNBu (4.01 mM) over a 10 day growth period, confirmed that N-acylation of GlcN abolished the inhibitory effect of GlcN (Figure 2A). Interestingly, when the N-position of GlcN was butyrylated, supplementation of the media with the purified GlcNBu compound resulted in a significant stimulation in

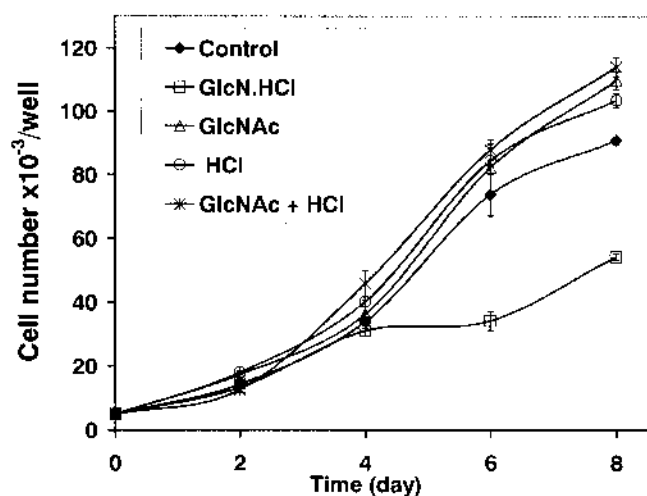


Figure 1. AD BAC proliferation in the presence of GlcN analogs. Chondrocytes were seeded at  $5 \times 10^3$  cells/well, and after a day they were treated with 1.25 mM of GlcN.HCl, GlcNAc, HCl, or GlcNAc + HCl. Cells were harvested at time intervals and counted. Data points represent the mean from 3 replicate wells each counted 3 times (standard error bars are included for all points, but some fall within symbols).

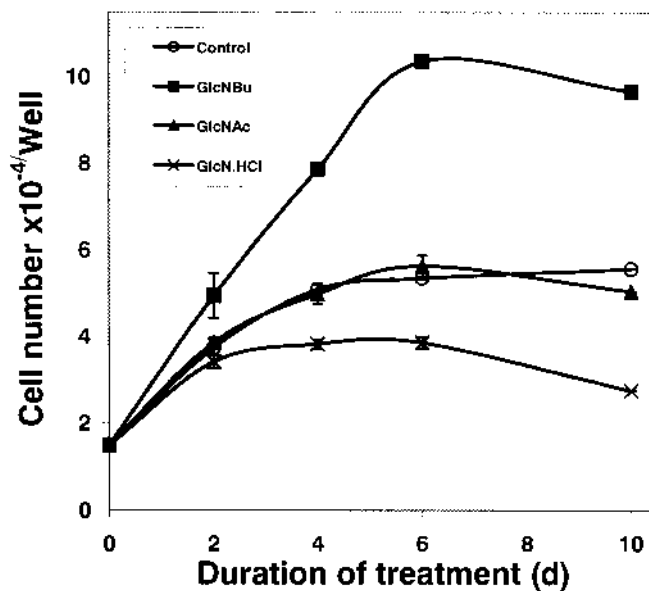


Figure 2A. AD BAC proliferation in the presence of GlcN analogs. Chondrocytes were seeded at  $5 \times 10^3$  cells/well, and after a day they were treated with 1.25 mM of GlcN.HCl, GlcNAc, GlcNBu, or Control (Glc +). Cells were harvested at time intervals and counted. Data points represent the mean from 3 replicate wells each counted 3 times (standard error bars are included for all points, but some fall within symbols).

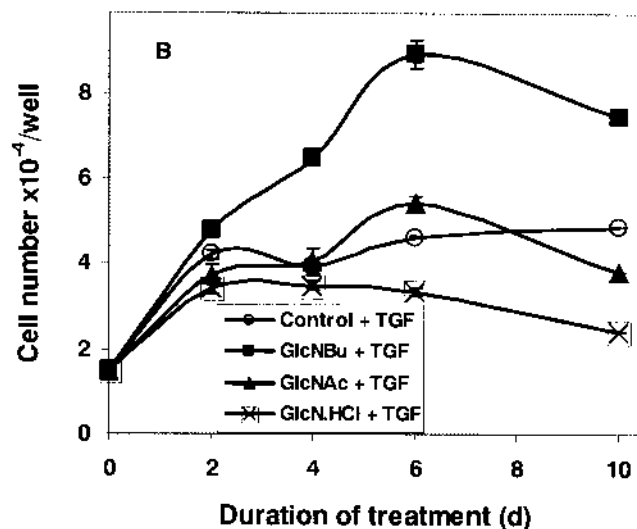


Figure 2B. AD BAC proliferation in the presence of GlcN analogs and TGF- $\beta$ . Conditions were identical to those in Figure 2A, except that TGF- $\beta$  10 ng/ml was added at the beginning of the incubation period. Data points and standard errors are as for Figure 2A.

chondrocyte proliferation over the controls. Addition of TGF- $\beta$  to the cultures only slightly modified the proliferative responses (Figure 2B). Dose-response studies at 6 and 12 day growth periods indicated that the stimulatory effect of GlcNBu on chondrocyte proliferation could not be accounted for by the butyryl moiety, since sodium butyrate at  $\geq 2$  mM inhibited cell proliferation, as did GlcN.HCl

(Figure 3). No antiproliferative effects of GlcNBu were observed up to 6 mM during these growth periods.

The specificity of GlcN functional groups on chondrocyte proliferation was explored further, in the absence of Glc in the culture media. Data obtained from 8 day treatments of chondrocytes with 0.5–5 mM GlcN.HCl showed a dose-dependent decrease in the final cell numbers (Figure 4A). Interestingly, GlcN.HCl in the absence of Glc still inhibited cell proliferation as observed in the presence of Glc. In contrast, GlcNBu increased cell number compared to the controls (Figure 4A). The addition of GlcNAc resulted in small increases in cell numbers over controls. Also, the morphology of cells treated with GlcN.HCl was different from those of Glc, GlcNBu, and GlcNAc. BAC incubated with GlcN.HCl, especially > 0.5 mM, appeared more fibroblastic compared to other treatments (data not shown). The proportion of dead cells was similar for the different treatments. Rates of proliferation and final cell densities achieved were highly reproducible for replicate cultures for BAC isolated from the cartilage of any one animal, as indicated by the small standard errors in the data. However, cell isolates from different animals, of varying weight and herd origin, did show differences in absolute values for the proliferative responses, as expected. The reversal of the inhibitory effects of GlcN (at the higher concentrations) when the amino group of GlcN is N-acylated was entirely consistent for all isolates, although we observed differences in the magnitude of stimulatory responses of the GlcNAcyls in cultures derived from different animals.

The pattern of DNA synthesis, utilizing [<sup>3</sup>H]-thymidine incorporation (Figure 4B), was generally similar to the BAC

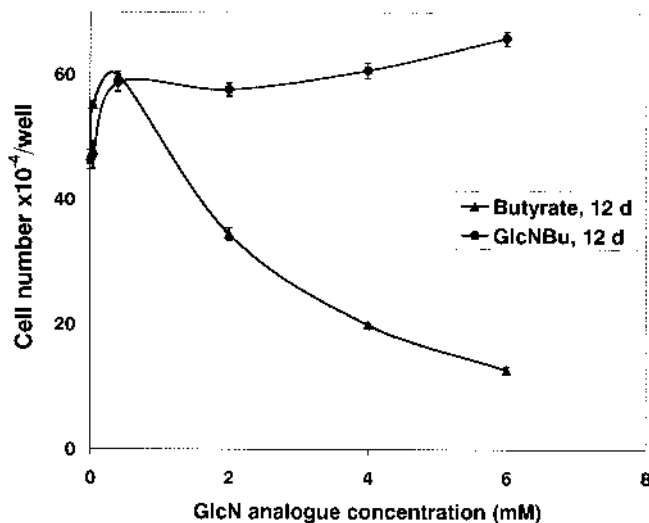


Figure 3. Effect of varying concentrations of GlcNBu and butyrate on AC BAC proliferation. After plating BAC and allowing them to attach overnight ( $33 \times 10^3$  cells/well; 6-well plates), chondrocytes were treated with various concentrations (0.04, 0.4, 2, 4, and 6 mM) of GlcNBu and butyrate for 12 days, and cells were counted (standard error bars are included for all points, but some fall within symbols).

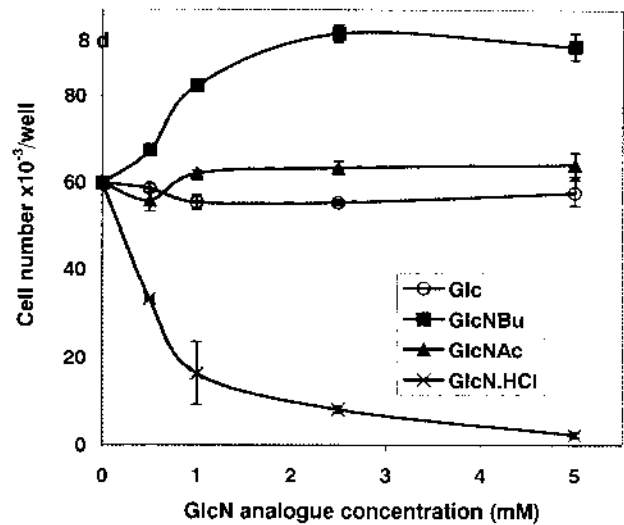


Figure 4A. Differential dose-dependent effect of GlcN analogs on AD BAC proliferation. After plating and attachment, the chondrocytes, cultured in the absence of glucose in the media, were treated with increasing concentrations of the various GlcN analogs for 8 days (0.5, 1, 2.5, and 5 mM). At that time cells were harvested and counted (standard error bars are included for all points, but some fall within symbols).

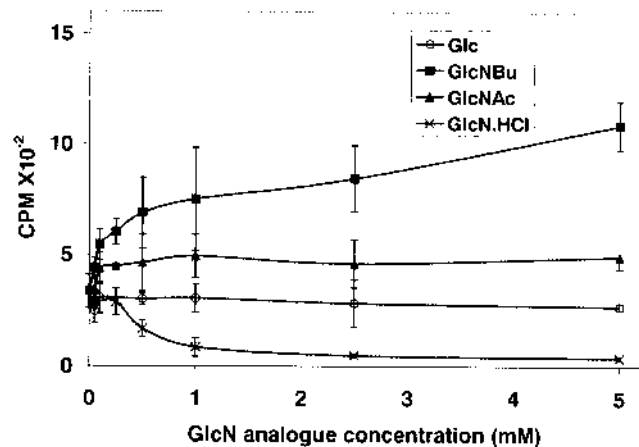
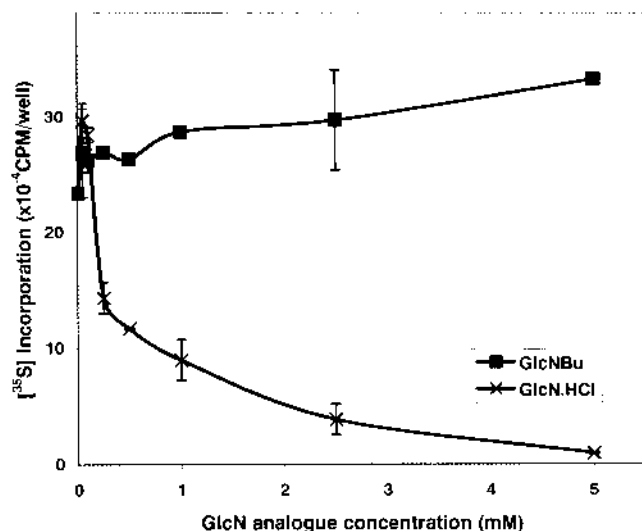


Figure 4B. GlcN analogs differentially affect [<sup>3</sup>H]-thymidine incorporation into DNA by AD BAC. After 1 day in culture, chondrocytes were incubated with fresh media containing Glc, GlcNBu, GlcNAc, or GlcN.HCl. At 3 days, media were replaced with fresh media containing [<sup>3</sup>H]-thymidine and increasing concentration of Glc analogs. Incorporation of [<sup>3</sup>H]-thymidine into total DNA for each well was determined after 6 h of radiolabeling for each concentration of the added GlcN derivatives. Data and standard errors shown are for the mean of 3 separate determinations.

proliferation data, with respect to the relative responses of the GlcN analogs. Labeling at 3 or 7 days of culture and pulsing periods with the label for 6 h or 18 h (see Materials and Methods) resulted in differences in total [<sup>3</sup>H] incorporation into chondrocyte DNA, but the relative effects for the GlcN analogs were similar, as shown for 3 day cultures labeled for 6 h (Figure 4B).

Since PG are the only macromolecules in the extracellular matrix of articular cartilage that contain a substantial number of covalently bound sulfate groups<sup>31</sup>, the convenient Alcian blue precipitation method<sup>29</sup> was used to estimate net PG synthesis over a 4 day labeling period. Experiments were carried out using confluent BAC cultures, treated with varying concentrations of Glc, GlcNBu, GlcNAc or GlcN.HCl, and [<sup>35</sup>S]-sulfate, as described above. Analyses of the net PG synthesis, as assessed by incorporation of [<sup>35</sup>S] into PG, revealed a concentration-dependent decrease in the net PG synthesis (per well) as a result of supplementation of the media with GlcN, for concentrations  $\geq 0.5$  mM (Figure 5). Lower concentrations resulted in a small stimulation of net PG synthesis. The addition of GlcNBu resulted in a relatively small stimulation of PG synthesis (compared to no addition). However, there were large (statistically significant) increases of PG synthesis for the higher concentrations for the GlcN to GlcNBu comparison. This “reversal” of the inhibition of PG synthesis by increasing concentrations of GlcN was not specific to GlcNBu, since other N-acylated GlcN, including GlcNAc, also reversed the GlcN mediated inhibition (data not shown).

**BAC proliferation and PG synthesis under AI conditions.** BAC proliferation and PG synthesis was also evaluated under AI conditions, utilizing primary cultures of chondrocytes grown in alginate beads, as described in Materials and Methods. These conditions tend to promote the differentiated cartilage phenotype, but generally showed much slower rates of proliferation compared to the AD conditions (Figure



**Figure 5.** N-butyrylation reverses the GlcN mediated inhibition of incorporation of [<sup>35</sup>S] into PG. Confluent chondrocyte cultures were treated with [<sup>35</sup>S]-sulfate (10  $\mu$ Ci/ml) and various concentrations of GlcNBu and GlcN.HCl for 4 days (0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 mM). The newly synthesized PG were precipitated from the media on cellulose acetate strips, employing the Alcian blue method, followed by scintillation counting of the stained spots (standard error bars are included for all points, but some fall within symbols).

6). Varying concentrations of GlcN (0.5–5 mM) had no effect on proliferation after 1 or 2 weeks. Small but progressive degrees of inhibition of growth were noted after 3 and 4 weeks with increasing concentrations of GlcN (Figure 6). Similar experiments with the N-acylated derivatives reversed these minor inhibitions, but did not result in significant stimulation of proliferation over controls (data not shown).

Cumulative PG synthesis per cell with increasing periods of incubation, evaluated by [<sup>35</sup>S]-sulfate labeling, was also studied both inside the alginate beads and in medium outside the beads (Figures 7A, 7B, respectively). The total amount of PG (per well) accumulating inside the beads was much higher than that in the medium outside the beads, reaching about a 40-fold difference by the end of 3 weeks of incubation. The [<sup>35</sup>S] radioactivity incorporated into the PG, accumulating within the beads at the end of the 3 week incubation period (on a per cell basis), was similar to controls for the cultures incubated in the presence of GlcN, GlcNAc, and GlcNBu, at the 0.05 and 0.5 mM concentrations (Figure 7A). There was a trend of increasing PG per cell at the 0.05 concentration for GlcN, GlcNAc, and GlcNBu that was not seen at the higher concentration of the aminosugars. If these results are expressed as total radioactivity incorporated into the PG inside all the beads in each well, there was relatively less radioactivity in GlcN treated cultures than in the GlcNAc and GlcNBu treated cultures at the end of the 3 weeks of culture (compare with Figure 6). The labeled PG appearing in the media of the alginate bead cultures (Figure 6B) likely represent a leaching out of PG from the beads and appear to reflect the pattern of PG labeling within the beads.

**HAC proliferation and PG synthesis, AD conditions.** Monolayer HAC cultures showed a slower rate of proliferation than did similarly seeded BAC cultures. HAC responses to additions of the experimental compounds also occurred at a slower rate than did BAC cultures. Cell numbers remained similar from all conditions up to Day 8, when non-significant differences in total cell number begin to appear, indicating a roughly 2-fold decrease in the GlcN treated compared to the GlcNBu treated cultures (data not shown). Net PG synthesis per cell for the HAC under AD followed a slow rate of increase for Days 2–8, followed by more rapid synthesis for Days 8–14 (Figure 8). The small differences observed between treatment conditions did not achieve statistical significance.

**HAC cDNA microarray.** Analysis of gene transcription level data was cross-referenced against the corresponding data from the “flipped” data. The inclusion of flip-fluor data to the analysis reduces the possibility that a gene is identified as regulated due to variation in labeling efficiency and hybridization efficiency.

The gene list consisted of two 19,000 cDNA arrays per experimental condition (either GlcN vs Glc or GlcNBu vs Glc). From the 76,000 examined cDNA spots, 50 were iden-

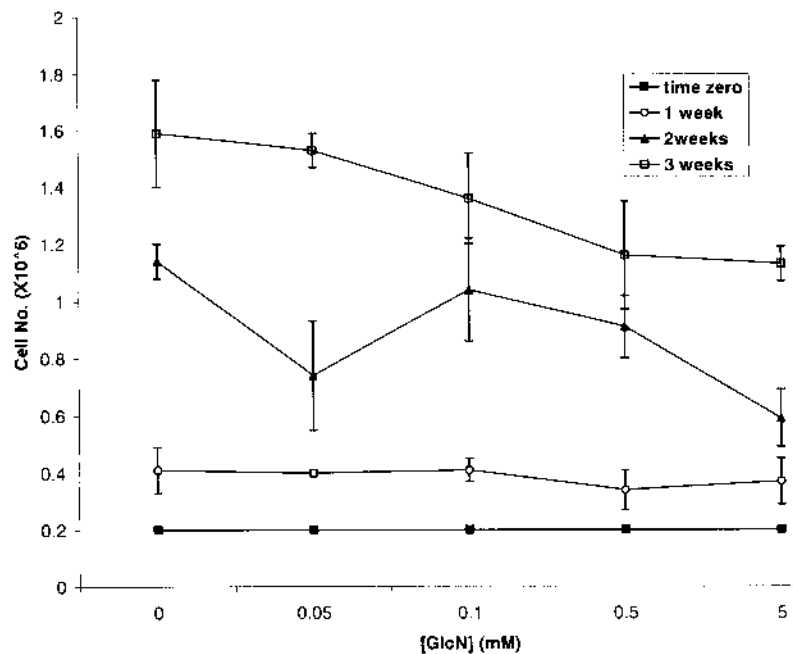


Figure 6. Effect of GlcN and N-Acylated derivatives on BAC proliferation under AI conditions. Primary cultures of BAC (passage 0) were seeded in alginate beads under the different concentrations of GlcN and N-acylated derivatives. Cells were counted after 3 weeks of growth.

tified as meeting all requirements for significant transcription regulation.

The final gene list, from 19,000 EST per chip, was composed of 43 genes upregulated by GlcNBu, 5 genes downregulated by GlcNBu, and 2 genes downregulated by GlcN. No genes survived the sorting procedure from the GlcN upregulated data set (Table 1).

Regulated genes of interest included collagen 6 $\alpha$ 1, COX 15, RAR $\alpha$ , and TATA binding protein from the upregulated GlcNBu data set, galactosidase  $\beta$ 1 from the GlcNBu downregulated data set, and chitinase 3-like 1 from the GlcN downregulated data set. Collagen type II and aggrecan were not significantly regulated by these additions.

Mapping of genes listed in Table 1 to their chromosomal location revealed a trend of transcriptional upregulation of genes found at or near chromosome region 1q21. These genes are cathepsin K, glutamate-ammonia ligase, and ribosomal protein S27. Chitinase 3-like 1, found at 1q32, was downregulated by additions of GlcN.

## DISCUSSION

We found that GlcN.HCl (0.1–5.0 mM) had a concentration-dependent antiproliferative effect on chondrocytes, seen best in anchorage-dependent and less so under anchorage-independent culture conditions. Our data show that the inhibitory effect of GlcN.HCl was not associated with the HCl moiety and appeared to be due to the GlcN molecule itself. Also, the growth kinetics of the chondrocytes in the presence of 1.25 mM GlcN indicate that initial growth rates,

up to 4 days, were similar to the controls and only after that did the growth rate flatten out (Figure 1). This pattern is similar to that seen in density-dependent inhibition of growth, which may be mediated by extracellular signals and/or the addition of peptide growth factors such as TGF- $\beta$  to AD cultures reported for other systems<sup>31,32</sup>. However, the addition of TGF- $\beta$  did not change the pattern of initial growth rates significantly, or the density-dependent inhibition of growth in the BAC grown in the presence of GlcN, GlcNAc, or GlcNBu (Figures 2A, 2B). Generally, the GlcN antiproliferative effect was reversed by N-acylation, including N-acetylation and N-butyrylation, and the latter also stimulated chondrocyte proliferation. The decreased BAC proliferation, by equivalent concentrations of butyrate, indicates that the butyrate moiety (i.e., on its own) is not responsible for the observed stimulation of proliferation by GlcNBu. The mechanism by which N-acylation reverses the antiproliferative effect of GlcN is not understood. However, the inhibition of cell growth by GlcN, but not by the N-Acyl derivatives, appears to be accounted for by a decline in cell division, as illustrated by the [<sup>3</sup>H]-thymidine labeling data (Figure 4B), rather than cell death.

GlcN is known to have a variety of effects on cell and animal physiology. It is toxic to some experimentally induced tumors in rodents<sup>33</sup>. It has also been reported that exogenous GlcN induces insulin resistance in cultured adipocytes in a manner similar to that caused by hyperglycemia, but at a 40-fold lower concentration than that required for Glc<sup>34</sup>. Also, aminosugars added in high con-



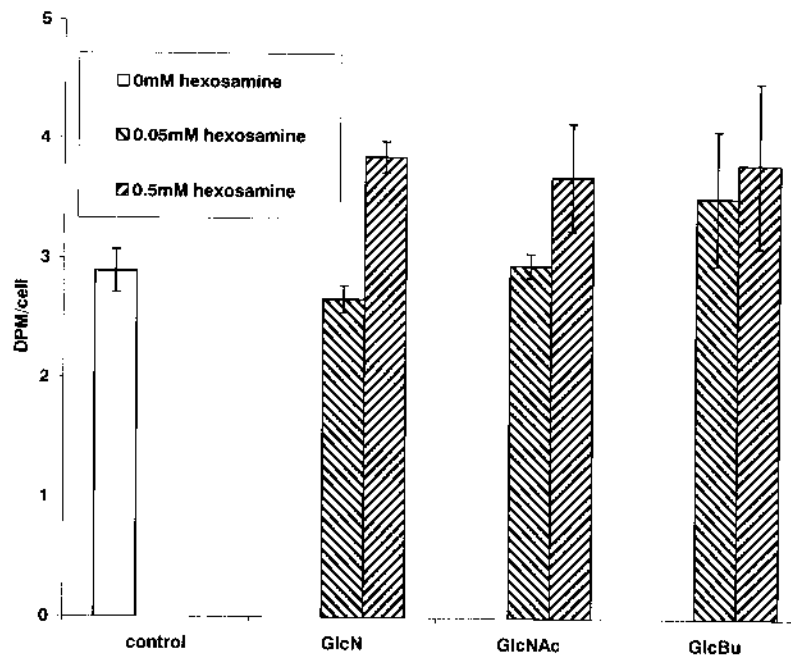


Figure 7A. Effect of GlcN and N-Acylated derivatives on PG synthesis under AI conditions, within alginate beads. Primary cultures of BAC were grown in alginate beads for 3 weeks, as described in Figure 6. Cultures were labeled with [<sup>35</sup>S]-sulfate for 5 days before harvesting (Days 17–21), and radioactivity incorporated in PG within the beads was quantified.

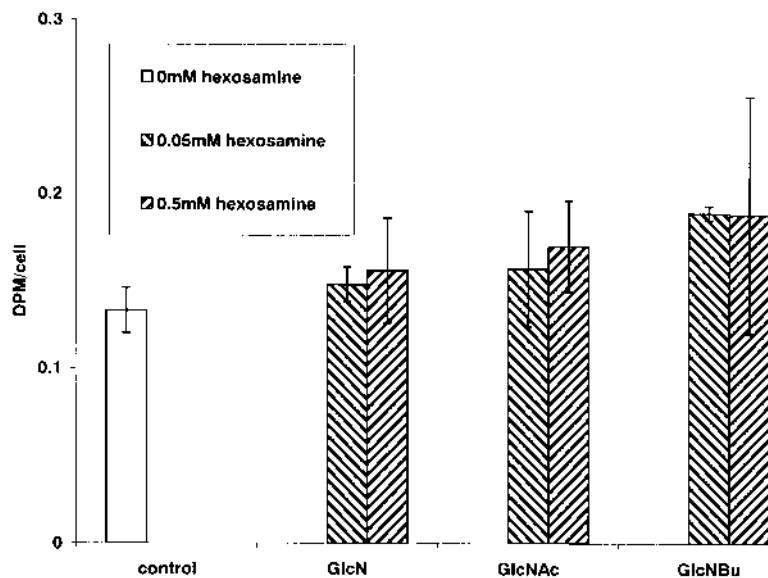


Figure 7B. Effect of GlcN and N-Acylated derivatives on PG synthesis under AI conditions, in the medium, outside the beads. Methods and results are from the same experiment as in Figure 7A, except that radioactivity incorporated is in the PG of the medium outside the alginate beads.

centrations to cartilage culture systems (maximal inhibition for mannosamine at 1.35 mM and 5–10 times higher concentrations for GlcN) have been shown to inhibit the activity of the PG-degrading enzyme aggrecanase<sup>35,36</sup>. GlcN.S also reduced phospholipase A<sub>2</sub> and collagenase activities in

human osteoarthritic chondrocytes in culture<sup>37</sup>. As well, the addition of GlcN to equine cartilage explants in high concentrations (25 mg/ml) inhibited PG and metalloproteinase release in the media<sup>38</sup>. Similarly, the addition of GlcN.S in mg/ml concentrations inhibited PG degradation, an effect

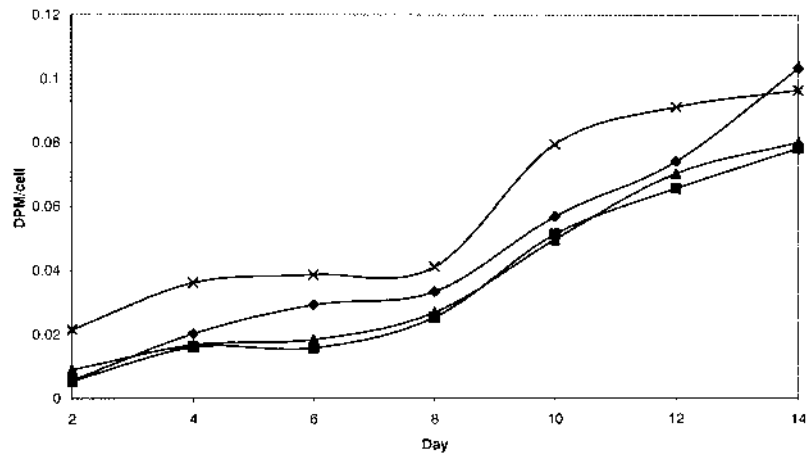


Figure 8. Net PG synthesis by HAC under AD conditions. Replicate cultures were labeled with [<sup>35</sup>S]-sulfate, 48 h before each timepoint indicated, and incorporated radioactivity was quantified. Cumulative incorporated radioactivity is indicated for each timepoint.

Table 1. Summary of regulated genes present on both “flipped” arrays following additions of 0.1 mM GlcN or GlcNBu to HAC cultures.

GlcNBu Treated HAC	
Upregulated genes	
Alpha-fetoprotein	Log <sub>2</sub> 2.17
Calsenilin	2.85
Cathepsin K	2.43
CGI-19 protein	2.09
Exportin 1	3.55
FK506-binding protein	2.66
Glutamate-ammonia ligase	2.57
Glutathione S-transferase A <sub>2</sub>	2.46
MGC16202	2.32
MGC5618q	2.35
KIAA1673	2.05
Leucine-rich repeat-containing 2	2.25
Mitofusin 1	2.53
Myosin light chain 6	2.32
Neogenin	2.67
Neural precursor cell expressed, developmentally downregulated 8	2.02
Nuclear RNA export factor 1	2.37
Pregnancy-associated plasma protein A	2.83
Procollagen-lysine 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2	2.36
Protein phosphatase 2	2.59
Reticulon 1	2.26
Ribosomal protein S27	2.00
SON DNA binding protein	2.07
Sphingomyelin phosphodiesterase 1	2.99
Downregulated genes	
Immunoglobulin kappa constant	2.05
Ninein	2.00
SWI/SNF related, actin dependent regulator of chromatin, b1	2.03
GlcN.HCl Treated HAC	
Downregulated genes	
Chitinase 3-like 1	2.44
Ras homolog gene family, member C	1.15

not found with N-acetyl GlcN<sup>39</sup>. A recent report<sup>40</sup> showed that high concentrations of GlcN (up to 10 mM) inhibited degradation of radiolabeled PG from cartilage explant cultures, but did not provide longterm suppression of aggrecan loss induced by retinoic acid. Thus, quite high concentrations of GlcN have been used to achieve inhibition of the enzymes responsible for PG degradation in culture.

In other studies, chondrocyte clusters from OA cartilage showed small increases of PG content of culture media and clusters (on the basis of DNA) with increasing concentrations of GlcNS (1–100 μg; Rotta Research Laboratories, Monza, Italy)<sup>39</sup>, although the DNA synthesis of the clusters was similar in all groups and the DNA content of the clusters was not reported. More recently, GlcN.S (Rotta Research Laboratories) was tested for its effect on releasing PG from chondrocyte suspensions (on methacrylate coated culture plates)<sup>41</sup>. These investigators found increasing amounts of PG released into the medium by increasing concentrations of GlcNS added to the cultures, from 28% to 120% of control values for the range of added GlcN.S of 1–150 μM, and a reduction of matrix metalloproteinase (MMP)-3 protein. It is difficult to determine to what extent these effects are due to new PG synthesis (or release) as opposed to inhibition of degradation of presynthesized PG. It has recently been reported that GlcN.HCl (100 μg/ml) suppressed PGE<sub>2</sub> production from OA chondrocytes and MMP from normal but not OA chondrocytes<sup>42</sup>. These investigators emphasize the importance of defining cells and conditions in these types of experiments. Inhibition of PG degradation by GlcN and its reversal by GlcNAc has been reported in cartilage explant systems<sup>43,44</sup>.

Our results with BAC cultures also suggest that the addition of relatively high concentrations of GlcN inhibits total, net PG synthesis, in a dose-dependent manner. This inhibitory effect of GlcN is seen most prominently under

AD conditions and less so in the AI (alginate) system (compare Figures 5 and 7), and appears to be related to the effects on cell density at any given timepoint. However, even under AD conditions, the relationship of cell density to the amount of PG synthesized per cell is complex. In some AD systems, increasing cell density is associated with decreasing amounts of PG synthesized per cell, an association that is “uncoupled” by the addition of TGF- $\beta$ <sup>31</sup>. The advantages and disadvantages of AD and AI systems were outlined above, but generally speaking, for initial drug testing results from AI systems are easier to interpret. The mechanism by which N-acylation of GlcN abolishes the inhibitory effects seen at higher concentrations has not been elucidated. The addition of ATP or inosine failed to reverse the inhibitory effects (unpublished results). In a rat chondrocyte system, recent work<sup>45</sup> has indicated, by Northern blot, that GlcNBu upregulated collagen type II and aggrecan mRNA, effects not seen with the addition of GlcNAc or N-propionyl glucosamine.

In evaluating the biological effects of a change in the chemical structure of a drug (i.e., GlcN and GlcNBu comparison) it is important to examine different systems. The BAC and HAC AD cultures illustrated significant differences in the chondrocytes between these biological sources. The HAC (from the margin of OA joints) proliferated much more slowly and exhibited different kinetics than the BAC. This slower growth rate of the HAC probably also contributed to attaining similar cell densities in the presence of the test compounds at the concentration tested. Thus, it was possible to carry out the comparative gene expression studies at similar rates of growth and final cell densities in this system, and the differences in gene expression observed (Table 1) are less likely to be confounded by effects of the compounds on cell proliferation. The finding of a small number of HAC genes being downregulated by GlcN was not anticipated, but may be due to the conversion of endogenous Glc into GlcN through the hexosamine pathway. If this is the case, then distinct transcriptional signature and growth profile found with additions of GlcNBu to HAC cultures may be due to GlcNBu influencing a separate metabolic pathway, culminating in the differential regulation of a distinct set of genes. Other groups have found that GlcN can regulate the expression of genes, most notably the TGF- $\beta$ 1 gene<sup>16,18</sup>. Relatively high stringency analysis of the microarray findings suggests regulation of a number of other genes by GlcN and its N-acylated derivatives that are of interest for further studies. These include chitinase 3-like 1 (HC gp39)<sup>46</sup> and ribosomal protein S27<sup>47</sup>, which have been implicated in chondrocyte differentiation and repair. A less stringent evaluation of gene expression, which did not require the data to survive the “flip-fluor” requirement, was also conducted. With the lesser stringency criteria, chitinase 3-like 1 was upregulated by GlcNBu but not by GlcN. Northern blot analysis confirmed a modest (35%) increase

in chitinase 3-like 1 transcript with GlcNBu and downregulation with GlcN treatment, as well as small increases and decreases in exported protein in the chondrocyte medium, respectively.

Further, the identification of many chondrocyte-related genes, previously identified as necessary for healthy cartilage, from this list suggests that additions of GlcNBu to monolayer HAC cultures may constitute a positive influence. These upregulated genes include PG family members of biglycan and the chondroitin sulfate proteoglycan (versican); glucose metabolism enzymes UDP-glucose pyrophosphorylase 2 and UDP-N-acetylglucosamine pyrophosphorylase 1; tissue inhibitor of metalloproteinase 1 and 3; and TGF- $\beta$ . Van der Pouw Kraan, *et al*<sup>48</sup> demonstrated that generation of distinct gene expression signatures can be accomplished between subsets of RA as well as between RA and OA. However, they did not include an analysis of the “flipped” data, which would reduce the possibility of the gene expression signature occurring by chance or technical variation.

The *in vitro* systems chosen to investigate effects of drugs for cartilage repair need to be considered also in the context of joint pathology. Histopathological studies of whole OA joints have shown that when repair of damaged articular cartilage does occur in the human joint, as part of remodeling, it is primarily in the form of fibrocartilage<sup>49,50</sup>. In addition to these seminal pathological studies, more recent studies have indicated both type I and type II collagens are expressed in different regions of OA joints, such as related to osteophyte formation<sup>51</sup>, and the transformation of fibrocartilage to neocartilage then becomes prominent<sup>52</sup>. Biochemical analysis had indicated high levels of type I collagen in OA, but not normal human cartilage, and this is expressed primarily by the deep layers of fibrocartilaginous tissue<sup>53</sup>. The AD systems we used (dedifferentiated chondrocytes grown on plastic surfaces) show some of the markers for fibrocartilage, while the AI system expresses the more differentiated cartilage phenotype, but the issue of the “best system” for drug evaluation for cartilage repair has not been resolved. Further, a number of naturally occurring support matrices have been utilized as bioengineering scaffolding for chondrocyte growth<sup>54</sup>, but whether the gene pattern expressed in these supports provides definite indication of the success of the implants is not certain. The advantages and disadvantages of the AI and AD systems, in particular for drug evaluation, are shown in Table 2.

We found that *in vitro* activity of GlcN on chondroproliferation and PG synthesis may be dependent on whether the amino group of the N-GlcN molecule is N-acylated or free. The N-acylated GlcN represent a class of compounds with markedly different effects from the parent GlcN moiety. The N-acylated GlcN, in particular GlcNBu, are currently being tested in animal models, where they have shown promising effects. In view of the many genes that appear to be upregu-

lated, these *in vivo* effects will need to be evaluated at different metabolic levels and in various tissues in order to determine the correlation between the *in vitro* and *in vivo* findings.

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