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N-Butyryl Glucosamine Increases Matrix Gene Expression by Chondrocytes

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ABSTRACT

Proteoglycan synthesis is dependent on *N*-acetyl glucosamine (GlcNAc) produced by the hexosamine biosynthetic pathway or obtained exogenously. Although used therapeutically to relieve symptoms of osteoarthritis, the actions of glucosamine and its analogs on cartilage are poorly understood. The purpose of this study was to determine the effects on chondrocytes of *N*-acylated-glucosamine analogs bearing alkyl chains of different lengths. Chondrocytes isolated from neonatal rat femoral condyles were cultured in the presence of glucosamine analogs. GlcNAc, *N*-proprionyl glucosamine (GlcNPro), or *N*-butyryl glucosamine (GlcNBu) did not alter cell number, lactate dehydrogenase release, or metabolic acid production, consistent with lack of cytotoxicity. Treatment of chondrocyte cultures with GlcNBu for 6 days significantly increased levels of type II collagen and aggrecan mRNA as determined by Northern blot analysis. In contrast, GlcNAc and GlcNPro had no significant effect. A significant increase in type II collagen mRNA was induced by GlcNBu within 3 days. GlcNBu did not alter stability of type II collagen mRNA, suggesting it acts on gene transcription. We have previously shown that tumor necrosis factor-α (TNFα) decreases levels of type II collagen mRNA. However, chondrocytes pretreated with GlcNBu maintained type II collagen mRNA at control levels in the presence of TNFα. These results establish that the *N*-butyrylated analog of glucosamine but not GlcNAc promotes matrix gene expression by chondrocytes. Thus, GlcNBu has the potential for use as a chondroprotective agent in osteoarthritis.

N-Acetyl glucosamine (GlcNAc) produced by the hexosamine biosynthetic pathway or obtained exogenously is an essential building block for the glycosaminoglycan side chains of proteoglycans. Aggrecan, the most abundant proteoglycan in cartilage, is composed of multiple keratan sulfate and chondroitin sulfate glycosaminoglycan side chains attached to discrete regions of a core protein (Knudson and Knudson, 2001). In healthy cartilage, water is readily retained by the negatively charged glycosaminoglycan side chains. However, with age, the length of the glycosaminoglycan side chains is reduced, resulting in a loss of cartilage hydration (Buckwalter et al., 1994; Knudson and Knudson, 2001). The macrofibrillar collagen network composed of predominantly type II collagen imparts structural support to articular cartilage. This collagenous network restricts the osmotic swelling of the tissue, thereby inducing a pressure that helps counteract compressive forces and tissue deformation (Poole et al., 2001). Chondrocytes regulate the synthesis and breakdown of extracellular matrix components, including aggrecan and type II collagen, in response to mechanical signals, soluble mediators such as hormones and growth factors, and feedback interactions with extracellular matrix molecules (Hering et al., 1994). In degenerative joint diseases such as osteoarthritis, loss of cartilage is mediated by an increase in matrix metalloproteinase and aggrecanase activity (Billinghurst et al., 1997; Dahlberg et al., 2000; Mort and Billington, 2001) and suppressed synthesis of matrix molecules by chondrocytes (for review, see Sandell and Aigner, 2001).

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ABBREVIATIONS: GlcNAc, *N*-acetyl glucosamine; GlcN, glucosamine; GlcNPro *N*-proprionyl glucosamine; GlcNBu, *N*-butyryl glucosamine; GlcNHex, *N*-hexanyl glucosamine; GlcNPen, *N*-pentanyl glucosamine; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; α-MEM, α-minimal essential medium; DRB, 5,6-dichloro-1-β-D-ribo-furanosylbenzimidazole; SSC, standard saline citrate; TGF-β, transforming growth factor-β; NF-κB, nuclear factor-κB.

Administration of exogenous glucosamine (GlcN) is thought to promote glycosaminoglycan synthesis and to lengthen proteoglycan side chains by circumventing the rate-limiting enzymatic step in the conversion of glucose to GlcN and GlcNAc by glutamine:fructose-6-phosphate amidotransferase (McClain and Crook, 1996). Glucose and GlcN are both substrates of glucokinase (Van Schaftigen, 1995); however, the resulting phosphorylated product of glucosamine (glucosamine-6-phosphate) allosterically inhibits glucokinase (Virkamaki and Yki-Jarvinen, 1999), altering both glucose and subsequent GlcN metabolism. In contrast, glucokinase has a low affinity for GlcNAc (Miwa et al., 1994). GlcNAc kinase mediates the phosphorylation of GlcNAc and the product (GlcNAc-6-phosphate) does not affect glucokinase activity (Shikhman et al., 2001), allowing both glucose and glucosamine metabolism to proceed unimpeded. Thus, exogenous GlcNAc may be more advantageous than GlcN for promoting the biosynthesis of glycosaminoglycans.

Although used therapeutically to relieve symptoms of osteoarthritis, the actions of glucosamine and its analogs on cartilage are poorly understood (Towheed and Anastassiades, 2000). Exogenous GlcN may serve in an anti-inflammatory capacity reducing joint swelling and pain to levels comparable with those observed with nonsteroidal anti-inflammatory drugs (Lopes Vaz, 1982; Muller-Fassbender et al., 1994; Ruane and Griffiths, 2002). In degenerating cartilage, proinflammatory cytokines such as IL-1β and TNFα are associated with increased degradation of cartilage matrix (Sandy et al., 1998; Sekiya et al., 2000) as well as reduced matrix gene expression and synthesis in vitro (Goldring et al., 1994; Séguin and Bernier, 2003). Exogenous GlcN counteracts some of the deleterious effects of IL-1β on proteoglycan synthesis and turnover (Sandy et al., 1998; Gouze et al., 2002). Furthermore, exogenous GlcNAc reduces nitric oxide production induced by IL-1β and TNFα (Shikhman et al., 2001) and suppresses the synthesis of cyclooxygenase-2 by human chondrocytes in response to IL-1β (Largo et al., 2003). However, maintenance of cartilage is also dependent on the production of key matrix proteins such as type II collagen and aggrecan. The effect of exogenous GlcN and its analogs on the expression of key cartilage matrix genes by chondrocytes is unclear.

The present study investigated the effect of increasing alkyl chain length in acylated GlcN analogs on cartilage health. It is possible that longer alkyl chain length slows metabolism of the glucosamine analog. Moreover, increased hydrophobicity would promote passive diffusion of the analog across cell membranes. We investigated the effects of analogs on chondrocyte proliferation, metabolic acid production, expression of cartilage-selective matrix genes, and responsiveness to TNFα. We demonstrate that analogs having an alkyl chain length of up to four carbons are well tolerated by chondrocytes. However, only *N*-butyryl glucosamine (GlcNBu) and not analogs with shorter alkyl chains increases the steady-state levels of type II collagen and aggrecan mRNA compensating for the negative effects of TNFα on matrix gene expression.

Materials and Methods

Chemicals. GlcNAc, EGF, and TNFα were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Analogs of GlcNAc bearing longer acyl chains [i.e., *N*-proprionyl glucosamine (GlcNProp), GlcNBu, *N*-pentanyl glucosamine, and *N*-hexanyl glucosamine] were synthesized following the method previously described by Inouye et al. (1956) with the following modifications: 1) sodium methoxide was obtained from a commercial supplier as a 30% solution in methanol (Sigma-Aldrich) rather than formed in situ; and 2) the crude GlcNAcyl was recrystalized using a soxhlet extraction method that drastically reduced the amounts of solvent required. In brief, for the synthesis of GlcNBu [*N*-(2,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-3-yl)-butyramide], glucosamine hydrochloride (20 g; 93 mmol) was added to a solution of methanol (anhydrous, 150 ml) and sodium methoxide (30 weight % solution in methanol, 1 eq., 16.7 g, 17.39 ml) (Fig. 1). The solution was mixed gently for 5 min and the resulting sodium chloride precipitate was removed by filtration on a fine sintered glass filter. Butyric anhydride (1.2 equivalents, 111.6 mmol, 17.66 g = 18.2 ml; Aldrich Chemical Co., Milwaukee, WI) was then added in one portion with rapid stirring to the filtrate. After approximately 5 min, the solution turbidified, and precipitation of the product commenced. The reaction mixture was stirred for 4 h at room temperature and then cooled at 0°C overnight. For the other analogs, butyric anhydride was substituted with propanoic anhydride, pentanoic anhydride, and hexanoic anhydride (all from Aldrich Chemical Co.) for the synthesis of GlcNPro, GlcNPen, and GlcNHex, respectively. The crude GlcNBu was filtered and washed with 20 ml of cold methanol, followed by 20 ml of cold ethanol, and finally, 200 ml of diethyl ether. The remaining material was packed into a soxhlet extraction thimble and extracted with ethanol. The ethanolic mixture of product was then cooled overnight in a cold room, and the product was isolated by filtration. The product was washed with 10 ml of cold ethanol followed by 50 ml of diethyl ether. The title compound was a pure white, crystalline powder with a overall yield of approximately 80%. After freeze drying, the compound had a melting point of 212 to 213°C, which is consistent with the 212°C value cited in the literature (Inouye et al., 1956). The purity of the synthesized compound was confirmed by reverse phase HPLC, mass spectrometry, and ^{1}H NMR.

Culture of Chondrocytes. Primary articular chondrocytes were isolated from the medial and lateral femoral condyles of 1-day-old

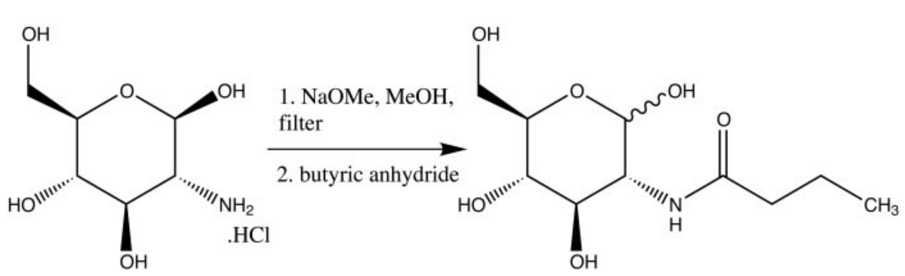

Fig. 1. Synthesis of GlcNBu. Glucosamine free base is formed by reaction of glucosamine hydrochloride with sodium methoxide. After removal of precipitated sodium chloride, the crude GlcNAcyl compound is then formed as a precipitate by reaction of the methanolic GlcN free base solution with a suitable anhydride (butyric anhydride for synthesis of GlcNBu). Isolation and recrystalization of this material then affords pure GlcNAcyl compound in good yield.

Sprague-Dawley rats (Charles River, St. Hyacinthe, QC, Canada) as described previously (Séguin and Bernier, 2003). These procedures were approved by the Animal Use Subcommittee of The University of Western Ontario Council on Animal Care. The cells were seeded at 4.25×10^4 cells/cm^2 on tissue culture plates (Falcon; BD Biosciences Discovery Labware, Bedford, MA) and cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES (Invitrogen Canada Inc., Burlington, ON, Canada) at 37°C in an atmosphere of 5% CO_2 in air.

Quantification of Cell Number and Viability. DNA content was assayed to determine cell number. Chondrocytes were plated in 96-well microtiter plates (Falcon) at a density of 6×10^3 cells/well with supplemented RPMI 1640 media with or without analog and cultured for 6 days. Medium was changed every 2 days. Cells were washed with 100 μl of PBS and then fixed in 3.7% formalin in PBS for 5 min. Cells were then washed with 20 mM NH_4Cl in PBS (100 μl/well) and permeabilized by treatment with 0.1% Triton X-100 in 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% gelatin, and 0.05% Nonidet P-40 for 30 min. SYBR Green I [50 μl, 1/1000 (v/v); Cambrex Bio Science Rockland, Inc., Rockland, ME] was added to each well and incubated overnight at 4°C, as described previously (Myers, 1998). Fluorescence was quantified at 520 nm (with excitation at 495 nm; Safire microplate reader; Tecan, San Jose, CA). Cell number was determined using a standard curve of fluorescence versus cell number.

Cell viability was monitored by release of lactate dehydrogenase. Chondrocytes were plated at 2.4×10^5 cells/well in a 48-well plate and cultured for 48 h. GlcNBu in fresh serum-free media was added and cells were cultured for an additional 48 h. Lactate dehydrogenase content in media was assessed using the Promega CytoTox-96 assay kit (Promega, Madison, WI) according to the manufacturer' s instructions. Total cell lactate dehydrogenase release was obtained by freeze-thawing the cell monolayer.

Measurement of Proton Efflux. Chondrocytes were seeded at 7 to 9×10^4 cells/cm^2 on porous polycarbonate membranes (Transwell, 12 mm in diameter, 3-μm pore size; Corning Glassworks, Corning, NY) in α-minimal essential medium (α-MEM; Invitrogen) containing HCO_3^- (26 mM) supplemented with fetal bovine serum (10%) and antibiotic solution. Cells were cultured for 48 h and then a further 24 h in serum-free α-MEM before acute treatment with the analogs. To investigate the effect of extended culture in the presence of glucosamine analogs on metabolic acid efflux, chondrocytes were cultured in 25-cm^2 flasks (Falcon) in RPMI 1640-supplemented medium in the presence or absence of glucosamine analogs until 80 to 90% confluent (6 days). Cells were harvested with trypsin and EDTA and seeded on porous polycarbonate membranes as described above. Cells were cultured in supplemented medium with or without corresponding analogs for 48 h and then a further 24 h in serum-free α-MEM with or without analogs before determining the resting proton efflux.

Measurement of proton efflux was conducted using a Cytosensor microphysiometer (Molecular Devices, Sunnyvale, CA) as described previously (Lui et al., 2002). Superfusion media supplemented with EGF (10 ng/ml) was used as a positive control. Nonspecific interactions of media or supplements with the silicon sensors were not detected when cultures were rendered nonviable by superfusion with 0.1% Triton X-100 in standard medium and then superfused with test solutions (Lui et al., 2002).

RNA Extraction and Northern Blot Analysis. Chondrocytes were plated in 60-mm dishes (1.2×10^6 cells/dish; Falcon) and cultured in supplemented RPMI 1640 medium for 2 days. Cells were then treated for 6 days with vehicle, GlcNAc, GlcNProp, or GlcNBu (1 or 10 mM). Culture medium was replaced every 2nd day. Before stimulation of cells with TNFα (Sigma-Aldrich), cells were incubated for 4 h in serum-free media. In some experiments, the inhibitor of de novo mRNA synthesis 5,6-dichloro-1-β-D-ribo-furanosylbenzimidazole (DRB, 3 mg/ml stock in ethanol) (Calbiochem, San Diego, CA) was added to cultures in serum-free RPMI 1640 medium with or without GlcNBu. Cells were incubated for 24 h in the presence of 10 μg/ml DRB. To assess the effects of GlcNBu on responses to TNFα, GlcNBu was removed after 6 days of treatment, and cells were incubated in serum-free media for 24 h before stimulation with TNFα (30 ng/ml) for 24, 48, or 72 h.

Total RNA was collected from cells using the acid-guanidinium-phenol-chloroform extraction method (TRIzol; Invitrogen). Total RNA (10 μg) was resolved on a 1.1% agarose gel containing formaldehyde. Equivalent loading of samples was verified by ethidium bromide staining before RNA was transferred to Nytran membranes (Schleicher & Schuell, Keene, NH). RNA was fixed to the Nytran membrane by baking at 80°C for 2.5 h under vacuum. Complementary DNA (cDNA) probes corresponding to the mouse C-propeptide of type II collagen (pKN225) (Krebsbach et al., 1996), rat aggrecan (p1353) (Doege et al., 1987), and 18S rRNA (Ambion, Austin, TX) were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) by a random-primed oligonucleotide method (Prime-a-gene labeling kit; Promega). We have previously verified the specificities of the type II collagen and aggrecan probes by sequencing. Membranes were prehybridized for one h at 42°C in ULTRAhyb (Ambion), before overnight hybridization with the desired probe at 5×10^5 cpm/ml of ULTRAhyb. Membranes were washed twice with 4× SSC and 0.1% SDS at 42°C for 15 min, followed by one 15-min wash with 0.5× SSC and 0.1% SDS at 52°C, and exposed to Hyperfilm-MP (Amersham Biosciences Inc., Baie d'Urfe, QC, Canada) at −80°C. Before reprobing, blots were stripped with 80% formamide, 10 mM Tris HCl, pH 8, 1 mM EDTA, and 1% SDS at 68°C for 1 h, followed by two 15-min washes with 4× SSC and 0.1% SDS at 42°C, and then one 15-min wash in 0.5× SSC and 0.1% SDS at 52°C. Levels of type II collagen and aggrecan were normalized to the expression levels of 18S rRNA.

Densitometry and Statistical Analysis. Data shown are representative of at least three independent experiments. Bands occurring on radiographic film were analyzed using Kodak Digital Science software (Eastman Kodak, Rochester, NY). Statistical differences were assessed by analysis of variance followed by the Tukey-Kramer post test at a confidence level of 95%, using Prism version 4.0 software (GraphPad Software Inc., San Diego, CA).

Results

Synthesis of Glucosamine Analogs and Analysis of GlcNBu. Glucosamine analogs having alkyl chains of different lengths were synthesized as described under *Materials and Methods*. The chemical properties of resulting compounds were confirmed by HPLC, mass spectrometry, and ^{1}H NMR. For GlcNBu, two anomers at carbon 1 (α and β) were identified (Fig. 1). The retention time of the two anomers on an LC 8 reverse phase HPLC column with a mobile phase of 50/50 MeOH/H_2O at a flow rate of 1.0 ml/min were 3.264 and 3.407 min, respectively. By mass spectrometry, using electropositive electrospray ionization, mass to charge ratios (*m/z*) were 288.2 (M+K$^+$), 272.1 (100%, M+Na$^+$), and 250.2 (M+H$^+$). The ^{1}H NMR (D_2O) spectrum revealed signals at (δ ppm) 5.02 d, ~0.5 H, β-anomeric H, J = 3.4 Hz; 4.53, d, ~0.5 H, α-anomeric H, J = 8.1 Hz; 3.8–3.2, m, 6H, sugar-H, $\mathbf{CH_2}$-OH; 2.1, td, 2H, $CH_3CH_2\mathbf{CH_2}$-CO-, J = 3.1 Hz, 6.9 Hz; 1.45, sextuplet, 2H, $CH_3\mathbf{CH_2}CH_2$-CO-, J = 6.9 Hz; 0.73, td, 3H, $\mathbf{CH_3}CH_2CH_2$-CO-, J = 1.5 Hz, 6.9 Hz. Taken together, the results of the HPLC and NMR analyses indicate that the final product obtained in D_2O is a mixture of α and β anomers in a ratio of approximately 50:50.

Differential Effects of Glucosamine Analogs on Chondrocyte Cell Number. The effect of analogs on chondrocyte population size was determined by measuring DNA content (Fig. 2A). Chondrocytes were cultured in the presence of individual analogs (0.1–25 mM) for 6 days. Over the concentration range examined, no significant change was observed with GlcNAc, GlcNPro, or GlcNBu. A significant reduction in cell number was observed after treatment with GlcNPen (0.1 and 25 mM). Cultures treated with GlcNHex had lower cell numbers, and this analog was toxic at 25 mM. To directly assess effects on chondrocyte viability, we assayed lactate dehydrogenase release in cultures treated with GlcNBu (1–25 mM) for 48 h (Fig. 2B). No release of lactate dehydrogenase was observed, ruling out an acute effect of this analog on chondrocyte viability.

Proton Production by Chondrocytes Was Not Altered by GlcNPro or GlcNBu. The availability of glucose and the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase of the hexosamine biosynthetic pathway contribute to the regulation of cellular metabolism by glucose (Singh et al., 2001). Glucose metabolism results in the production of lactic and carbonic acid that can be monitored by detecting proton efflux from the cells. To determine whether exposure to glucosamine analogs alters metabolic acid production by chondrocytes, the effects of acute (12-min) and prolonged (6-day) exposure to GlcNPro and GlcNBu on proton efflux were investigated. Rates of proton efflux were determined by microphysiometry. A positive control, EGF (10 ng/ml), induced a transient increase in proton efflux to levels ~35% above basal followed by a sustained elevation, as described previously (Lui et al., 2002). In contrast, acute treatment with 10 mM GlcNPro or GlcNBu did not alter proton efflux (Fig. 3). Similarly, prolonged culture of chondrocytes with 10 mM GlcNPro or GlcNBu had no significant effect on basal proton efflux (Table 1). These results indicate that exogenous glucosamine analogs do not influence glucose metabolism.

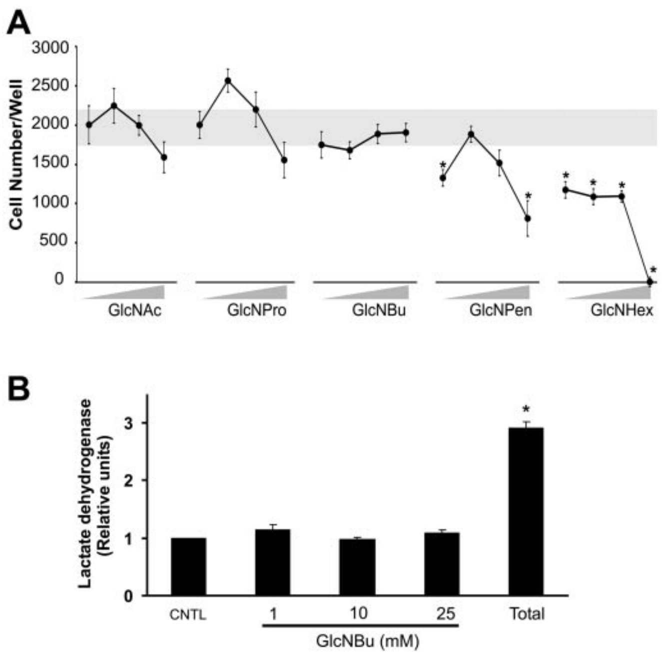

Fig. 2. Glucosamine analogs differentially alter the growth of rat articular chondrocytes. A, chondrocytes were cultured for 6 days in medium that was supplemented with 0.1, 1.0, 10, or 25 mM GlcNAc, GlcNPro, GlcNBu, GlcNPen, or GlcNHex, or not supplemented in the case of the controls. Medium was changed every 2nd day. Cell number was determined from nuclear DNA staining (SYBR Green I). Data represent mean cell number ± S.E.M. from three independent experiments with a minimum of three replicates each. The shaded area represents the range of cell numbers for untreated controls ± S.E.M. * indicates significant difference from control as determined by analysis of variance and a Tukey-Kramer post test ($p < 0.05$). B, GlcNBu does not alter cell viability. Chondrocytes were cultured for 2 days in medium supplemented with 1.0, 10, and 25 mM GlcNBu or not supplemented in the case of the controls. Release of lactate dehydrogenase was measured in the medium ($n = 3$ independent experiments). Total cell lactate dehydrogenase was obtained by freeze-thawing the cell monolayer.

TABLE 1
Basal levels of proton efflux were not significantly different from control levels in chondrocytes cultured in the presence of GlcNPro or GlcNBu

Pretreatment	Mean Basal Proton Efflux (± S.E.M.)	
	nmol of H^+/min/sample	
Control	0.183 ± 0.017	n=40
GlcNPro (10 mM)	0.136 ± 0.014	n=18
GlcNBu (10 mM)	0.158 ± 0.028	n=18

GlcNBu Increases mRNA Levels of Cartilage Matrix Genes. Whether these analogs could influence the phenotype of chondrocytes was next assessed by analysis of the mRNA levels of key phenotypic markers type II collagen and aggrecan (Fig. 4, A and B, respectively). Chondrocytes were cultured for 6 days in the presence of individual analogs (1 and 10 mM) or control medium. Levels of type II collagen and aggrecan mRNA were assessed by Northern blot analysis, yielding bands of the expected molecular size. These levels were not significantly altered by GlcNAc or GlcNPro, or by 1 mM GlcNBu (Fig. 4). However, treatment with 10 mM GlcNBu significantly increased levels of type II collagen mRNA (Fig. 4A). Similarly, levels of aggrecan mRNA were increased by treatment with 10 mM GlcNBu (Fig. 4B).

To determine the length of time required for up-regulation of type II collagen mRNA levels, chondrocytes were cultured in the presence of 10 mM GlcNBu for 24, 48, and 72 h. A significant increase in type II collagen mRNA was observed within 72 h (Fig. 5). These data suggest that the actions of GlcNBu are not due to an immediate effect on transcription, but they may reflect a change in turnover of type II collagen

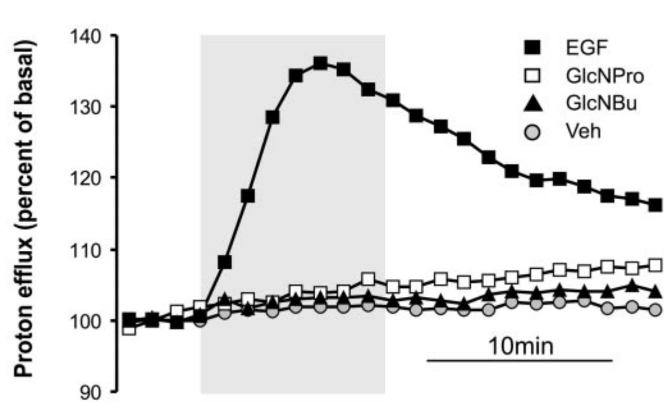

Fig. 3. Acute exposure to GlcNPro and GlcNBu does not alter proton efflux from rat articular chondrocytes. Chondrocytes were cultured on polycarbonate membranes and proton efflux was monitored using a microphysiometer. Cells were superfused with standard medium, and at 1.5-min intervals, superfusion was stopped for 30 s to measure acidification rate. Proton efflux (net efflux of H^+ equivalents) was calculated from the acidification rate and expressed as a percentage of basal proton efflux. Cells were superfused with 10 mM GlcNPro (n = 3), 10 mM GlcNBu (n = 3), vehicle (VEH) (n = 7), or the positive control, 10 ng/ml EGF (n = 10) in standard medium for 12 min where indicated by the shaded area.

mRNA or the involvement of secondary signals that regulate transcription.

To determine whether GlcNBu increased the stability of type II collagen mRNA, transcription was inhibited by DRB. Chondrocytes were cultured in the presence or absence of GlcNBu (10 mM) for 6 days and then treated with or without DRB for 24 h (Fig. 6). The levels of type II collagen mRNA after DRB treatment were not significantly different in control and GlcNBu-treated cells. Thus, GlcNBu does not seem to alter the stability or rate of turnover of type II collagen mRNA, but it likely causes an increase in its transcription.

The reversibility of the effect of 10 mM GlcNBu was assessed after withdrawal of the analog (Fig. 7). Upon removal of GlcNBu, levels of type II collagen mRNA remained significantly elevated above control levels for up to 72 h, indicating that treatment with GlcNBu has a long-lasting effect on the regulation of type II collagen mRNA.

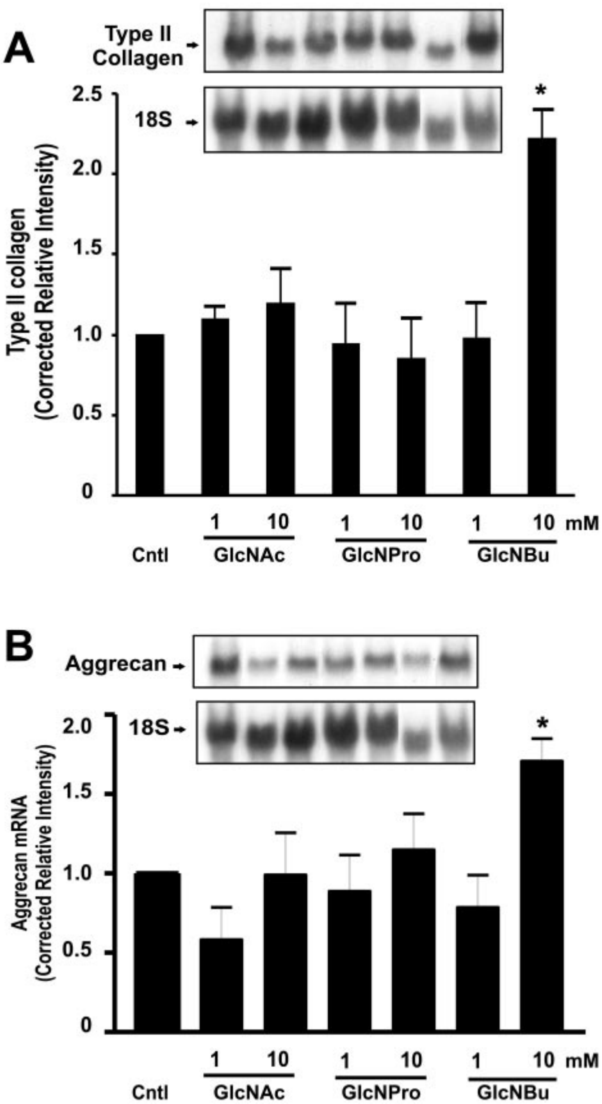

Fig. 4. GlcNBu increases levels of type II collagen and aggrecan mRNA. Chondrocytes were cultured for 6 days in the presence of 1 or 10 mM GlcNAc, GlcNPro or GlcNBu or control medium. Levels of type II collagen and aggrecan, and 18S rRNA were assessed by Northern blot analysis (A and B, top). The histograms show densitometric analysis of the relative intensity of bands corrected for levels of 18S rRNA. Data are means ± S.E.M. of five independent experiments for type II collagen (A) and three independent experiments for aggrecan (B). * indicates a significant difference from the control treatment ($p < 0.05$).

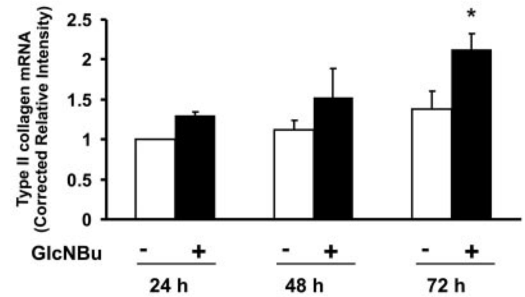

Fig. 5. GlcNBu increases type II collagen mRNA within 72 h. Chondrocytes were cultured for 2 days in control medium followed by medium with or without GlcNBu (10 mM) for 24, 48, or 72 h. mRNA levels were assessed by Northern blot analysis. Relative intensity of bands is presented as mean ± S.E.M. of levels of type II collagen mRNA corrected for levels of 18S rRNA and represents at least three independent experiments. Open columns represent control cells; closed columns represent GlcNBu-treated cells. * indicates a significant difference from the 24 h control ($p < 0.05$).

Effects of GlcNBu Can Compensate for TNFα-Induced Loss of Type II Collagen mRNA. We have previously shown that prolonged exposure of chondrocytes to TNFα causes a continual decline in type II collagen mRNA levels (Séguin and Bernier, 2003). Chondrocytes were pretreated with or without GlcNBu (10 mM) for 6 days, washed, and then treated with TNFα for 24 or 72 h in the absence of GlcNBu (Fig. 7). Both untreated and GlcNBu-pretreated chondrocytes responded to TNFα with a reduction in type II collagen mRNA. However, in GlcNBu-treated chondrocytes, the level of type II collagen mRNA in TNFα-treated cells did not fall below that of untreated chondrocytes. These results suggest that pretreatment with GlcNBu protects chondrocytes from TNFα-mediated loss of matrix gene expression.

Discussion

Degenerative cartilage disorders are characterized by both an increase in extracellular matrix breakdown and a lack of matrix replacement. In this study, we analyzed the ability of several analogs of GlcNAc to maintain or enhance the expression of matrix gene mRNA. Only GlcNBu was found to promote the expression of two key extracellular matrix proteins, type II collagen and aggrecan, by articular chondrocytes. GlcNBu was well tolerated by chondrocytes and did not alter metabolic acid production. Thus, GlcNBu acts selectively to facilitate matrix gene expression without perturbing cell survival. A critical finding of the current study is that pretreatment with GlcNBu (10 mM) compensated for the typical reduction in type II collagen mRNA after challenge with TNFα.

A therapeutic intervention to preserve cartilage should not negatively influence the cells responsible for the production and maintenance of the cartilage matrix. Of the five analogs investigated, the three with the shorter alkyl chains (GlcNAc, GlcNPro, and GlcNBu) did not alter the population growth of chondrocytes, whereas chondrocyte populations treated with analogs with longer alkyl chains failed to expand to the same extent. If the GlcNAc analogs share those transporters used by glucose and GlcN (Uldry et al., 2002), then analogs with longer alkyl chains may compete or block transporters for glucose

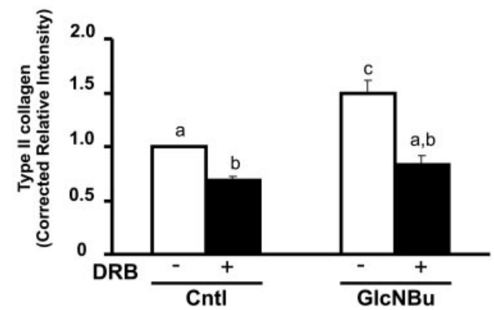

Fig. 6. Pretreatment with GlcNBu does not alter the turnover of type II collagen mRNA. Chondrocytes were cultured for 2 days in control medium followed by medium with or without GlcNBu (10 mM) for 6 days. After 6 days, cultures were treated with or without DRB (10 μg/ml) for 24 h. mRNA levels were assessed by Northern blot analysis. Relative intensity of bands is presented as mean ± SEM of levels of type II collagen mRNA corrected for levels of 18S rRNA for three independent experiments. Open columns represent control treatment, whereas closed columns represent DRB-treated cells. Treatments with the same letter are not significantly different.

hindering cell population growth. A deficiency in ATP was postulated to account for the reduction in aggrecanase activity after treatment of bovine chondrocytes with GlcN (Sandy et al., 1998). Again, through interference with glucose metabolism, exogenous GlcN might deplete the ATP stores of chondrocytes. Cellular metabolism results in the production of lactic and carbonic acids. Thus, proton efflux can be monitored as an indirect but sensitive measure of metabolic rate (Ajilore and Sapolsky, 1997; Yusim et al., 2000). Both the lack of change in proton efflux and cell number observed in the present study indicate that the short alkyl chain analogs of GlcNAc do not alter normal glucose metabolism by chondrocytes.

This is the first identification of an analog of GlcNAc, i.e., GlcNBu, that increases the levels of mRNA for cartilage-selective matrix genes. A number of mechanisms could produce such an increase, including up-regulation of autocrine factors supporting matrix gene expression, increased mRNA stability, and direct or indirect increases in transcription. It has been shown previously that exogenous GlcN working through the hexosamine biosynthetic pathway increased levels of TGF-β mRNA in addition to the increased production of the extracellular matrix components heparin sulfate and fibronectin in mesangial cells (Kolm-Litty et al., 1998). TGF-β promotes the synthesis of extracellular matrix by chondrocytes (Lee et al., 2000). However, if GlcNBu was similarly mediating its effects via TGF-β, then the other GlcNAc analogs should have produced similar effects, because the increase in TGF-β is independent of the presence of an *N*-alkyl chain. Thus, the mechanism of action of GlcNBu may involve modulation of the regulatory machinery controlling extracellular matrix mRNA production and turnover. The reported half-life of type II collagen mRNA is approximately 17 h in rabbit articular chondrocytes and 18 h in human costal chondrocytes (Galera et al., 1992; Goldring et al., 1994). mRNA stability is regulated by 3′-terminal deadenylation and steric protection of an endoribonuclease-sensitive site within the 3′ untranslated region of the mRNA (Waggoner and Liebhaber, 2003). However, we found that the GlcNBu-induced increase in type II collagen mRNA does not result from an increase in transcript stability. Furthermore, levels of type II collagen mRNA are still elevated upon withdrawal of GlcNBu, indicating a sustained alteration in the machinery. One of the products of the hexosamine biosynthetic pathway is uridine-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) that participates as a donor molecule in most glycosylation reactions and in post-translational modification of signaling molecules including transcription factors (Hanover, 2001). If taken through the biosynthetic pathway, GlcNBu may alter the stability or activity of key regulatory molecules that directly interact with the type II collagen and aggrecan promoters or indirectly changes secondary factors that interact with these promoters.

TNFα induces cartilage degeneration by both sustaining cytokine production and increasing expression of collagenases and aggrecanases (Dozin et al., 2002). Our previous studies demonstrated that TNFα reduces type II collagen mRNA levels via downstream targets of the mitogen-activated protein kinase kinase 1/2 pathway with contribution from NF-κB (Séguin and Bernier, 2003). Even a brief 4-h exposure of chondrocytes to TNFα is sufficient to initiate sustained activation of NF-κB and loss of mRNA for matrix molecules. Modulation of NF-κB, signaling might account for the effects of GlcNBu observed in the present study. GlcN analogs have been reported to have mixed effects on IL-1β-mediated activation of NF-κB, ranging from reductions in rat chondrocytes and human osteoarthritic chondrocytes (Gouze et al., 2001; Largo et al., 2003) to no change by GlcNAc in human chondrocytes (Shikhman et al., 2001). Preliminary investigation of intracellular signaling pathways revealed no effect of GlcNBu on NF-κB activation by TNFα in our cell model.

The results of this study suggest the potential use of this analog in vivo. Preliminary findings in animal models of osteoarthritis and inflammatory joint disease suggest that positive biological effects can be obtained in vivo through oral administration of GlcNBu (20–200 mg/kg/day) (J. Carran and T. Anastassiades, unpublished data). Furthermore, delivery of high concentrations of GlcNBu to articular cartilage may also be facilitated by intra-articular injection. Thus,

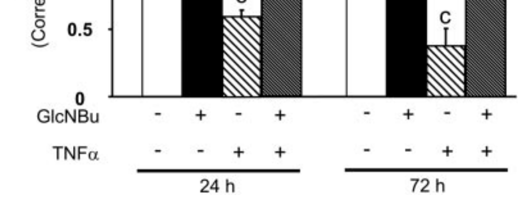

Fig. 7. Pretreatment of chondrocytes with GlcNBu compensates for TNFα-mediated loss of type II collagen mRNA. Chondrocytes were cultured for 6 days in the presence of 10 mM GlcNBu or control medium. GlcNBu was removed and cells were treated with 30 ng/ml TNFα or vehicle for 24 or 72 h. mRNA levels were assessed by Northern blot analysis. Relative intensity of bands is presented as mean ± SEM of levels of type II collagen mRNA corrected for levels of 18S rRNA from three independent experiments. Within each time period, treatments with the same symbol are not significantly different from each other.

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GlcNBu has potential for use clinically as a chondroprotective agent.

In summary, extending the alkyl chain of GlcN produced an analog that enhances the expression of cartilage matrix genes by chondrocytes. GlcNBu alone increased the levels of type II collagen and aggrecan mRNA and helped chondrocytes to offset the shutdown of type II collagen gene expression induced by TNFα. Promoting expression of these two key extracellular molecules and preventing chondrocyte dedifferentiation is critical for preserving cartilage tissue and promoting its repair at times of injury.

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