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Interleukin-1β Down-Regulates the Expression of Glucuronosyltransferase I, a Key Enzyme Priming Glycosaminoglycan Biosynthesis

Influence of Glucosamine on Interleukin-1β–Mediated Effects in Rat Chondrocytes

Jean-Noel Gouze, Karim Bordji, Sandrine Gulberti, Bernard Terlain, Patrick Netter, Jacques Magdalou, Sylvie Fournel-Gigleux, and Mohamed Ouzzine

Objective. **To assess the variations of galactose-β-1,3-glucuronosyltransferase I (GlcAT-I) expression related to the decrease in proteoglycan synthesis mediated by interleukin-1β (IL-1β) in rat chondrocytes, and to evaluate the influence of glucosamine on the effects elicited by this proinflammatory cytokine.**

Methods. **Rat articular chondrocytes in primary monolayer cultures or encapsulated into alginate beads were treated with recombinant IL-1β in the absence or presence (1.0–4.5 gm/liter) of glucosamine. Variations of GlcAT-I and expression of stromelysin 1 (matrix metalloproteinase 3 [MMP-3]) messenger RNA (mRNA) were evaluated by quantitative multistandard reverse transcriptase–polymerase chain reaction. In vitro enzymatic activity of GlcAT-I was measured by thin-layer chromatography, with radiolabeled UDP-glucuronic acid and a digalactoside derivative as substrates. Proteoglycan synthesis was determined by ex vivo incorporation of Na_2-$^{35}SO_4$. Nitric oxide synthase and cyclooxygenase activities were monitored by the evaluation of nitrite (NO^-_2) and prostaglandin E_2 (PGE_2) produced in the culture medium, respectively.**

Results. **IL-1β treatment resulted in a marked inhibition of GlcAT-I mRNA expression and in vitro catalytic activity, together with a decrease in proteoglycan synthesis. In addition, glucosamine was able to prevent, in a dose-dependent manner, the inhibitory effects of IL-1β. In the same way, the amino sugar reduced NO^-_2 and PGE_2 production induced by IL-1β. Finally, the up-regulation of stromelysin 1 (MMP-3) mRNA expression by IL-1β was fully prevented by glucosamine.**

Conclusion. **The results of this study suggest that the deleterious effect of IL-1β on the anabolism of proteoglycan could involve the repression of GlcAT-I, a key enzyme in the biosynthesis of glycosaminoglycan. Glucosamine was highly effective in preventing these IL-1β–mediated suppressive effects. The amino sugar also prevented the production of inflammatory mediators induced by the cytokine. This action could account for a possible beneficial effect of glucosamine on osteoarthritic articular cartilage.**

Glycosaminoglycans (GAG) are ubiquitously distributed among tissues, usually in covalent linkage to proteoglycan core proteins (1). They are linear polymers of repeating disaccharides containing hexosamine and hexuronic acid. In the case of keratan sulfates (KS), hexuronic acid is replaced by galactose. GAG are in-

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volved in the regulation and maintenance of cell proliferation, cytodifferentiation, and tissue morphogenesis (2,3). They also play a central role in matrix assembly and are especially essential in maintaining the structural and functional integrity of cartilaginous tissue. In particular, sulfated GAG attached to proteoglycans, such as aggrecan, the most abundant proteoglycans in cartilage, contribute to the unique biomechanical properties of this tissue (4). Hexuronic acid–containing GAG are bound to serine residues in the core protein via the common carbohydrate sequence GlcA-β-1,3-Gal-β-1,3-Gal-β-1,4-Xyl-β-1-*O*-Ser, forming the so-called GAG–protein linkage region (1,3). This tetrasaccharide linkage region is shared by chondroitin sulfates, heparan sulfates, and dermatan sulfates.

In contrast, KS are *N*-linked to asparagine in KS-I proteoglycans of cornea, or *O*-linked to serine or threonine via an *N*-acetylgalactosamine residue in skeletal KS-II (5). GAG biosynthesis is initiated by the addition of a xylose moiety to specific serine residues in the core protein. This reaction is followed by the stepwise addition of 2 galactose and 1 glucuronic acid residues, and subsequently by the alternate addition of hexosamine and hexuronic residues. Each step is catalyzed by a specific glycosyltransferase using the corresponding UDP-sugar as donor substrate. The addition of the first glucuronic acid residue on the trisaccharide linkage intermediate is catalyzed by a galactose-β-1,3-glucuronosyltransferase (GlcAT-I), of which the human complementary DNA (cDNA) has recently been cloned by Kitagawa et al (6). GlcAT-I is a member of a family of enzymes catalyzing the transfer of glucuronic acid to a β-linked galactose residue. These glucuronosyltransferases are involved in the biosynthesis of GAG chains as well as in the formation of HNK1, a carbohydrate epitope present on various glycolipids and glycoproteins. They are thought to be different from other glucuronosyltransferases involved in the formation of the repeating disaccharidic unit of GAG (7).

GlcAT-I plays a crucial role in priming hexuronic acid–containing GAG synthesis, and any change in activity could likely affect the rate of synthesis of GAG chains and consequently proteoglycan structure and properties. Joint diseases such as osteoarthritis (OA) or rheumatoid arthritis (RA) are characterized by quantitative and qualitative modifications of proteoglycans and of their GAG chains, leading to alteration of the cartilaginous matrix (8,9). An imbalance between the biosynthesis and degradation of matrix components leads to a progressive destruction of the tissue, finally causing complete damage of the articular surface.

There is convincing evidence that interleukin-1β (IL-1β), a cytokine released by synovial cells and invading macrophages in inflamed joints, plays a decisive role in OA and RA (10). Indeed, the synovial fluid of patients with RA and OA contains elevated levels of IL-1β (11). It is accepted that this cytokine initiates a number of events leading to cartilage damage. This process involves inhibition of biosynthesis of the main matrix components, in particular proteoglycans, together with promotion of their degradation. Cartilage breakdown induced by IL-1β is initiated by the release of matrix metalloproteinases (MMPs), such as stromelysin and collagenase, and by a reduction in the concentration of tissue inhibitors of MMPs (12). Moreover, the proinflammatory effects of IL-1β are attributed to a variety of mediators such as prostaglandin E_2 (PGE_2), produced by the induction of cyclooxygenase 2 (COX-2), or nitric oxide (NO), formed by inducible NO synthase (iNOS) in activated chondrocytes (13,14).

Various studies suggest that an inhibition of proteoglycan synthesis by IL-1β, rather than a stimulation of proteoglycan breakdown, causes the depletion of proteoglycans in cartilage (15,16). However, much research has focused on IL-1β–mediated degradation of proteoglycans, in particular aggrecan, whereas the mechanisms underlying the inhibition of proteoglycan anabolism are much less documented. In this study, we addressed the question of a possible action of this cytokine on the messenger RNA (mRNA) expression and activity of GlcAT-I, a key enzyme involved in the priming of GAG biosynthesis. We show, for the first time, that IL-1β represses both mRNA expression and enzyme activity of GlcAT-I and that these effects are concomitant with a loss of proteoglycan synthesis in rat chondrocytes.

Recent attention to the symptomatic relief afforded by the treatment of OA with glucosamine has spurred new basic scientific research into the effect of this agent on cartilage (17). We found that glucosamine could efficiently prevent the IL-1β–mediated repression of GlcAT-I expression and of proteoglycan anabolism in a dose-dependent manner. In addition, glucosamine was able to antagonize the production of inflammatory mediators induced by IL-1β. These actions could account for the effects of glucosamine derivatives in OA treatment.

MATERIALS AND METHODS

Chemicals. Uridine 5′-diphosphate (UDP)–glucuronic acid (sodium salt) and collagenase B were purchased from

Boehringer-Mannheim (Mannheim, Germany) and UDP U-^{14}C-glucuronic acid (180 mCi/mmole) was from Isotopchim (Ganagobie-Peyruis, France). Na_2-$^{35}SO_4$ was obtained from Amersham (Les Ulis, France). D(+)glucosamine, D(+)glucose, galactosylpyranosyl-β-1,3-D-thio-galactopyranose (Gal-S-Gal), and pronase were supplied by Sigma-Chimie (St Quentin Fallavier, France). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, gentamicin, reverse transcriptase (RT), and restriction enzymes were obtained from Gibco-BRL (Cergy Pontoise, France). *Taq* polymerase was supplied by Eurobio (Les Ulis, France), and oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Soluene 350 was obtained from Packard (Rungis, France). Recombinant IL-1β was purchased from Pepro-Tech (Tebu, Le Perray en Yvelines, France).

Chondrocyte cultures and treatments. Male Wistar rats (130–150 gm; Charles River, Saint-Aubin-lès-Elboeuf, France) were housed under controlled temperature and lighting conditions with food and water ad libitum. Articular cartilage isolated from femoral head cap pieces was aseptically dissected, and chondrocytes were obtained after digestion of cartilage fragments in pronase (2% [weight/volume] in 0.15*M* NaCl) followed by an overnight digestion in collagenase B (1.5% [w/v] in DMEM without serum) (18). Experiments were performed with first-passage cultures, 6 days after collagenase treatment. For this purpose, chondrocytes were grown to confluence in 25-cm^2 flasks ($\sim 4 \times 10^6$ chondrocytes per flask) in 5 ml of complete medium (DMEM supplemented with L-glutamine [2 m*M*], gentamicin [50 mg/ml], FBS 10% [volume/volume]). In a first set of experiments, cells were then cultured for 6 hours in FBS-free medium containing glucose (1.0 gm/liter) and glucosamine (1.0, 2.0, or 4.5 gm/liter), and finally stimulated with IL-1β (25 units/ml) for 12 hours. In a second set of experiments, cells were cultured for 6 hours in FBS-free medium containing glucose (4.5 gm/liter) or glucosamine (4.5 gm/liter), and finally stimulated with IL-1β (25 or 250 units/ml) for 12 hours. For RNA isolation and enzymatic assays, cells were harvested, washed once in phosphate buffered saline (Gibco-BRL), pelleted by centrifugation, and kept at -80°C prior to analysis.

For evaluation of proteoglycan synthesis by Na_2-$^{35}SO_4$ incorporation and measurement of NO and PGE_2 production in the culture medium, chondrocytes were suspended in sterile, filtered, low-viscosity alginate solution (1.2% [w/v]) at 6×10^6 cells/ml) and slowly extruded through a 22-gauge needle into a 100-m*M* $CaCl_2$ solution (19). After 2 washes with 0.15*M* NaCl, the encapsulated chondrocytes were maintained in a complete culture medium for 6 days in a humidified atmosphere with 5% CO_2 at 37°C. The IL-1β and glucosamine treatments were performed as described above, and alginate beads were then incubated in medium supplemented with heat-inactivated FBS (2% [v/v]) and 10 μCi/ml of Na_2-$^{35}SO_4$ for 4 hours. Encapsulated chondrocytes were then extensively washed with 0.15*M* NaCl and solubilized in Soluene 350 (0.5*M* quaternary ammonium hydroxide in toluene) overnight. The amount of radiolabeled sulfate that incorporated sulfated GAG was quantitated by liquid scintillation counting (20,21). NO production and PGE_2 assays were performed on samples prepared from chondrocytes cultured under the same conditions but without radiolabeled sulfate (see below).

Enzymatic assays. Membrane-enriched fractions were prepared from chondrocytes by differential ultracentrifugations, according to the procedure of Hogeboom (22), and stored in 100-m*M* HEPES buffer (pH 7.4) containing 0.25*M* sucrose. Protein content was determined by the method of Bradford (23), with bovine serum albumin as standard. Glucuronidation activity for GlcAT-I was measured by the method of Bansal and Gessner (24), using Gal-S-Gal as reporter substrate and radiolabeled UDP–glucuronic acid. Briefly, incubations (40 μl total volume) containing 0.1 m*M* Gal-S-Gal, 0.3 m*M* UDP–glucuronic acid (0.2 μCi), 20 μg microsomal protein, 10 m*M* $MgCl_2$, and 10 m*M* $MnCl_2$ in 100 m*M* sodium acetate buffer (pH 5.0) were carried out at 37°C for 1 hour. Incubations were terminated by addition of 40 μl ethanol, and reaction products were separated by thin-layer chromatography (TLC) on silica plates (Whatman, Clifton, NJ) with *N*-butanol, acetone, acetic acid, aqueous ammonia, and water as the developing solvent system. After scraping the radioactive area visualized by autoradiography, the amount of products formed was quantitated by liquid scintillation counting.

Analysis of GlcAT-I and stromelysin 1 mRNA expression by quantitative RT–polymerase chain reaction (PCR). A partial coding sequence for rat GlcAT-I cDNA was cloned by RT-PCR using chondrocyte poly(A+)RNA as a template. Two degenerate oligonucleotides were designed based on information from amino acid sequence alignment of rat galactose-β-1,3-glucuronosyltransferase (25) with putative proteins in *Caenorhabditis elegans* and *Schistosoma mansoni*, as described by Kitagawa et al (6). These primers were used for PCR amplification of a 210-bp fragment corresponding to nucleotides 560–770 of the human cDNA sequence (GenBank accession number AB009598). This fragment was further extended at the 5′ end by rapid amplification of cDNA ends (5′ RACE system; Gibco-BRL), according to the manufacturer's instructions. The obtained sequence corresponded to nucleotides 268–770 of the human GlcAT-I cDNA and exhibited high homology with an expression sequence tag in the data bank corresponding to the mouse orthologous GlcAT-I.

This sequence information was used to design appropriate oligonucleotide primers for the quantitative multistandard RT-PCR assay developed to evaluate variations of GlcAT-I mRNA levels in rat chondrocytes (26). This method takes advantage of both GlcAT-I and β-actin sequence conservation between mouse and rat species (26). The protocol allowed us to normalize the amounts of GlcAT-I mRNA with respect to those of β-actin mRNA in each sample. Total RNA samples extracted from rat cells were mixed with a constant amount of total RNA prepared from mouse tissue, thus bringing together competitive mouse β-actin with gene sequences of interest and acting as a multistandard source. The mixture was reverse-transcribed using hexamer random primers. Separate PCR amplifications for GlcAT-I, stromelysin 1 (MMP-3), and β-actin were then undertaken with sense and antisense primers corresponding to conserved domains in rat and mouse sequences, allowing the amplification of both sequences with the same efficiency.

For RT-PCR amplification of mouse and rat GlcAT-I, the sense primer extended from nucleotides 268 to 290 and the antisense primer from nucleotides 592 to 620 of the cDNA sequence. These oligonucleotide sequences were identical in

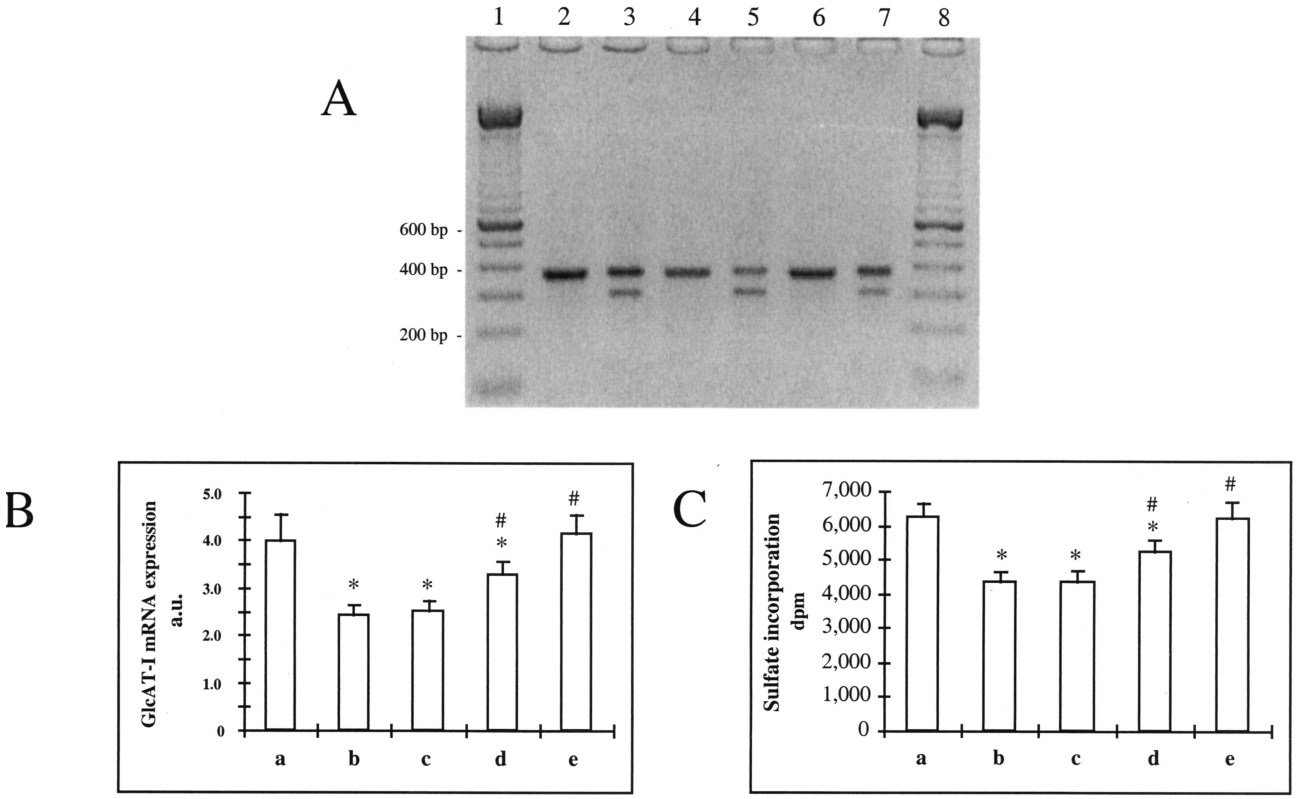

Figure 1. Effect of interleukin-1β (IL-1β) on galactose-β-1,3-glucuronosyltransferase I (GlcAT-I) mRNA expression and proteoglycan synthesis in rat chondrocytes cultured in the presence of increasing doses of glucosamine. **A**, Electrophoretic profile of GlcAT-I reverse transcriptase–polymerase chain reaction (RT-PCR) products amplified from mRNA of rat chondrocytes treated as described below and mixed with mouse mRNA as described in Materials and Methods. Lanes 2, 4, and 6 represent uncut RT-PCR products and lanes 3, 5, and 7 represent *Bam* HI–digested amplicons. Lanes 1 and 8 correspond to molecular weight markers. Lanes 2 and 3, 4 and 5, and 6 and 7 correspond to lanes a, b, and e in Figure 1B after quantitation by scanning densitometry. **B**, Quantitation of the GlcAT-I mRNA expression level. Chondrocytes were maintained in monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine, gentamicin, and fetal bovine serum (FBS) as described in Materials and Methods. Cells were cultured for 6 hours in serum-free medium containing glucose (1.0 gm/liter) and glucosamine (1.0, 2.0, 4.5 gm/liter; c, d, and e, respectively). Treatment with IL-1β (0 units/ml, lane a; 25 units/ml, lanes b–e) for 12 hours was then performed in the same medium. Values correspond to quantitation of the mRNA expression level after scanning densitometry analysis of RT-PCR products as illustrated on **A** and are presented in relative arbitrary units (a.u.). **C**, Variations of proteoglycan synthesis. Chondrocytes were encapsulated in alginate beads and maintained in culture in DMEM supplemented with glutamine, gentamicin, and FBS as described in Materials and Methods. Cells were cultured for 6 hours in medium containing glucose (1.0 gm/liter) and glucosamine (1.0, 2.0, 4.5 gm/liter; lanes c, d, and e, respectively) and 2% (v/v) FBS. Treatment with IL-1β (0 units/ml, lane a; 25 units/ml, lanes b–e) for 12 hours was then carried out in the same medium. Proteoglycan synthesis was evaluated by the measurement of radiolabeled sodium sulfate incorporation in dpm, as described in Materials and Methods. Results are the mean and SD of 3 different assays. $* = P < 0.05$ versus control value (glucose-containing medium, lane a) by Fisher's *t*-test. $\# = P < 0.05$ versus control value (IL-1β treatment, lane b) by Fisher's *t*-test.

rat and mouse, thus allowing the coamplification of both mRNAs. For amplification of rat and mouse β-actin mRNA, the sense primer extended from nucleotides 600 to 623 and the antisense primer from nucleotides 1143 to 1170 of the rat sequence (GenBank accession number X80130). Each amplification product was then distinguished by restriction-site polymorphism: the mouse GlcAT-I RT-PCR product was *Bam* HI digested into 2 fragments (121 and 304 bp), while the rat product remained uncut. A similar approach was used to differentiate mouse and rat β-actin amplification products. Digestion at the single *Xba* I restriction site present in the mouse sequence produced a 144-bp and a 425-bp fragment, whereas the rat β-actin RT-PCR product sequence remained uncut.

For amplification of MMP-3, the mouse and rat sense primers extended from nucleotides 680 to 702 and the antisense primer extended from nucleotides 1366 to 1388 according to the rat sequence (GenBank accession number X02601). The RT-PCR amplification products were distinguished by restriction site polymorphism: the mouse stromelysin 1 product

was *Hha* I digested into 2 fragments (318 and 390 bp), while the rat product remained uncut.

Restriction endonuclease–digested RT-PCR products were resolved by agarose gel electrophoresis stained with ethidium bromide. The bands were visualized under ultraviolet light and photographed using a computer-assisted camera. Quantitation of each band was performed by densitometry analysis with National Institutes of Health software. Results are expressed as the ratio (analyzed $gene_{rat}$:β-$actin_{rat}$) $\times$ (β-$actin_{mouse}$:analyzed $gene_{mouse}$), in arbitrary units.

Nitrite assay. NO production was determined spectrophotometrically by measuring the accumulation of nitrite (NO^-_2), a stable breakdown product of NO, in the culture medium by the Griess reaction using sodium nitrite as standard (27). Briefly, 100 μl of culture supernatant was mixed with 100 μl of Griess reagent (sulfanilamide (1% [w/v]) in 2.5% H_3PO_4 and *N*-naphthylethylenediamine dihydrochloride (0.1% [w/v]) in H_2O) for 5 minutes in 96-well plates. Absorbance at 550 nm was measured on an MR5000 microplate reader (Dynatech, Ann Arbor, MI). Nitrite concentration was calculated from a standard curve of sodium nitrite. Nitrite production was assessed as cumulative nitrite released during incubation with IL-1β and/or glucosamine.

PGE_2 assays. PGE_2, the main metabolite of COX in chondrocytes, was measured in supernatants by an enzyme-linked immunosorbent assay method according to the manufacturer's instructions (Dynatech). This assay showed no cross-reactivity with other prostanoids. Sensitivity was <30 pg/ml. Results were expressed in ng/ml.

Statistical analysis. After comparison of data by analysis of variance, different groups were compared using Fisher's *t*-test. Assays were made in triplicate. *P* values less than 0.05 were considered significant.

RESULTS

Effect of glucosamine on IL-1β–mediated repression of GlcAT-I mRNA expression and proteoglycan anabolism. To investigate the effect of IL-1β and/or glucosamine on GlcAT-I expression, rat articular chondrocytes were isolated, maintained in culture, placed in glucose- and/or glucosamine-containing medium, and challenged with IL-1β. In order to analyze GlcAT-I mRNA expression, we cloned a partial cDNA coding sequence corresponding to 268–770 bp of the human GlcAT-I coding sequence, using mRNA isolated from rat chondrocytes as the template. The rat cDNA sequence obtained showed 88% homology with the human cDNA (6). The newly cloned sequence was used to design appropriate primers for quantitative RT-PCR assay of GlcAT-I mRNA expression in rat chondrocytes, as described in Materials and Methods. A representative RT-PCR experiment is illustrated in Figure 1A.

The effects of increasing doses of glucosamine (1–4.5 gm/liter) on GlcAT-I mRNA expression and on proteoglycan synthesis in chondrocytes cultured in the presence of 1 gm/liter glucose and treated with IL-1β (25 units/ml) were investigated. Treatment of chondrocytes with IL-1β resulted in a significant decrease in GlcAT-I mRNA expression, as illustrated in Figure 1A (compare upper bands in lanes 3 and 5). Quantitation of the variation in mRNA level by scanning densitometry indicated a 38% decrease of GlcAT-I mRNA following IL-1β treatment (Figure 1B, compare lane b with lane a). In addition, as expected, IL-1β produced an inhibition in proteoglycan synthesis (32%; Figure 1C, compare lane b with lane a). Interestingly, the addition of glucosamine prevented IL-1β–mediated inhibitory effects on GlcAT-I mRNA expression (Figure 1B) and on proteoglycan synthesis (Figure 1C) in a dose-dependent manner. A partial restoration of GlcAT-I mRNA expression level and proteoglycan synthesis was observed with the dose of 2.0 gm/liter (Figures 1B and C, compare lane d with lane c), whereas the highest dose of glucosamine (4.5 gm/liter) fully counteracted the inhibitory effects of IL-1β on both variables (Figure 1A, compare lane 7 with lane 5; Figures 1B and C, compare lane e with lane c). The latter dose was therefore selected for the subsequent experiments.

Effect of glucosamine on IL-1β–mediated repression of GlcAT-I expression and activity and proteoglycan anabolism. Figure 2 illustrates the effects of 2 doses of IL-1β (25 units/ml and 250 units/ml) on the GlcAT-I mRNA expression level, GlcAT-I catalytic activity, and on proteoglycan anabolism in chondrocytes cultured in the presence of glucose or glucosamine. Culture of chondrocytes in either glucose- or glucosamine-containing medium did not significantly modify the GlcAT-I mRNA expression level (Figure 2A, compare lane d with lane a). In contrast, IL-1β treatment at a dose of 25 units/ml resulted in a large decrease in GlcAT-I mRNA expression (48%) in rat chondrocytes cultured in glucose-containing medium (Figure 2A, compare lane b with lane a). Increasing the dose of cytokine (to 250 units/ml) did not further enhance this effect (Figure 2A, compare lane c with lane b). Interestingly, the inhibitory effects of Il-1β on GlcAT-I mRNA expression were fully prevented when chondrocytes were cultured in a glucosamine-containing medium (Figure 2A, compare lanes e and f with lane d).

In order to analyze GlcAT-I enzyme activity in membrane-enriched fractions from chondrocytes (Figure 2B), we adapted a TLC method based on the use of radiolabeled UDP–glucuronic acid (24). GlcAT-I activity toward Gal-S-Gal, whose structure mimics that of the natural oligosaccharidic substrate of this enzyme (28), was optimized in terms of pH and concentration of divalent ions. GlcAT-I exhibited a maximum specific activity at pH 5.0 in the presence of 10 m*M* Mn^{2+} and 10

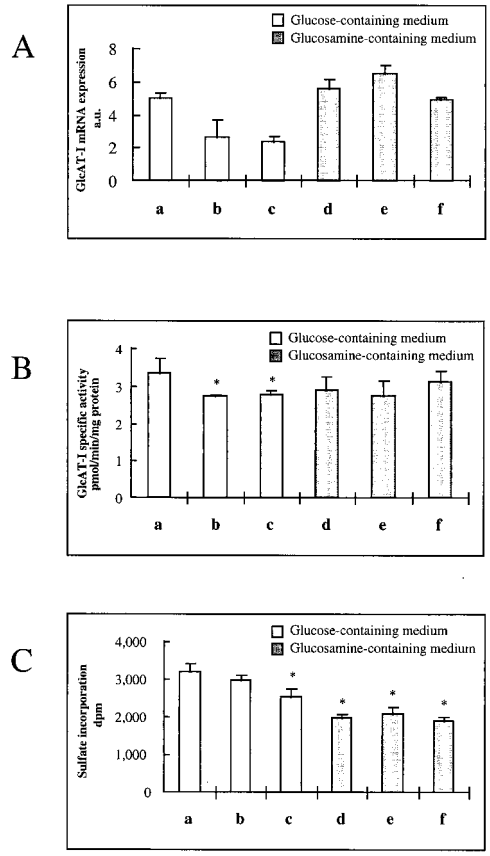

Figure 2. Effect of IL-1β on GlcAT-I mRNA expression and activity and on proteoglycan synthesis in rat chondrocytes cultured in the presence of glucose or glucosamine. Chondrocytes were maintained in monolayer cultures in DMEM supplemented with glutamine, gentamicin, and FBS as described in Materials and Methods. Cells were maintained for 6 hours in serum-free medium containing glucose (4.5 gm/liter; lanes a–c) or glucosamine (4.5 gm/liter; lanes d–f). Treatment with IL-1β (0 units/ml, lanes a and d; 25 units/ml, lanes b and e; 250 units/ml, lanes c and f) for 12 hours was then performed in the same medium. Variations in GlcAT-I mRNA expression **(A)** and enzyme activity toward Gal-S-Gal **(B)** were evaluated by quantitative RT-PCR and thin-layer chromatography, respectively, as described in Materials and Methods. For the determination of the variations of proteoglycan biosynthesis **(C),** chondrocytes were encapsulated in alginate beads and maintained in culture in DMEM supplemented with glutamine, gentamicin, and FBS as described in Materials and Methods. Cells were cultured for 6 hours in medium containing glucose (4.5 gm/liter; lanes a–c) or glucosamine (4.5 gm/liter; lanes d–f) and 2% (v/v) FBS. Treatment with IL-1β (0 units/ml, lanes a and d; 25 units/ml, lanes b and e; and 250 units/ml, lanes c and f) for 12 hours was then performed in the same medium. Proteoglycan synthesis was evaluated by the measurement of radiolabeled sodium sulfate incorporation as described in Materials and Methods. Results are the mean and SD of 3 different assays. $* = P < 0.05$ versus control value (glucose-containing medium, lane a) by Fisher's *t*-test. See Figure 1 for definitions.

m*M* Mg^{2+} (data not shown). The presence of glucosamine instead of glucose in the chondrocyte culture medium did not significantly modify GlcAT-I activity (Figure 2B, compare lane d with lane a). In contrast, as with the mRNA expression level, treatment of chondrocytes cultured in glucose-containing medium with each of the 2 doses of IL-1β led to a decrease in GlcAT-I activity (18%; Figure 2B, compare lanes b and c with lane a), whereas the same treatments had no significant effect when performed in the presence of glucosamine (Figure 2B, compare lanes e and f with lane d).

Furthermore, Figure 2C shows that IL-1β produced a significant concentration-dependent inhibition of proteoglycan synthesis, up to 20% with the highest dose of cytokine (compare lanes b and c with lane a). In contrast, no inhibitory effect of IL-1β on proteoglycan anabolism was observed when the cytokine treatment was performed in the presence of glucosamine (Figure 2C, compare lanes e and f with lane d). On the other hand, glucosamine alone (4.5 gm/liter) produced a significant decrease in proteoglycan synthesis (Figure 2C, compare lane d with lane a).

Taken together, our results provide evidence that IL-1β was able to down-regulate both the mRNA expression and enzyme activity of GlcAT-I in rat chondrocytes and that this effect was concomitant with the inhibition of proteoglycan synthesis. In addition, we found that the cytokine-mediated effects on both GlcAT-I expression and proteoglycan synthesis were prevented when IL-1β was administered in the presence of glucosamine.

Effects of IL-1β on mRNA expression of stromelysin 1 in chondrocytes cultured in the presence or absence of glucosamine. Our results clearly suggested a possible antagonist effect of glucosamine toward the deleterious action of IL-1β on the synthesis of proteoglycan. This prompted us to examine the action of the amino sugar toward the inducing effect of this cytokine on the expression of enzymes involved in the degradation of the cartilaginous matrix. For this purpose, changes in the level of mRNA coding for stromelysin 1 (MMP-3), which is involved in the degradation of the core protein of aggrecan and the collagen network, were evaluated by quantitative RT-PCR, as described for GlcAT-I. Treatment of rat chondrocytes with IL-1β led to a dose-dependent increase in the stromelysin 1 mRNA level, reaching 77% at the highest dose (Figure 3, compare lanes b and c with lane a). In contrast, when the IL-1β challenge was carried out in the presence of glucosamine, no significant variation of stromelysin 1 mRNA expression level was observed (Figure 3, compare lanes e and f with lane d). On the

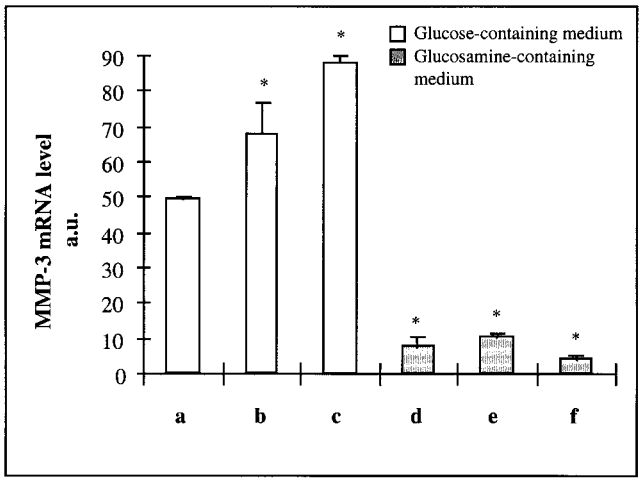

Figure 3. Effect of IL-1β induction on stromelysin 1 mRNA expression in rat chondrocytes. Chondrocytes were maintained in monolayer culture with DMEM supplemented with glutamine, gentamicin, and FBS as described in Materials and Methods. Cells were treated as described in the legend to Figure 2. Quantitation of the stromelysin 1 mRNA level was performed as described in Materials and Methods. Results are the mean and SD of 3 different assays. * = $P < 0.05$ versus control value (glucose-containing medium, lane a) by Fisher's *t*-test. See Figure 1 for definitions.

other hand, in chondrocytes cultured in glucosamine alone, the mRNA expression level of stromelysin decreased significantly (Figure 3, compare lane d with lane a).

Effects of IL-1β on NO synthesis and PGE_2 production in chondrocytes cultured in the presence or absence of glucosamine. In order to investigate the influence of glucosamine on IL-1β–mediated effects, we further examined the variations of known mediators of the inflammatory response, i.e., NO and PGE_2. As expected, treatment of chondrocytes with IL-1β in the presence of glucose induced a strong dose-dependent enhancement of NO production, reaching 200% at a dose of 250 units/ml of cytokine (Figure 4A, compare lanes b and c with lane a). Interestingly, incubation of chondrocytes with IL-1β in a glucosamine-containing medium resulted in an increase in NO production that did not exceed 48% with the highest dose of cytokine (Figure 4A, compare lanes e and f with lane d). It is noteworthy that no significant difference in constitutive NO release was observed when chondrocytes were cultured in the presence of glucosamine, when compared with glucose (Figure 4A, compare lane d with lane a).

On the other hand, as shown in Figure 4B, the production of PGE_2 by chondrocytes cultured in glucosamine-containing medium was significantly lower than that measured when chondrocytes were maintained in glucose-containing medium (mean ± SD 0.62 ± 0.04 μg/ml and 1.63 ± 0.36 μg/ml, respectively; compare lane d with lane a). This observation could result from an inhibitory effect of glucosamine on COX-1, the noninducible isoform. Furthermore, as with NO, IL-1β induced a strong dose-dependent increase in PGE_2 production in the presence of glucose (Figure 4B, compare lanes b and c with lane a). In contrast, this increase was small when the challenge with cytokine was performed in glucosamine-containing medium (142% at 250 units/ml versus 4,600%; compare lane f with lane d and lane c with lane a, respectively).

DISCUSSION

GlcAT-I is a key UDP-glycosyltransferase enzyme involved in the priming of hexuronic acid–GAG chain synthesis. The cDNA coding for the human form

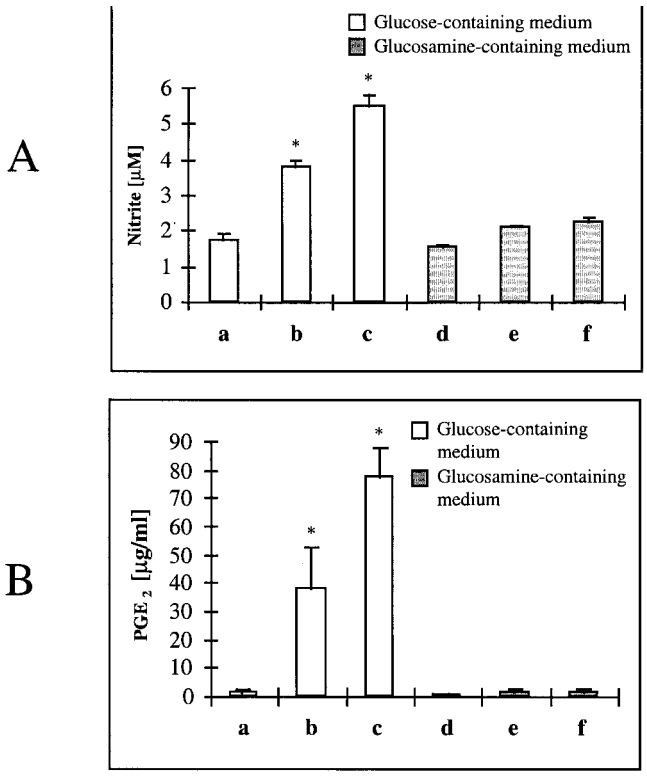

Figure 4. Effect of interleukin-1β (IL-1β) induction on proinflammatory mediators in rat chondrocytes. Chondrocytes encapsulated in alginate beads were cultured and challenged with IL-1β as described in the legend to Figure 2. Production of nitric oxide (**A**) and prostaglandin E_2 (PGE_2) (**B**) in the culture medium was determined as described in Materials and Methods. Results are the mean and SD of 3 different assays. * = $P < 0.05$ versus control value (glucose-containing medium, lane a) by Fisher's *t*-test.

of this enzyme was recently isolated (6), but no information on its regulation is yet available. By cloning the rat orthologous cDNA, we generated appropriate tools for the analysis of GlcAT-I mRNA expression in cultured rat chondrocytes. Furthermore, the development of a specific assay based on the use of a disaccharidic derivative, analogous to the natural digalactosidic substrate of this enzyme (28), allowed us to follow its catalytic activity simultaneously.

A significant finding of this study is that IL-1β markedly down-regulates GlcAT-I expression as well as its enzyme activity in rat chondrocytes. The concomitant inhibition of GlcAT-I expression and proteoglycan synthesis raises the possibility that the reduced expression of this UDP-glucuronosyltransferase responsible for the biosynthesis of the common tetrasaccharidic linkage of GAG could contribute to the deleterious effect of the proinflammatory cytokine on proteoglycan synthesis. Based on the reduced incorporation of radiolabeled precursor sugars, it has been previously suggested that the biosynthesis of GAG chains is affected in IL-1β–challenged chondrocytes (15). However, this is the first demonstration of a direct repressive effect of IL-1β on a glucuronosyltransferase involved in a key step of GAG biosynthesis. It is reasonable to hypothesize that this effect may lead to a reduced content of GAG chains available for further sulfation, thus contributing to the reduced incorporation of radiolabeled sulfate in proteoglycans, traditionally described after IL-1β treatment either in vivo (21) or in cultured cells (29).

Glucosamine is a major component of the repeating disaccharide motif of GAG chains involved in the assembly of the cartilaginous matrix. This amino sugar has recently received attention in the literature (30) and in some clinical studies as a treatment for OA (31,32). The efficacy of glucosamine has been attributed to stimulation of the synthesis of GAG chains and other glycoconjugates (17), although experimental data supporting this hypothesis are limited. In this study, we show that if glucosamine did not by itself increase proteoglycan synthesis, it efficiently prevented the IL-1β–mediated decrease in proteoglycan synthesis in stimulated rat chondrocytes. These results are consistent with those of Bassler et al (17), who reported that glucosamine restored proteoglycan production by human chondrocytes from OA cartilage.

It has been suggested that exogenous addition of glucosamine circumvents glutamine fructose-1-phosphate transaminase, which is responsible for the formation of amino sugars. Since this enzyme is rate-limiting in the hexosamine pathway, a supply of glucosamine provides precursors for glycosyltransferases involved in GAG biosynthesis. In addition, we show here that the mechanisms underlying glucosamine action involve the regulation of GlcAT-I. Our data are strengthened by the fact that the antagonistic effects of glucosamine on IL-1β inductive events were observed at the levels of both GlcAT-I mRNA and enzyme-specific activity. This effect of glucosamine, possibly coordinated with the stimulation of aggrecan core protein synthesis, as suggested by other studies (17), may provide a basis for the restoration of proteoglycan content in IL-1β–stimulated chondrocytes. In accordance with this suggestion, we show that glucosamine is able to antagonize the IL-1β–induced expression of stromelysin 1 as well. Taken together, the data indicate that the corrective effects of glucosamine toward IL-1β–mediated deregulation of enzymes involved either in the catabolism or in the anabolism of proteoglycans may account, at least in part, for the beneficial effect of the drug on pathologic cartilage.

The signaling pathways that mediate the effects of IL-1β and other chondrocyte regulatory factors have only been partially characterized. However, it is accepted that the proinflammatory action of IL-1β involves the mobilization of several transduction pathways, such as the mitogen-activated protein kinase cascade. These various signaling pathways control directly or indirectly the activation of several transcription factors such as activator protein 1 and nuclear factor κB (33). Activation of these factors leads to the up- or down-regulation of the transcriptional activity of a battery of genes associated with inflammation, such as COX-2 and iNOS. In order to provide further insight into the mechanisms underlying glucosamine action, we investigated its influence on the production of mediators of the inflammatory process. Consistent with previous data, our experiments show an enhancement of PGE_2 and NO production in rat chondrocytes treated with IL-1β, likely due to the induction of COX-2 (34) and iNOS (13), respectively.

Interestingly, we found that glucosamine consistently prevents the influence of IL-1β on these variables, further emphasizing a specific effect of the amino sugar in the IL-1β–mediated response. However, the mechanisms that govern the antagonist action remain unclear. Glucosamine could potentially act at many different levels. Complementary to our studies, Sandy et al (30) recently provided evidence for an inhibitory effect of glucosamine on IL-1β–stimulated aggrecanase expression. They suggested that this capacity may involve the inhibition of glycosylphosphatidylinositol (GPI)–linked

proteins (35). Such a putative GPI-anchored component may also be part of an upstream receptor/signaling pathway and may regulate the transcription of various genes. In the same way, glucosamine has also been shown to rapidly induce several genes at the transcription level (36). In fact, our study provides evidence that glucosamine modifies the mRNA expression level of GlcAT-I and stromelysin 1. Several studies indicate that the hexosamine pathway may be involved in glucosamine regulation of gene expression (36). Whether this signaling pathway is responsible for glucosamine-mediated effects on GlcAT-I in IL-1β–stimulated chondrocytes requires further investigation.

In conclusion, the results of the present study demonstrate that IL-1β down-regulates the expression of GlcAT-I, a pivotal enzyme of GAG polysaccharide chain biosynthesis, at both the mRNA and enzyme activity levels, possibly contributing to the loss of proteoglycan synthesis elicited by the cytokine. Furthermore, we show that glucosamine not only corrected these effects, but also antagonized the production of various proinflammatory mediators activated by IL-1β, such as NO and PGE_2. Taken together, these results suggest that the pharmacologic effects of glucosamine in the treatment of OA and RA may involve the antagonist action of this amino sugar on the signaling events mediated by IL-1β.

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