Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 or retinoic acid can be inhibited by glucosamine

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A rat chondrosarcoma cell line and bovine cartilage explants have been used to study the control of aggrecan degradation by chondrocytes treated with interleukin-1 (IL-1) or retinoic acid (RA). Aggrecan fragment analysis with anti-neo-epitope antibodies suggests that aggrecanase (an as yet unidentified enzyme) is the only aggrecan-degrading proteinase active in these cultures. With rat cells, aggrecanase converts the aggrecan core protein into two major G1-domain-bearing products (60 kDa with a C-terminal Glu-373, and 220 kDa with a C-terminal Glu-1459). Both products were quantified on a standardized Western analysis system with a G1-specific antibody. Immunoblots were analysed by scanning densitometry and the sensitivity, linearity and reproducibility of the assay were established. With rat cells the aggrecanase response to IL-1 was optimal at about 2 mM

glutamine, but was progressively inhibited at higher concentrations, with about 90 % inhibition at 10 mM glutamine. Such inhibition by glutamine was not, however, observed with bovine explants. On the other hand, marked inhibition of aggrecanase-dependent cleavage was observed with both rat cells and bovine explants when D(+)-glucosamine was included at concentrations above 2 mM. Inhibition was apparently not due to cytotoxicity or interference with IL-1 signalling, since biosynthetic activity was not inhibited and inhibition of the aggrecanase response was also obtained when RA was used as the catabolic stimulator. Possible mechanisms for the inhibition of the aggrecanase response by glucosamine in chondrocytes treated with IL-1 or RA are discussed.

INTRODUCTION

Chondrocytes in mature articular cartilage maintain a steady-state tissue content of aggrecan by controlling the rates of deposition and proteolytic removal of this component. Anabolic growth factors such as insulin-like growth factor-1 and transforming growth factor- β promote the synthesis and deposition of aggrecan [1,2], whereas catabolic agents such as interleukin-1 β (IL-1 β), tumour necrosis factor α (TNF α), oncostatin M, fibronectin fragments and retinoic acid (RA) provoke a chondrocyte-mediated catabolic response which results in depletion of aggrecan from the tissue [3–7]. The aggrecan degradative pathway has been studied *in vivo* [8,9], in cartilage explants [3] and chondrocyte culture systems [10]. While there are clear correlations between the expression of matrix metalloproteinases (MMPs) and the rate of aggrecan degradation in a range of situations [11,12], and evidence of MMP degradation of aggrecan in mature human cartilage [13], the only proteinase that has been directly implicated in all catabolic systems under study is an unidentified activity termed aggrecanase.

Aggrecanase has not yet been cloned, but the available evidence from inhibitor studies in explant cultures [14,15] suggests that it may be a metalloproteinase. Other studies [16] have led to the conclusion that aggrecanase is the terminal activity of a proteinase cascade which involves both cysteine proteinases and metalloproteinases. A significant limitation of many of the inhibitor studies has been that the activity being monitored has not been

specifically identified as aggrecanase. Instead, the measurement of aggrecan fragment release from explants, measured as chondroitin sulphate (CS), has been widely interpreted as a direct measure of aggrecanase activity.

Aggrecanase activity, as originally described [3,17,18], can however only be specifically assayed by the quantification of aggrecan catabolic products with precisely defined cleavage sites. One approach to this is the assay of aggrecan fragments containing the G1 domain, which bear the aggrecanase-generated C-terminal residues Glu-373, Glu-1459, Glu-1564 or Glu-1664 (rat residue numbering). In this paper we describe a method for the quantitative analysis of two such G1-bearing products of aggrecanase activity in cultures of rat chondrosarcoma cells treated with IL-1 β . These species, of molecular mass 220 kDa and 60 kDa, are generated by cleavage at the Glu-1459–Gly-1460 and Glu-373–Ala-374 bonds respectively, and represent the only G1-bearing products that accumulate in this cell system. Optimization of the IL-1 β response by assay of these specific products has shown that, in this cell system, aggrecanase activity is highly regulated by the exogenous supply of either glutamine or glucosamine.

The inhibitory effects of glutamine and glucosamine in rat cells were also seen with RA as the catabolic stimulator. Further, bovine cartilage explants treated with IL-1 α or RA also accumulate aggrecanase-generated G1 fragments [3,14], and this process was markedly inhibited by glucosamine, but not by glutamine. Taken together, these results suggest the existence of

Abbreviations used: AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; CS, chondroitin sulphate; DMEM, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; IL-1, interleukin-1; i.p.d., integrated pixel density; MMP, matrix metalloproteinase; RA, retinoic acid; TNF, tumour necrosis factor.

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a previously undescribed pathway for the control of aggrecanase by glucosamine or its metabolites in chondrocytes.

EXPERIMENTAL

Materials

Insulin (bovine pancreas), RA (all *trans*) and D(+)-glucosamine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) (powder) and culture-tested distilled water were from Life Technologies (Grand Island, NY, U.S.A.). Fetal bovine serum was from HyClone (Logan, UT, U.S.A.). Human recombinant TNF α and IL-1 α and mouse recombinant IL-1 β were from Genzyme (Boston, MA, U.S.A.). α_2 -Macroglobulin (bovine plasma) was from Boehringer Mannheim (Chicago, IL, U.S.A.). Goat anti-(rabbit IgG) (peroxidase-conjugated) was from Calbiochem (San Diego, CA, U.S.A.). The ECL detection system, biotinylated molecular mass markers and streptavidin-(horseradish peroxidase) conjugate were from Amersham (Arlington Heights, IL, U.S.A.), and were used according to the manufacturer's instructions. The production, characterization and use of the aggrecan G1 domain antiserum (anti-ATEGQV) and the anti-G3 antiserum (anti-TYKHRL) have been described [19]. Antisera anti-NITEGE and anti-KEEE and recombinant human MMP-3 were supplied by Merck (Rahway, NJ, U.S.A.). Chase ABC (protease-free) was from Seikagachu. Lactate was determined on papain digests with the Sigma kit, and DNA was determined fluorimetrically on papain digests of whole cultures [19a].

Experiments with rat cells

Cells from a rat chondrosarcoma cell line termed LTC were obtained from Dr. J. Kimura [10], and were plated at 100 cells per 100 mm dish in 5 ml of growth medium. After 4 days, single colonies (about 8 cells per colony) were isolated by cloning ring and trypsinized for replating. Clonal lines were tested for aggrecan synthesis and aggrecanase-mediated degradation on treatment with cytokines. Several responsive clones were sub-cloned and one resulting line (clone 11) was chosen for the studies described.

For catabolic studies, clone 11 cells up to passage 45 were plated at about 40 000 cells per 0.5 ml of growth medium in 48-well plates and maintained for 3 or 4 days, in which time they deposited 15–30 μ g of glycosaminoglycan (GAG) per cell layer. The growth medium was Gibco DMEM powder (formulation 12800, which contains 25 mM glucose) dissolved in Gibco bottled water and supplemented with sodium bicarbonate (3.7 g/l), glutamine (2 mM), ascorbic acid (50 mg/l), gentamycin (50 mg/l) and Hyclone fetal bovine serum (10%), pH 7.4.

For cytokine response experiments, these cultures were switched to the appropriate catabolic medium as follows. Wells were washed with 2 $\times$ 0.5 ml of catabolic medium (containing the appropriate glucose concentration) and then were maintained in 0.5 ml of catabolic medium for 1–2 h. Wells were then washed with another 0.5 ml of catabolic medium to remove residual serum proteins and nutrients. To the washed wells was added 200 μ l of the test medium (catabolic medium containing supplements and test agents), and after 1 h the cytokine was added from a concentrated stock solution. Cultures were maintained for up to 5 days without medium change.

For biosynthetic experiments, isotopes (10 μ Ci of 35 S, 20 μ Ci of [3 H]Pro or 20 μ Ci of [3 H]GlcNH $_2$) were added from concentrated stocks either 1 h after addition of IL-1 β on day 0, or to cultures without medium change on days 1–5. For determination of [35 S]sulphate and [3 H]glucosamine incorporation, the cultures

were terminated by papain digestion and samples were fractionated on Sephadex G-50 columns in 4 M guanidinium chloride/0.1 M sodium acetate/20 mM Mes/0.5% CHAPS, pH 7.0. For [3 H]proline incorporation, cultures were terminated by digestion for 2 h with Chase ABC (protease-free) in 50 mM Tris/HCl/50 mM sodium acetate/10 mM EDTA, pH 7.6, and extracted in 4 M guanidinium chloride/0.1 M sodium acetate/20 mM Mes/0.5% CHAPS, pH 7.0, for 2 h; the soluble fraction was fractionated on Sephadex G-50 as above.

For Western analysis, total cultures (generally 20–50 μ g of GAG in 220 μ l of medium) were digested with Chase ABC as above. Samples were centrifuged at 1460 *g* for 10 min in a bench-top centrifuge and portions of the clear supernatant (about 10 μ l) were analysed by Western blot as previously described [10,19]. The anti-ATEGQV, anti-NITEGE, anti-TFKEEE and anti-TYKHRL antisera were used at 1:5000 dilution and the peroxidase-conjugated goat anti-(rabbit IgG) was detected with the ECL kit from Amersham. Film exposure times ranged from 5 s to 5 min to provide quantifiable images.

Experiments with bovine cartilage explants

Explant culture methods were based on previous work [3]. Briefly, about 1 g of cartilage from the metacarpal-phalangeal joints of calves (1–2 weeks old) was sliced finely, washed in PBS and maintained for 3–5 days in 20 ml of explant medium (see below) supplemented further with 2 mM glutamine and 20 mM Hepes. The tissue was then washed (3 $\times$ 10 ml) with the appropriate catabolic medium (glucose concentration adjusted) and distributed into 48-well plates at about 25 mg wet wt. per 250 μ l of catabolic medium. Additions of glutamine and glucosamine were made and, after about 1 h, human IL-1 α (1 ng/ml) or RA (10 μ M) was added. Every 2–3 days the medium was collected and replaced with appropriate freshly prepared supplements. After 15 days the cultures were terminated by collection of medium and extraction of the tissue at 4 °C for 48 h with 500 μ l of 4 M guanidinium chloride/10 mM Mes/50 mM sodium acetate, pH 6.8 [containing 5 mM EDTA, 0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 5 mM iodoacetic acid and 1 μ g/ml pepstatin]. The extract was collected and the residual GAGs were solubilized by addition of 500 μ l of 0.1 M NaOH. Portions of the medium collections, guanidine extracts and NaOH extracts were analysed to determine the effect of the culture period on the distribution of GAGs between the tissue and the medium.

For analysis of the aggrecan fragments in each culture, samples of 50 μ l of each medium collection and 100 μ l of the appropriate guanidinium chloride extract were combined and added to 3 vol. of ice-cold ethanol/5 mM sodium acetate before storage at –20 °C for 16 h. Precipitates were collected by centrifugation at 4 °C in a Beckman Microfuge run at maximum speed for 20 min. The bulk ethanol was carefully removed and the samples were dried *in vacuo* before the addition of 250 μ l of 50 mM sodium acetate/50 mM Tris/10 mM EDTA, pH 7.6. A portion of each sample (containing 22 μ g of GAG) was taken for digestion with Chase ABC (protease-free; 0.3 unit/mg of GAG) for 2 h at 37 °C. Samples were then adjusted to 4 mM AEBSF and 7 mM *N*-ethylmaleimide before digestion with keratanase I (0.01 unit/mg of GAG) for 1 h at 37 °C, followed by Western analysis with anti-ATEGQV (for total aggrecan G1) or anti-NITEGE (for aggrecanase-generated G1). The G1-NITEGE catabolic product was the expected doublet of 62 kDa and 68 kDa previously observed in bovine explants [10,19]. The content of the G1-NITEGE doublet was determined from a standard curve [integrated pixel density (i.p.d.) for both bands against μ g of

GAG equivalents] generated with a 15-day-medium pool from IL-1 α -treated bovine explants. For quantification of Western blots, the radiographic images were captured on a Hewlett-Packard Scanjet 3C scanner with DeskScan II software and products were quantified with NIH Image 7.5 software. Data were obtained only in the linear detection range with the scale set at a pixel aspect ratio of 750 and 28.3 pixels per cm. The i.p.d. value for each product was determined in triplicate and a mean value used to determine the amount of GAG equivalents (μ g).

General methods

The explant medium was Gibco DMEM powder (formulation 23800, which is glucose-free) dissolved in Gibco bottled water and supplemented with glucose (25 mM), sodium bicarbonate (3.7 g/l) sodium pyruvate (110 mg/l), pyridoxine hydrochloride (4 mg/l) and gentamycin (50 mg/l). The catabolic medium was explant medium without Hepes buffer and adjusted to the appropriate glucose concentration (5–25 mM). The GAG content was assayed in papain digests, as CS equivalents, by using Dimethylmethylene Blue [15] with shark CS as standard.

RESULTS

Quantification of aggrecanase-mediated cleavage of aggrecan by rat cells

Rat chondrosarcoma cells degraded aggrecan in response to a range of stimulatory agents, including TNF α , oncostatin M, RA and IL-1 β (Figure 1). In every case, Western analysis of the total cultures indicated that aggrecan proteolysis resulted exclusively from the action of aggrecanase. The aggrecan present before treatment, or after 5 days of culture in catabolic medium without cytokine, was a single 350 kDa band which reacted strongly with both the anti-G1 (lane 1) and anti-G3 (lane 2) antisera, and which therefore represents the full-length core protein. After treatment with any of the cytokines (TNF α , oncostatin M, RA or IL-1 β) for 5 days, two fragments were generated at about 220 kDa and 60 kDa, both of which reacted with the anti-G1 antiserum (lanes 3–6), but neither of which reacted with the anti-G3 antiserum (lane 7), indicating that they had undergone proteolysis resulting in loss of the G3 domain. The 350 kDa species in these samples still reacted with the anti-G3 antiserum,

Figure 1 Cytokine stimulation of aggrecan degradation by rat chondrosarcoma cells: Western blot identification of degradation products with anti-peptide and anti-neo-epitope antibodies

Cultures were grown for 3 days and then either terminated (CONT) or treated for 5 days with TNF α (10 ng/ml), oncostatin M (OSM; 50 ng/ml), RA (10 μ M) or IL-1 β (100 ng/ml) before termination. Cultures were terminated by chondroitinase digestion and analysed by Western blotting with the anti-G1 antiserum (anti-ATEGQV), the anti-G3 antiserum (anti-TYKHRL) or the C-terminal specific antisera (anti-TFKEE and anti-NITEGE), as indicated.

Figure 2 Standardization of the Western blot method

Various amounts of the IL-1 β -treated sample (from Figure 1) were analysed by Western blotting with the anti-G1 antiserum (anti-ATEGQV) and the bands were quantified by scanning densitometry with NIH Image software. The graph shows the relationship between the amount loaded [as GAG equivalents (μ g)] and the i.p.d. determined for each of the three species (350 kDa, 220 kDa and 60 kDa). The Western blot has been compressed for presentation.

being the remaining intact full-length core protein. The C-terminal residue of the 220 kDa species was identified as Glu-1459 on the basis of reactivity with the anti-TFKEE antiserum alone (lane 8), and the C-terminal residue of the 60 kDa species was identified as Glu-373 on the basis of reactivity with the anti-NITEGE antiserum alone (lane 9). The same 350, 220 and 60 kDa species had each previously been identified in aggrecan preparations from rat chondrosarcoma tumour tissue [19].

To examine the control of the cell-mediated catabolic response to IL-1 β , we developed a quantitative densitometric immuno-analysis system to determine the relative amounts of G1 reactivity present in each of the three aggrecan species (350 kDa, 220 kDa and 60 kDa). For standardization of this assay, a sample equivalent to that shown for the IL-1 β -treated culture (Figure 1, lane 6) was used. A loading range from 0.12 to 1.2 μ g of GAG (CS equivalents) was analysed on a single gel and the relationship between the amount loaded and the G1 reactivity (determined as i.p.d.) for each of the three species was determined (Figure 2). The quantifiable response region for all three species was from about 0.3 μ g to about 1 μ g of CS (or about 5–17 ng of protein for each species) in this particular sample. The 350 kDa and 220 kDa species showed very similar response curves, with saturation at about 22000 i.p.d. units. The 60 kDa species showed saturation at a higher value (about 29000 i.p.d. units), suggesting a less restricted epitope accessibility at high loadings of this species.

To examine the linearity and reproducibility of this assay, we digested chondrosarcoma cultures for 24 h with a range of concentrations (50–800 ng) of MMP-3. MMP-3 cleaves the Asn-341–Phe-342 bond of aggrecan [13] and so is a reasonable model of the aggrecanase-dependent cleavage of the Glu-373–Ala-374 bond. The 350 kDa substrate and the 55 kDa MMP product (in this case G1 with a C-terminus of Asn-341) were quantified by densitometric analysis, and the results (not shown) illustrated that the concentration of product (μ g equivalents of 55 kDa product) and the amount of MMP-3 exhibited an essentially linear relationship over the range 150–800 ng of MMP-3; fur-

thermore, a variation of less than 3 % from the mean i.p.d. value was seen between replicates at each concentration.

Optimization of the aggrecanase response of rat cells to IL-1 β

To study aggrecanase induction by IL-1 β in these cells in more detail, we first examined the effect of the extent of matrix deposition on the subsequent catabolic response. For this, IL-1 β was added in catabolic medium to cultures of about 40000 cells that had been maintained in growth medium (including 10 % serum) for periods between of 2 and 8 days before study. It was found that the aggrecanase response was detectable only when the cultures had been maintained for about 3–4 days, at which time the cell layers contained about 15–30 μg of GAG and about 200000 cells. The non-responsiveness after more than 4 days of matrix deposition was apparently related to the entrapment of serum α_2 -macroglobulin in the cell layer matrix during culture in serum. Indeed, when the amount of α_2 -macroglobulin in different cell layers was assessed by Western blot (results not shown), the aggrecanase response was found to be good only in those cultures with a very low α_2 -macroglobulin content. Moreover, exogenous bovine α_2 -macroglobulin (at about 0.5 unit/ml) was a potent inhibitor of the IL-1-induced response. Interference from the small amount of α_2 -macroglobulin in the layers at 4 days could, however, be readily eliminated by thorough washing and desorption of the cell layer before cytokine addition (see the Experimental section).

When the cell layers were washed with medium containing ascorbic acid (50 $\mu\text{g}/\text{ml}$), it was repeatedly observed that the inclusion of ascorbate markedly reduced the subsequent response of the cells to IL-1 β . However, when ascorbate was omitted from the wash phase, but added at 50 $\mu\text{g}/\text{ml}$ with IL-1 β , it did not markedly affect the aggrecanase response. Therefore the inhibitory effect of ascorbate does not appear to be due to its capacity to act as an intracellular antioxidant. Also, when insulin was included with IL-1 β , the aggrecanase response was inhibited by about 25 % at 10 ng/ml insulin and by 100 % at 100 ng/ml. This insulin effect is consistent with the finding that the IL-1 α -induced degradation of aggrecan in cartilage explants can be inhibited by insulin-like growth factor-1 [20].

Effect of glutamine supply on the aggrecanase response in rat cells

The extent of the aggrecanase response was also influenced by the effective glutamine concentration of the catabolic medium; thus, because of chemical decomposition, the actual concentration in stored medium can be markedly lower than the starting concentration [21]. In a series of experiments with the mixed population of rat chondrosarcoma cells (LTC), we found that variability in the extent of the aggrecanase response could be attributed to alterations in the glutamine concentration of the medium. Thus the response was poor if glutamine-containing medium was prepared and stored for periods up to 6 weeks before use. On the other hand, the response was markedly reduced in freshly prepared glutamine-containing medium if 2 mM glutamine was added daily during the period of treatment with IL-1 β .

To examine this in more detail, clone 11 cells were plated in serum-rich medium and allowed to proliferate and deposit matrix for 4 days. After this time (day 0 of the catabolic experiment), the capacity of cells to degrade aggrecan over the next 5 days was examined in catabolic medium supplemented with glutamine concentrations in the range 0.05–2.5 mM. In control cultures without IL-1 β , there was no evidence of degradation and only

Figure 3 Effect of glutamine supply on the aggrecanase response to IL-1 β in rat cells

Cultures were treated with IL-1 β at the total glutamine concentrations shown. After 5 days the total product was digested with chondroitinase and portions were analysed by Western blotting with the anti-G1 antiserum (anti-ATEGQV). The graph shows the relationship between glutamine concentration and the content per culture (μg of GAG equivalents) of total G1 and of G1 present in the three aggrecan species (350 kDa, 220 kDa and 60 kDa). The Western blot has been compressed for presentation.

the 350 kDa species was present at all glutamine concentrations and all times. However, in the presence of IL-1 β , the extent of aggrecan degradation as determined by Western analysis was found to be markedly dependent on glutamine supply (see Figure 3A). At low concentrations the major species present after IL-1 treatment was the 350 kDa substrate, whereas at 1.5 mM and 2.5 mM glutamine this substrate had been totally degraded and the products of 220 kDa and 60 kDa were present. The data from the Western blots were next converted (using the standard curve; Figure 2) into μg of GAG equivalents for the 350 kDa, 220 kDa and 60 kDa products, and the results are plotted in Figure 3(B) below the corresponding Western blot. In keeping with the known structures of these species, generation of the 220 kDa and 60 kDa products was always associated with loss of the 350 kDa substrate. The total amount of immunoreactive aggrecan in the system (Total G1; Figure 3) was also enhanced by glutamine in this range, with a maximum increase of about 1.5-fold at 1.5 mM glutamine relative to that at 0.05 mM glutamine.

Inhibition by glutamine of the aggrecanase response in rat cells

To investigate further the role of glutamine in the aggrecanase response, we next examined the effect of further glutamine addition above the catabolic optimum of 1.5 mM. This additional glutamine caused a dose-dependent inhibition of aggrecan degradation, as demonstrated by Western analysis with anti-ATEGQV (Figure 4A). As expected, the decrease in abundance of the 220 kDa and 60 kDa species was accompanied by an increase in the 350 kDa substrate. The amounts of GAG equivalents (from the standard curve; Figure 2) of the 220 kDa and 60 kDa products in each lane were next used to calculate the percentage

Figure 4 Inhibitory effect of glutamine on the aggrecanase response in rat cells

Cultures were treated with IL-1 β at the glutamine concentrations shown. After 5 days the total product was digested with chondroitinase and portions were analysed by Western blotting with the anti-G1 antiserum (anti-ATEGQV). The gels (A) were used to determine the percentage inhibition caused by glutamine addition in the production of the 220 kDa and 60 kDa aggrecanase products (B). Also shown are the inhibition curves for these species determined with anti-TFKEEE (220 kDa) and anti-NITEGE (60 kDa) antibodies. The gel image has been compressed for presentation.

inhibition of degradation (where the amount of each product in the sample at zero glutamine addition was taken to indicate 100% degradation for that product), and these values were plotted below the appropriate lane of the Western blot (Figure 4B). Marked inhibition of the generation of both products was obtained with 2.5 mM glutamine added (4 mM total glutamine). It was notable that the percentage inhibition at each concentration was much greater for the 60 kDa product than for the 220 kDa product, and this was confirmed by analysis of the same samples with antisera specific for the C-terminal sequences of these two species. Thus when the 220 kDa and 60 kDa species were assayed with the antisera anti-TFKEEE and anti-NITEGE respectively, the percentage inhibition data (Figure 4B, open symbols) agreed very closely with the data obtained with the anti-ATEGQV antiserum (Figure 4B, closed symbols).

Inhibition by glucosamine of the aggrecanase response in rat cells

The metabolic fate of glutamine in chondrocytes has not been fully established [22]; however, it is clear that a significant proportion is used in the conversion of fructose 6-phosphate into glucosamine 6-phosphate. Glucosamine 6-phosphate is in turn required for the biosynthesis of UDP-*N*-acetylglucosamine (and UDP-*N*-acetylgalactosamine), which are the essential hexosamine donors in the biosynthesis of CS (on aggrecan) and hyaluronan. Therefore we investigated whether the inhibition of aggrecanase by glutamine (Figure 4) might operate through its metabolism to glucosamine 6-phosphate. We first examined whether the aggrecanase-mediated response to IL-1 β was affected by exogenous glucosamine. These experiments were performed in 5 mM glucose, since the medium glucose concentration used in

Figure 5 Inhibitory effect of glucosamine on the aggrecanase response in rat cells

Cultures (all at 1.5 mM glutamine and 5 mM glucose) were treated with IL-1 β at the glucosamine concentrations shown. After 5 days the total culture was digested with chondroitinase and portions were analysed by Western blotting (A) with the anti-G1 antiserum (anti-ATEGQV). Panel (B) shows the percentage inhibition caused by glucosamine compared with control production (at zero glucosamine) of the 220 kDa and 60 kDa aggrecanase products. The Western blot image has been compressed for presentation.

the glutamine experiments (25 mM) was considered too high to allow efficient glucosamine uptake by the glucose transporter system [23].

Glucosamine was indeed found to have a marked dose-dependent inhibitory effect on the aggrecanase response (Figure 5A); the degree of inhibition is shown in the corresponding quantitative analyses directly below each Western blot (Figure 5B). Glucosamine at 5 mM produced a 50% inhibition of the formation of the 60 kDa product, and this product was undetectable at 15 mM glucosamine. As with glutamine, the inhibitory effect of glucosamine was much more marked for the 60 kDa product than for the 220 kDa product. These inhibition curves for glucosamine were essentially unchanged when the experiment was repeated at 10 mM glucose; however, in the range 15–25 mM glucose, the degree of inhibition was much reduced, consistent with glucose and glucosamine competing for the same uptake process.

Mechanism of inhibition of the aggrecanase response by glutamine and glucosamine in rat cells

To investigate the possible mechanism of action of glutamine and glucosamine, we first examined the effect of these compounds on general metabolic activity, as assessed by protein synthesis ($[^3\text{H}]$ proline incorporation) and GAG synthesis ($[^{35}\text{S}]$ sulphate incorporation) over the 5-day culture period. Variation in the glutamine concentration (Figure 6, top panel) had a marked effect on biosynthesis in both control and IL-1 β -treated cultures. Glutamine enhanced general protein synthesis, with a 2–3-fold increase at 2.5 mM relative to 0.05 mM glutamine, but little effect at higher concentrations. This enhancement was seen in both controls and IL-1 β -treated cultures, although there was a consistent inhibition by IL-1 β relative to controls of about 25%.

Figure 6 Effects of glutamine and glucosamine on biosynthetic parameters of control and IL-1 β -treated cultures of rat chondrocytes

The top panel shows the effect of glutamine on the synthesis of protein (^3H) and sulphated GAG (^{35}S) in control and IL-1 β -treated cultures. The middle panel shows the effect of glucosamine on the same parameters. The bottom panel shows the effects of glutamine and glucosamine on lactate production ($\mu\text{g/culture}$) in control and IL-1 β -treated cultures. See the text for details.

Glutamine over a concentration range of 0.05–15 mM also enhanced GAG synthesis in both control and IL-1 β -treated cultures, and again there was a consistent inhibition by IL-1 β relative to controls of about 50%. These results (Figure 6, top panel) show that the profound inhibition of aggrecanase by glutamine addition (Figure 4) cannot be explained by a loss of cell viability or a general metabolic inhibition, since the synthesis of protein and GAG was maintained at a high rate in cultures with total glutamine concentrations up to 15 mM.

Glucosamine (1.5–15 mM) had no marked effect on protein or GAG synthesis when added to either control or IL-1 β -treated

cultures at 1.5 mM glutamine (Figure 6, middle panel). This shows that the inhibition of aggrecanase by glucosamine (Figure 5) also cannot be due to a general metabolic inhibition of the cells. This conclusion was supported by analysis of the effect of glutamine and glucosamine on the total lactate produced in these cultures due to anaerobic glycolysis. (Figure 6, bottom panel). Increasing the glutamine concentration from 0.05 to 15 mM had no marked effect on lactate production in control cultures, and actually increased its production in IL-1 β -treated cultures; addition of glucosamine (1.5–15 mM) had no marked effect on lactate production in either control or IL-1 β -treated cultures.

These biosynthetic studies also showed that the inhibitory effects of glutamine and glucosamine are apparently not due to these compounds blocking the IL-1 β signalling pathway; thus the inhibitory effects of IL-1 β on both protein and GAG synthesis were not affected by either high glutamine (Figure 6, top panel) or high glucosamine (Figure 6, middle panel). In addition, the stimulatory effects of IL-1 β on lactate production were not affected by high concentrations of either compound (Figure 6, bottom panel). The DNA content of these cultures (results not shown) was also not markedly affected by the inclusion of glutamine or glucosamine at concentrations that markedly inhibited the aggrecanase response to IL-1 β .

To investigate further the possibility that glutamine and glucosamine were affecting the generation of aggrecanase products by interference with IL-1 β signalling, we repeated the experiments with 10 μM RA as the catabolic stimulator. In these experiments the amount of 60 kDa product was greatest at about 1.5 mM glutamine, and its production was inhibited by glutamine loading. The percentage inhibition was 25% (at 5 mM glutamine), 42% (at 10 mM glutamine) and 56% (at 15 mM glutamine). Further, glucosamine also produced a concentration-dependent inhibition of RA-stimulated catabolism when added to cultures with 5 mM glucose and 1.5 mM glutamine. The amount of 60 kDa product was similar at 0.5, 1.5, 2.5 and 5 mM glucosamine, but was inhibited by 85% at 10 mM glucosamine and by 100% at 15 mM glucosamine.

Effects of glutamine and glucosamine on the aggrecanase response in bovine cartilage explants

To determine whether the results with rat chondrosarcoma cells are generally applicable to mammalian chondrocytes, we next examined the effect of glutamine on the response of bovine explants to IL-1 α and also the effect of glucosamine on the responses to both IL-1 α and RA in bovine cartilage explants. In this system [14] the response was measured both as the percentage release of GAG from the tissue into the medium and also as the formation of the aggrecanase-generated G1-NITEGE doublet in the total culture (medium plus tissue). The inhibition data obtained by these two methods agreed very closely (Figure 7), confirming that aggrecanase cleavage of the Glu-373–Ala-374 bond is primarily responsible for GAG release in this system. Most interestingly, glutamine did not affect the response to IL-1 α at any concentration between 0 and 15 mM. Thus, in clear contrast with the rat chondrosarcoma cell system (Figures 3 and 4), glutamine was not required for, nor was it inhibitory to, the aggrecanase response in bovine cartilage explants.

However, much as was seen with the rat cells, glucosamine produced a dose-dependent inhibition of the aggrecanase response in bovine cartilage explants at 2.5 mM glutamine, and this occurred when the tissue was treated with either IL-1 α or RA (Figure 7). A 50% inhibition was typically seen at about 3 mM glucosamine, as determined by G1-NITEGE formation (Figure 7A) or GAG release (Figure 7B). Moreover, a very similar

Figure 7 Effects of glutamine and glucosamine on the aggrecanase response to IL-1 α and RA in bovine explants

Cultures (all at 5 mM glucose) were treated with IL-1 α (1 ng/ml) or RA (10 μ M) at the glutamine or glucosamine concentrations shown. Glucosamine-containing cultures were all at 2.5 mM glutamine. The medium was changed every 2–3 days, and after 15 days the products (see the Experimental section) were analysed by Western blotting with the anti-NITEGE antiserum. Panel (A) shows typical blots from IL-1 α -treated cultures treated with glucosamine. Panel (B) shows the inhibition by glucosamine of the aggrecanase response to IL-1 α when measured as G1-NITEGE formation (■) or as GAG release (□), and the inhibition by glucosamine of the aggrecanase response to RA when measured as G1-NITEGE formation (▲) or as GAG release (△). Also shown is the effect of glutamine on the aggrecanase response to IL-1 α when measured as G1-NITEGE formation (●) or as GAG release (○). In each case the percentage inhibition was calculated from the values obtained in the same experiment in the absence of cytokine (negative control) and with cytokine without inhibitor (positive control). Typical values for negative controls were 20% GAG release and an estimated 3% of total aggrecan G1 as G1-NITEGE. Typical values for positive controls were 78% GAG release and an estimated 70% of total aggrecan G1 as G1-NITEGE. The data shown are from a single experiment which was repeated twice with very similar results.

inhibition curve was also seen when explants were treated with IL-1 α at either 0.5 mM or 1.5 mM glutamine (results not shown).

DISCUSSION

The molecular details of aggrecan degradation in the extracellular matrix have been studied widely in the past few years. Particularly important has been the development of antibodies for specific product characterization, and the description of an apparently novel proteinase, aggrecanase, which plays a central role in this process in cartilage matrix. The cell system described in the present work provides an opportunity to examine the aggrecanase process in a system that contains a single, apparently homogeneous, full-length aggrecan core protein as the starting material. Such experiments are difficult to perform in cartilage explants due to the presence in fresh tissue of C-terminally truncated, partially degraded, forms of aggrecan. In addition, the clonal cell line (clone 11) used here appears to be a good model for cartilage aggrecan degradation, since clone 11 cells have now been found to degrade aggrecan core in response to a range of compounds that have previously been shown to promote aggrecan catabolism *in vivo* and/or in explant cultures [3–10].

Interestingly, the catabolic pathways activated in clone 11 cells by all four agents (TNF α , oncostatin M, RA and IL-1 β) appear to be identical in terms of the cleavage products generated from the aggrecan substrate. Only two stable G1-bearing products appear in all cases (see Figure 1), and these represent the aggrecanase-mediated cleavages at Glu-1459 and Glu-373 [19].

On the other hand, this analysis clearly does not describe the fate of all aggrecan fragments generated, since the G3-bearing species released as a result of the cleavages described were not detected with this antiserum. However, analysis of these samples with an anti-G3 antiserum (results not shown) supports the interpretation made above, and shows that the CS-bearing region is cleaved in at least four sites, consistent with the pattern of aggrecanase-mediated cleavage that has been identified in the tumour tissue itself [19].

A significant concern regarding the interpretation of many previous catabolic studies using explant systems has been the limited amount of data on the structure of the products and identification of the degradative agent(s) involved. Since the release of aggrecan or aggrecan fragments from cartilage explants can be promoted by any proteinase, or by reactive oxygen species [24] that can cleave the aggrecan core protein or the hyaluronan network, it has been difficult to make firm conclusions regarding the nature of the activity which is under study. With this in mind, we have now described an immunological assay (Figure 2) which can directly quantify the specific products of aggrecanase action at both the Glu-1459 and Glu-373 bonds. This assay system can be readily applied to the products of both cell culture and explant systems [10,14].

Glutamine supply to rat chondrosarcoma cells was found to markedly influence the aggrecanase response to IL-1 β . Promotion of the response by increasing the glutamine concentration from 0.05 to 1.5 mM (Figure 3) may be related to the stimulatory effect of glutamine on general biosynthetic activity in this concentration range (Figure 6, top panel). In particular, the marked glutamine-dependent increase in protein synthesis might be a reflection of the production of proteins that are essential for aggrecanase synthesis, translocation or activation. Surprisingly, an increase in the glutamine concentration above 2.5 mM markedly inhibited the aggrecanase response to IL-1 β . Since glutamine can be readily metabolized to glutamate [22] and presumably to glutathione in these cells, we therefore tested the effects of these two compounds on the aggrecanase response. However, this did not provide an explanation, since glutamate had no effect on the catabolic response when added up to 15 mM, and the concentration of glutathione in cell extracts was not markedly influenced by glutamine supply (results not shown). Similarly, the effect did not appear to be due to a change in nitric oxide metabolism, since the level of nitrite in these cultures was also not markedly altered by glutamine supply (results not shown).

On the other hand, we obtained evidence that the effect of glutamine might be due to a change in the hexosamine metabolism of the cells; the inclusion of glucosamine in catabolic cultures also resulted in a profound concentration-dependent inhibition of the aggrecanase response (Figure 5). Interestingly, the capacity of glucosamine to inhibit the response was itself inhibited by increasing the glucose concentration in the medium; this supports the view that the effect of glucosamine is dependent on efficient uptake by the cellular glucose transport system, a process which would be competitively inhibited by high concentrations of glucose. As expected, the inhibitory effect of glutamine, which is transported by the amino acid uptake pathway, was not markedly influenced by the glucose concentration in the medium. Further evidence that the two compounds are operating via a common mechanism comes from the observation that both produce a more profound inhibition of production of the 60 kDa product relative to the 220 kDa product. This finding also suggests that the catabolic mechanism of aggrecanase is a sequential proteolytic process, with the 220 kDa product representing an intermediate in the pathway between the 350 kDa substrate and the 60 kDa end-product.

Biosynthetic studies (Figure 6) suggested that neither compound produces its effect by a general inhibition of cellular activity or by blocking the signalling pathway of IL-1 β . Further, to examine the extent to which inhibition of the aggrecanase response by glutamine and glucosamine is a property of normal epiphyseal chondrocytes, we tested the effects of these compounds in a bovine cartilage explant system (Figure 7). In these cultures, which model the *in vivo* situation for joint cartilages more closely, we found that glutamine was ineffective as an inhibitor, whereas glucosamine exhibited similar inhibitory activity as found with the chondrosarcoma cells. This finding clearly supports the argument that glucosamine (or its metabolites) is the effective agent and that the glutamine-induced inhibition in chondrosarcoma cells is due to its rapid conversion into glucosamine, a process which is probably accelerated in these cells relative to other chondrocytes [22]. Moreover, we found that the inhibitory effect of glucosamine in bovine cells was seen with either IL-1 α or RA as the catabolic stimulant, a finding which suggests strongly that the cellular site of action of glucosamine (or its metabolites) is downstream of nuclear signalling events. It is very unlikely that glucosamine could be acting as an inhibitor of both the IL-1 β and RA signalling pathways, which operate by very divergent mechanisms. On the other hand, analysis of the effects of glutamine and glucosamine on the activity of a range of pre-nuclear signalling events induced by IL-1 and RA is needed to eliminate this possibility completely.

If, as seems likely, the inhibition of the aggrecanase response is a consequence of metabolic changes that accompany a marked increase in the intracellular glucosamine concentration, then this may be explained in a number of ways. Firstly, an unrestricted supply of glucosamine might result in a rapid increase in the intracellular pools of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine, the limiting precursors for hyaluronan and aggrecan CS synthesis. While IL-1 β has been shown in the present work to markedly inhibit aggrecan CS synthesis, it may have a stimulatory effect on hyaluronan synthesis [25]; it is therefore possible that the aggrecan/hyaluronan matrix synthesized and deposited in the pericellular matrix during the period of IL-1 β or RA treatment is markedly enriched in hyaluronan relative to aggrecan. This hyaluronan-rich matrix might not represent a suitable substrate for aggrecanase action.

Alternatively, an unrestricted supply of glucosamine might lead to the depletion of cellular energy stores. This might result from a rapid usage of ATP for glucosamine 6-phosphate production [26,27], a direct inhibitory effect of glucosamine on glucose transport [23] or a broad down-regulation of the glucose transport system [28]. Such an energy depletion may then inhibit a subset of cellular activities that are specifically required for the aggrecanase response. Indeed, if a specific energy distribution effect is operating here, such as that seen for hepatocytes in hypoxia [31], it might not have been detected in our general biosynthetic studies of protein and GAG synthesis (Figure 6).

Finally, in our most recent experiments (J. D. Sandy, D. Gamett, V. Thompson and C. Verscharen, unpublished work) with both the rat chondrosarcoma and bovine explant systems, we have shown that mannosamine is also a very effective inhibitor of the aggrecanase response. Since both glucosamine and mannosamine are effective inhibitors of glycosylphosphatidyl-

inositol anchor synthesis [32], these results open up the interesting possibility that the aggrecanase catabolic unit includes a glycosylphosphatidylinositol-linked component.

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REFERENCES

- Luyten, F. P., Hascall, V. C., Nissley, S. P., Morales, T. I. and Reddi, A. H. (1988) Arch. Biochem. Biophys. **267**, 416–425
- Morales, T. I. and Roberts, A. B. (1988) J. Biol. Chem. **263**, 12828–12831
- Sandy, J. D., Neame, P. J., Boynton, R. E. and Flannery, C. R. (1991) J. Biol. Chem. **266**, 8683–8685
- Malfait, A. M., Verbruggen, G., Veys, E. M., Lambert, J., De Ridder, L. and Cornelissen, M. (1994) J. Rheumatol. **21**, 314–320
- Hui, W., Bell, M. and Carroll, G. (1996) Cytokine **8**, 495–500
- Homandberg, G. A., Hui, F. and Wen, C. (1996) Biochim. Biophys. Acta **1317**, 134–142
- Plaas, A. H. K. and Sandy, J. D. (1993) Matrix **13**, 135–147
- Sandy, J. D., Flannery, C. R., Neame, P. J. and Lohmander, S. F. (1992) J. Clin. Invest. **89**, 1512–1516
- Lohmander, S. L., Neame, P. J. and Sandy, J. D. (1993) Arthritis Rheum. **36**, 1214–1222
- Lark, M. W., Gordy, J. T., Weidner, J. R., Ayala, J., Kimura, J. H., Williams, H. R., Mumford, R. A., Flannery, C. R., Carlson, S. S., Iwata, M. and Sandy, J. D. (1995) J. Biol. Chem. **270**, 2550–2556
- Woessner, J. F. (1995) in Osteoarthritic Disorders (Kuettner, K. and Goldberg, V., eds.), pp. 281–290, American Academy of Orthopedic Surgeons, Rosemont, IL
- Okada, Y., Shinmei, M., Tanaka, O., Naka, K., Kimura, A., Nakanishi, I., Bayliss, M. T., Iwata, K. and Nagase, H. (1992) Lab. Invest. **66**, 680–690
- Flannery, C. R., Lark, M. W. and Sandy, J. D. (1992) J. Biol. Chem. **267**, 1008–1014
- Bonassar, L. J., Sandy, J. D., Lark, M. W., Plaas, A. H. K., Frank, E. H. and Grodzinsky, A. J. (1997) Arch. Biochem. Biophys. **344**, 404–412
- Mort, J. S., Dodge, G. R., Roughley, P. J., Liu, J., Finch, S. J., Dipsquale, G. and Poole, A. R. (1993) Matrix **13**, 95–102
- Buttle, D. J., Handley, C. J., Ilic, M. Z., Saklatvala, J., Mrata, M. and Barrett, A. J. (1993) Arthritis Rheum. **36**, 1709–1717
- Loulakis, P., Shrikhande, A., Davis, G. and Maniglia, C. A. (1992) Biochem. J. **284**, 589–593
- Ilic, M. Z., Handley, C. J., Robinson, H. C. and Mok, M. T. (1992) Arch. Biochem. Biophys. **294**, 115–122
- Sandy, J. D., Plaas, A. H. K. and Koob, T. J. (1995) Acta Orthop. Scand. **66**, 26–32
- Kim, Y.-J., Sah, R. L., Doong, J. and Grodzinsky, A. (1988) Anal. Biochem. **174**, 168–176
- Rayan, V. and Hardingham, T. E. (1994) Matrix Biol. **14**, 263–271
- Ozturk, S. S. and Palsson, B. O. (1990) Biotechnol. Prog. **6**, 121–128
- Spencer, C. A., Palmer, N. and Mason, R. M. (1990) Biochem. J. **265**, 911–914
- Plagemann, P. G., Wohlhueter, R. M., Graff, J., Erbe, J. and Wilkie, P. (1981) J. Biol. Chem. **256**, 2835–2842
- Bates, E. J., Harper, G. S., Lowther, D. A. and Preston, B. N. (1984) Biochem. Int. **8**, 229–235
- Yung, S., Coles, G. A. and Davies, M. (1996) Kidney Int. **50**, 1337–1343
- Yagita, M., Nakajima, M. and Saksela, E. (1989) Cell. Immunol. **122**, 83–95
- Tesorieri, G., Vento, R. and Calvaruso, G. (1975) Biochim. Biophys. Acta **385**, 58–67
- Marshall, S., Bacote, V. and Traxinger, R. (1991) J. Biol. Chem. **266**, 4706–4712
- Reference deleted
- Reference deleted
- Hayashi, K., Ochiai, T., Ishinoda, Y., Okamoto, T., Maruyama, T., Tsuda, K. and Tsubouchi, H. (1997) J. Gastroenterol. Hepatol. **12**, 249–256
- Yuan-Tsang, P., Kamitani, T., Bhuvanewaran, C., Hallaq, Y., Warren, C., Yeh, E. T. H. and Elbein, A. D. (1992) J. Biol. Chem. **267**, 21250–21255