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IL-1β Induces the Degradation of Equine Articular Cartilage by a Mechanism That Is Not Mediated by Nitric Oxide

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Proteoglycan degradation was induced in young equine articular cartilage explants cultured for eight days in the presence of 50ng/ml recombinant human interleukin-1β. Degradation was initiated after 6 hours of exposure to the cytokine. This was accompanied by an induction of nitric oxide synthesis and a decrease in the incorporation of [^{35}S]sulphate into the glycosaminoglycan chains of proteoglycans. The addition of 1mM N-iminoethyl-L-ornithine (an inhibitor of nitric oxide synthase) to the explant cultures in the presence of rhIL-1β suppressed the synthesis of NO and restored proteoglycan synthesis to control levels. However, treatment of explants with LNIO did not overcome proteoglycan degradation. These results indicate that although IL1β regulates both proteoglycan synthesis and degradation in equine cartilage explants, only the inhibition of proteoglycan synthesis is mediated by nitric oxide.

Articular cartilage is a major component of the articular joint and the mechanical properties of the tissue depend on the composition and structure of the macromolecules in the extracellular matrix. This is composed predominantly of proteoglycans, collagens and other non-collagenous proteins. The collagens form an interconnecting network from which the cartilage derives its tensile strength. The sulphated glycosaminoglycan chains on the proteoglycans (mainly aggrecan) produce an anionic environment which is highly hydrated and provides resistance to the compressive forces acting on the tissue. The progressive loss of matrix macromolecules is a characteristic feature of many joint diseases and is brought about by inhibition of their synthesis by the chondrocytes and by an increased rate of enzymatic degradation of the existing matrix architecture.

Growth factors and cytokines are important regulators of many aspects of chondrocyte metabolism. The pro-inflammatory cytokine, interleukin-1 (IL-1), is produced by a variety of cells and is synthesised within arthritic joints by synoviocytes and chondrocytes, where it is thought to have a role in the loss of cartilage matrix. It has been shown to inhibit proteoglycan synthesis and promote their degradation in human (1, 2), bovine (3), porcine (4), murine (5) and equine (6, 7) cartilage. The mechanism(s) by which IL-1 inhibits the synthesis of matrix components and accelerates proteinase-mediated matrix degradation are still not fully understood, but one potential candidate for the signalling mechanism which induces these effects is the free radical gas, nitric oxide (NO) (reviewed by Farrel and Blake) (8). This highly reactive agent is enzymically synthesised by constitutive and induced forms of the enzyme, nitric oxide synthase (NOS). Chondrocytes contain the inducible form of this enzyme (9) which is capable of synthesising large amounts of NO in response to IL-1 (10, 11, 12, 13). The suppression of proteoglycan synthesis is reported to occur via more than one mechanism, but at least one of these was shown to depend on the synthesis of NO (14, 15, 16). IL-1 also initiates the syntheses of matrix metalloproteinases by chondrocytes and synovial cells (17, 18) and these can be activated by NO in articular cartilage (19). In the present study, we have investigated to what extent the age of the chondrocyte influences the effect of NO on proteoglycan metabolism.

MATERIALS AND METHODS

Materials. Dulbecco's Modification of Eagles Medium (DMEM) containing glutamax and 44mM $NaHCO_3$, penicillin/streptomycin, gentamycin and non-essential amino acids were from LTI, Paisley, Scotland. Foetal calf serum (FCS) was obtained from Labtech, Uckfield, Sussex and Falcon tissue culture plates were from Marathon,

[1] Corresponding author. Fax: 01707 660671. Abbreviations used: NO, nitric oxide; IL-1, interleukin-1; LNIO, N-iminoethyl-L-ornithine.

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Park Royal, London, UK. Sephadex G25 (PD10 columns) was purchased from Pharmacia, St Albans, Herts, UK. Hybond N+ and $Na_2{}^{35}SO_4$ were purchased from Amersham International, Amersham, Bucks, UK. Recombinant human interleukin-1β (rhIL-1β) was a gift from Roussel, UCLAF, Swindon, Wilts, UK. L-N-(1-iminoethyl) ornithine (LNIO) was obtained from Calbiochem-Novabiochem (UK) Ltd, Nottingham, UK. All other chemicals were of analytical grade.

Cartilage sampling and culture conditions. Articular cartilage was obtained at post mortem from weight bearing surfaces of the metacarpophalangeal joints of thoroughbred horses within 6h of death. All specimens were macroscopically normal and free from degenerative lesions. Full thickness pieces were maintained for 18h in DMEM supplemented with 100U/ml penicillin, 100mg/ml streptomycin, 10ml/l non-essential amino acids and 10% FCS in a humidified atmosphere of 5% CO_2 in air at 37°C. Cartilage pieces were diced into 1mm explants and washed three times in DMEM. Explants were distributed to give approximately 100mgs tissue/well and equilibrated in 1ml DMEM without FCS for 48h. LNIO (1mM), rhIL-1β (50ng/ml) and LNIO (1mM) + rhIL-1β (50ng/ml) were added to triplicate cultures for various times over a 48h period. At the end of each culture period, proteinase inhibitors (20) were added to media which was then stored at −20°C prior to analysis. Explants were cryosectioned at 20μm and extracted with 10 vols 4M GnHCl, 50mM sodium acetate, pH 6.8 containing proteinase inhibitors for 18h at 4°C. Extracts were subsequently dialysed into 50mM sodium acetate, pH 6.8 containing proteinase inhibitors prior to analysis.

Rates of proteoglycan synthesis. $Na_2{}^{35}SO_4$ (20μCi/ml final concentration) was added to appropriate wells in triplicate for a known time period (up to 6h) prior to the end of treatment. The spent media were removed and the explants were digested in 0.5mls of 0.1M sodium acetate, 2.4mM EDTA, 0.012M cysteine, pH 5.8, containing 0.7U papain for 18h at 60°C. For the quantitative evaluation of ^{35}S-labelled proteoglycans, aliquots of digests and media were eluted on Sephadex G25 in PD10 columns under dissociative conditions (21). The radioactivity of newly synthesised proteoglycans was measured by scintillation counting.

Proteoglycan release. Proteoglycan release was assessed in triplicate cultures by measurements of sulphated glycosaminoglycans (GAG) in papain digested explants and media, using the dimethylmethylene blue dye-binding assay (22). Results were expressed as a cumulative release of GAG for each time point relative to the total GAG present, in excess of control values.

Nitrite release. Nitrite, (NO_2), a stable end-product of nitric oxide metaboism, was measured in the media of triplicate cultures using the spectrophotometric method based on the Greiss reaction (23). The absorbance was measured at 570nm with a Bio-Rad 2550EIA plate reader.

Gel electrophoresis. Volumes of extracts and media containing 9μg GAG were drop dialysed against distilled water, lyophilised and reconstituted in 10μl 10mM Tris base, 0.25mM Na_2SO_4, pH 6.8, containing 8M urea. Samples were allowed to dissociate overnight at 4°C prior to electrophoresis on large pore composite agarose/polyacrylamide gels, pre-equilibrated overnight in 10mM Tris, 0.25mM Na_2SO_4, 4M urea, pH 6.8 (24). Gels were stained in 0.2% toluidine blue (w/v) in 3% acetic acid and destained in several changes of 3% acetic acid.

Hydroxyproline determination. Aliquots of papain digested explants were acid hydrolysed overnight in 6M HCl at 110°C. The hydroxyproline content was measured using Chloramine T and Erlich's reagent (25). The absorbance was measured at 570nm with a plate reader.

Statistical analysis. Students t-test for unpaired data was used for statistical analysis and $p \leq 0.05$ was considered significant.

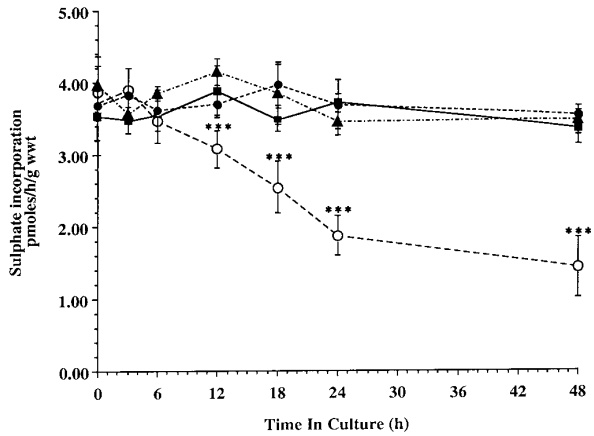

FIG. 1. Proteoglycan Synthesis in Explant Cultures of 4 Year Old Equine Articular Cartilage over 48 Hours. Explants were cultured in the presence of 50ng/ml rhIL-1β (– – – ○ – – –), 1mM LNIO (– – – ▲ – – –), 50ng/ml rhIL-1β + 1mM LNIO (– – – ● – – –) or no treatment as control (– – – ■ – – –). Values shown are means ± SEM (n=3). *** = $p \leq 0.001$ as compared to control values using an un-paired students t-test.

RESULTS

Incubation of equine cartilage explants with rhIL-1β caused an inhibition of proteoglycan synthesis by chondrocytes after a lag phase of approximately 6 hours (Fig. 1). The cytokine also stimulated a concomitant increase in the concentration of NO, measured as nitrite, in the same culture supernatants (Fig. 2). Both of these biosynthetic events were inhibited when L-NIO (1mM) was included with IL-1β in the incubation medium (Figs. 1 and 2). Cartilage explants incubated in the absence of IL-1β or in the presence of L-NIO alone maintained a constant rate of proteoglycan synthesis and did not generate much NO over the same incubation period. These findings were reproduced by equine cartilage explants regardless of the age of the animal (Table 1).

In contrast, the extent of proteoglycan degradation and release of glycosaminoglycans from IL-1β-stimulated explants did depend on the age of the specimen. Significant degradation and release of glycosaminoglycan was only observed when immature cartilage was treated with IL-1β and the cytokine had very little effect on proteoglycan catabolism by cartilage explants from mature animals (Fig. 3). Furthermore, when L-NIO was included with IL-1β in cultures of immature equine cartilage, it did not inhibit the release of glycosaminoglycans from the tissue (Fig. 4).

Composite agarose/polyacrylamide gel electrophoresis was used to fractionate proteoglycans extracted from the cartilage and also the proteoglycan degradation products that were released into the culture medium. Proteoglycans extracted from untreated explants migrated in the gel as two major toluidine blue staining

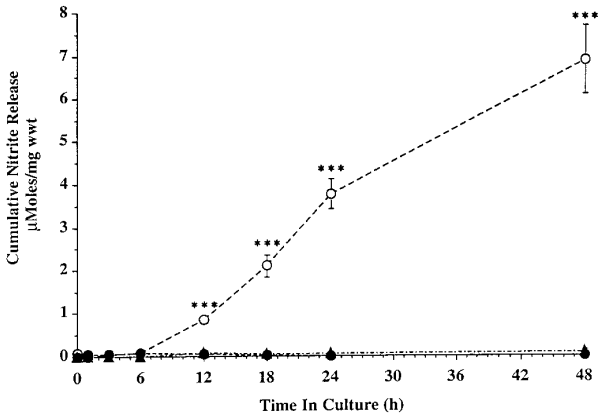

FIG. 2. The Cumulative Release of Nitrite into the Media of Explant Cultures of 4 Year Old Equine Articular Cartilage Over 48 Hours. Explants were cultured in the presence of 50ng/ml rhIL-1β (– – –○– – –), 1mM LNIO (– – –▲– – –), 50ng/ml IL-1β + 1mM LNIO (– – –●– – –) or no treatment as control. Values shown are means ± SEM (n=3). The corresponding control values have been subtracted from each time point. *** = $p \leqslant 0.001$ as compared to control values using an unpaired students t-test.

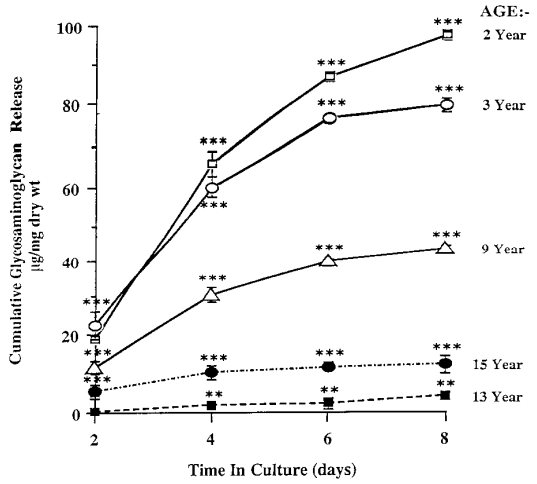

FIG. 3. The Cumulative Release of Glycosaminoglycan from an Age Range of Equine Articular Cartilage Explants Cultured for Eight Days in the Presence of 50ng/ml rhIL-1β. Values shown are means ± SEM (n=3) of GAG released. The corresponding control values have been subtracted from each tie point. ** = $p \leqslant 0.05$, *** = $p \leqslant 0.001$ as compared to control values using an un-paired students t-test.

bands and this pattern was unchanged after 48 hrs in culture (Fig. 5). However, the extracts of IL-1β treated cartilage contained additional, faster migrating, proteoglycan fragments (Fig. 5) which were also observed in the incubation medium as soon as 6 hrs after cytokine treatment (Fig. 6). Furthermore, this change in proteoglycan structure was not affected by the inclusion of L-NIO in the culture medium.

DISCUSSION

In a previous study of equine articular cartilage, Platt and Bayliss (6) demonstrated that rhIL-1β inhibits proteoglycan synthesis at all ages. In the study described here, IL-1β also induced nitric oxide production by equine chondrocytes, a finding that is consistent with the response of articular chondrocytes from other species. In marked contrast, the ability of iNOS inhibitors to abrogate the inhibitory effect that IL-1 has on proteoglycan synthesis, appears to be species dependent. For example, isolated human chondrocytes cul-

TABLE 1

The Effect of Animal Age on the Regulation of Proteoglycan Synthesis

Age (years)	Cytokine	GAG synthesis (mmoles/h/mg hydroxyproline)	% of control
2	Control	326	—
	IL-1	83	25
	IL-1 + L-NIO	339	104
9	Control	214	—
	IL-1	83	39
	IL-1 + L-NIO	197	92
15	Control	247	—
	IL-1	161	65
	IL-1 + L-NIO	247	100

Note. Explants were cultured in the presence of 10ng/ml IL-1β, 1mM L-NIO or no treatment (control) as indicated. Values shown are the means of 3 determinations.

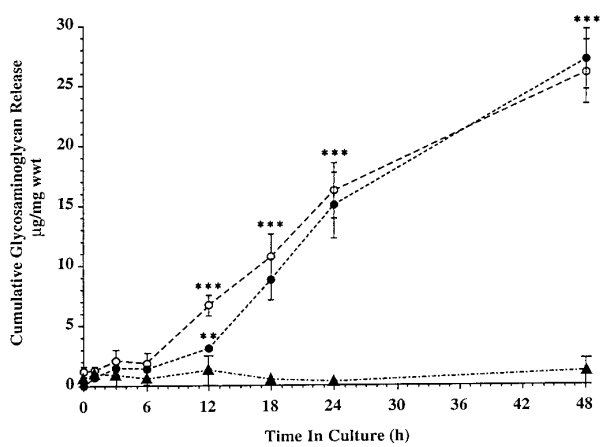

FIG. 4. The Cumulative Release of Glycosaminoglycan into the Media of Explant Cultures of 4 Year Old Equine Articular Cartilage over 48 Hours. Explants were cultured in the presence of 50ng/ml rhIL-1β (– – –○– – –), 1mM LNIO (– – –▲– – –), 50ng/ml rhIL-1β + 1mM LNIO (– – –●– – –) or no treatment as control. Values shown are means ± SEM (n=3) of GAG released. The corresponding control values have been subtracted from each time point. ** = $p \leqslant 0.05$, *** = $p \leqslant 0.001$ as compared to control values using an un-paired students t-test.

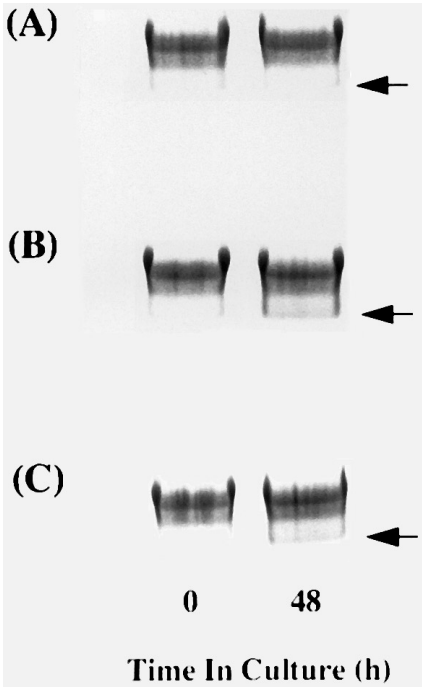

FIG. 5. The Visualisation of Proteoglycan Populations Present in Extracts of 4 Year Old Equine Articular Cartilage Explants Cultured for 48 Hours in the Presence of: (A) No Treatment, (B) 50ng/ml rhIL-1β, and (C) 50ng/ml rhIL-1β + 1mM LNIO by Composite Agarose Polyacrylamide Gel Electrophoresis. Aliquots of extracts at 0 and 48 hours of culture containing 10 mg radiolabelled GAG was dissociated in 4M urea and electrophoresed on composite agarose polyacrylamide gels. Aggrecan populations were visualised by toluidine blue staining. The arrow donates the positions of new, faster migrating proteoglycans.

tured in alginate showed only a limited response to the iNOS inhibitor N^G-monomethyl L-arginine (L-NMA) (14) and explants of bovine articular cartilage were completely non-responsive (26). However, a study which used lapine cartilage explants (15) showed that inhibition of iNOS by the same inhibitor completely restored proteoglycan synthesis. It was, therefore, unexpected to find that N-iminoethyl-L-ornithine (L-NIO) could completely overcome the inhibitory effect of IL-1, in every specimen examined regardless of its age. The reason for the discrepancy in the published studies is unclear, but apart from obvious species differences, the different culture conditions (isolated cells vs. intact cartilage) and the greater specificity and potency of L-NIO compared to L-NMA, are likely to be significant contributing factors.

Interleukin-1 is also a potent stimulator of cartilage degradation and it induces the catabolism of extracellular matrix components. In particular, the high molecular weight proteoglycan, aggrecan, is rapidly degraded and released from the tissue when explants are treated with the cytokine. Changes in the electrophoretic migration pattern of aggrecan released from immature equine cartilage are consistent with this observation. Furthermore, the inability of L-NIO to prevent IL-1 mediated proteoglycan loss, indicates that nitric oxide is not an intracellular mediator of this process. This was an unexpected finding given that the production of matrix metalloproteinases is blocked by iNOS inhibitors (19) and that in bovine cartilage explants, proteoglycan loss from the tissue is also inhibited (26). However, we were able to show in some experiments using lower concentrations of cytokine, that NO itself could partially inhibit proteoglycan loss (results not shown), suggesting that the actions of NO on cartilage catabolism are not straightforward and may depend on the local concentration of NO and the length of time that the chondrocytes are exposed to it; a finding supported by the recent work of Stefanovic-Racic et el (26). There are also zonal variations in the response of chondrocytes to IL-1 (27) which could influence the measured response in explant culture. The complexity of these interactions probably account for many of the variable responses of cartilage from different species that have been described in the literature.

The results of the present investigation demonstrate

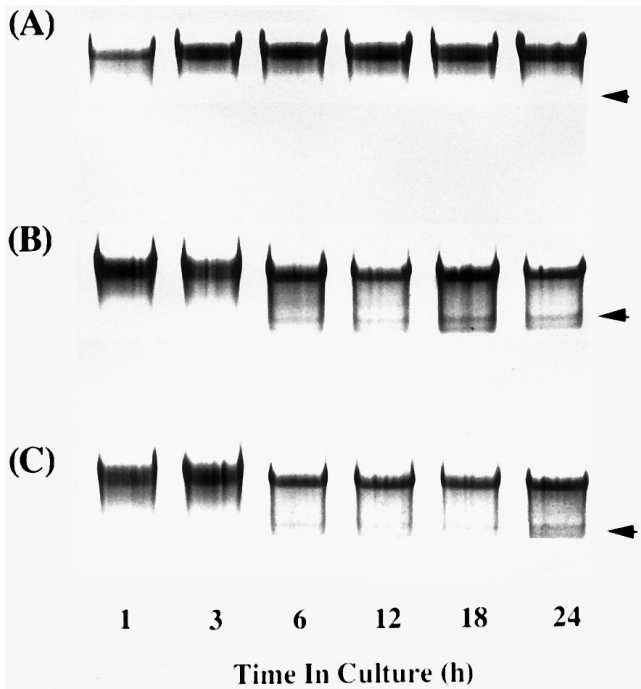

FIG. 6. The Visualisation of Proteoglycan Populations Released into the Media of 4 Year Old Equine Articular Cartilage Explants Cultured for 24 Hours in the Presence of (A) No Treatment, (B) 50ng/ml rhIL-1β and (C) 50ng/ml rhIL-1β + 1mM LNIO by Composite Agarose/Polyacrylamide Gel Electrophoresis. Aliquots of media from various time points over the culture period containing 10mg radiolabelled GAG were dissociated in 4M urea and electrophoresed on composite gels. Aggrecan populations were visualised by toluidine blue staining. The arrow donates the position of new, faster-migrating proteoglycans.

that NO is a major transducer of the anabolic signal activated by IL-1 in immature and mature chondrocytes and that this free radical, therefore, has a major role in the control of cartilage matrix homeostasis, but its role in cartilage catabolism is less obvious, especially in adult tissue. The mechanism(s) by which NO mediates the IL-1 signal in chondrocytes is still not clear, but is likely to involve the synthesis of a variety of factors including the IL-1 receptor antagonist (IL-1Ra) (28). Abnormalities in the regulation of these factors has been proposed as exacerbating events in the development of osteoarthritis (29). Understanding how NO regulates proteoglycan synthesis and degradation is an important step towards developing new therapeutic strategies to treat these degenerative diseases.

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