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Insulin-like growth factor 1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to cytokines

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A model system of explanted cartilage has been used *in vitro* to determine whether insulin-like growth factor 1 (IGF 1), which promotes matrix formation is effective in the presence of cytokines such as interleukin 1 (IL1) and tumour necrosis factor (TNF), which induce net matrix depletion. IGF 1 induced a dose-dependent 2.5-fold stimulation of proteoglycan synthesis, with a half-maximal dose of 25 ng/ml. A similar relative increase occurred in response to IGF 1 (10–100 ng/ml) in cartilage cultured also with IL1 or TNF (5–500 pM). There was no detectable qualitative change in the average molecular size or charge of the aggregating proteoglycan synthesized by explants exposed to IGF 1 alone or with IL1 or TNF. The increased production of prostaglandin E_2, which is initiated when IL1 or TNF bind to the chondrocytes, was the same in the presence or absence of IGF 1. The time taken for 50% of pre-labelled proteoglycan to be released from the explants ($t_{\frac{1}{2}}$) increased in the presence of IGF 1 (100 ng/ml) from 21 to 32 days in control cultures and from 8 to 26 days in cartilage cultured with IL1 (50 pM). It is concluded that IGF 1 enhances the synthesis of aggregating proteoglycan in cartilage exposed to cytokines and can directly decrease both the basal and the cytokine-stimulated degradation of proteoglycan in cartilage.

INTRODUCTION

The function of articular cartilage is to withstand high pressure, absorb shock and prevent the transmission of stress to the underlying bone. It is the hyaluronate–proteoglycan complexes within the matrix which allow the cartilage to undergo rapid reversible deformation (Kempson *et al.*, 1976). These aggregates are compressed into articular cartilage at a concentration of more than 60 mg/ml into one-fifth of the volume they would occupy in a more dilute solution. Their essential feature is the presence of the glycosaminoglycans as fixed negative charges which become hydrated to create an immense swelling pressure (Urban *et al.*, 1979; Maroudas & Bannon, 1981). The Type II collagen fibres, which have a high tensile strength, form a three-dimensional framework to restrain the proteoglycan gel and limit expansion of the tissue. Loss of cartilage matrix leads to impairment of joint function, pain and immobility, which are common features of arthritic disease.

Cultured cartilage explants can be grown in a defined medium, and synthesize matrix components characteristic of those found *in vivo* (Benya & Nimni, 1979; Hascall *et al.*, 1983; Tyler, 1985*b*). The chondrocytes in cultured cartilage have also been shown to regulate the turnover of proteoglycans to promote a net increase in response to depletion of the matrix (Sandy *et al.*, 1980), as occurs *in vivo* (Thomas, 1956; Pettipher *et al.*, 1986). This system therefore provides an attractive model for studying the mechanisms underlying alteration in extracellular-matrix accumulation associated with degenerative diseases.

Two classes of cytokine, interleukin 1 (IL1) and tumour necrosis factor (TNF), have been shown to cause a dramatic shift in the normally stable matrix equilibrium to induce a rapid net depletion of proteoglycan. They achieve this by stimulating the chondrocytes to increase their rate of proteoglycan degradation (Tyler, 1985*a*; Krakauer *et al.*, 1985; Saklatvala *et al.*, 1985; Pettipher *et al.*, 1986; Saklatvala, 1986) and selectively decrease proteoglycan synthesis (Tyler, 1985*b*; Saklatvala, 1986; Benton & Tyler, 1988). IL1 and TNF have been identified at sites of cartilage erosion in patients (Nouri *et al.*, 1984; di Giovine *et al.*, 1988), and are likely to be important mediators of cartilage destruction *in vivo*. In the presence of such cytokines, the ability of chondrocytes to maintain adequate levels of proteoglycan will determine whether the joint can continue to function efficiently. The ability of insulin-like growth factor 1 (IGF 1) to enhance matrix synthesis in normal cartilage is well established *in vivo* (Schoenle *et al.*, 1982) and *in vitro* (Guenther *et al.*, 1982; McQuillan *et al.*, 1986*b*). The purpose of this study was to determine whether IGF 1 could still promote proteoglycan accumulation in cartilage exposed to cytokines such as TNF and IL1.

METHODS

Materials

Iscove's Modified Eagle's Medium and supplements were from Gibco, Paisley, Scotland, U.K. Chondroitin sulphate and chondroitinase ABC were from Seikagaku Kogyo Co., Chuo-ku, Tokyo 103, Japan. 1,9-Dimethyl Methylene Blue was from Serva Feinbiochemica, Heidelberg, Germany. Sepharose CL-2B and Sephacryl S-200 were from Pharmacia, Uppsala, Sweden. Hyaluronate was generously given by Dr. E. A. Balazs (Biomatrix, Ridgefield, NJ, U.S.A). [^{35}S]Sulphate was from Amersham International (Amersham, Bucks., U.K.). All

Abbreviations used: IL1, interleukin 1; TNF, tumour necrosis factor; IGF 1, insulin-like growth factor 1.

other reagents were of analytical grade. Bovine fibroblast growth factor was from R + D Systems Inc., Minneapolis, MN, U.S.A. IGF 1 (human recombinant) was generously given by Ciba–Geigy, Basel, Switzerland. IL1α (human recombinant) was from Roche, Welwyn Garden City, Herts., U.K., and TNFα (human recombinant; Genzyme) was purchased from Koch–Light, Haverhill, Suffolk, U.K.

Tissue culture

Explants of articular cartilage were made by cutting a long thin strip (approx. 12 mm × 2 mm × 0.3 mm) from the condylar ridge of the metacarpophalangeal joints of freshly slaughtered pigs aged about 7 months. Care was taken to exclude the underlying marrow. The slices were cultured in Iscove's medium supplemented with bovine serum albumin (6 μg/ml), human transferrin (5 μg/ml) and streptomycin (100 units/ml) in a humidified atmosphere of CO_2/air (1:19) at 37 °C for 48 h. To estimate proteoglycan synthesis, six equivalent halves were cultured per ml with one medium change for 72 h in increasing amounts of IL1 (0–5000 pM; Roche) or TNF (0–500 pM; Genzyme) in the presence of IGF 1 (0–200 ng/ml; Ciba–Geigy). [^{35}S]Sulphate (10 μCi/ml) was added to each culture for the last 3 h. The radiolabelled explants were washed in ice-cold Iscove's medium, and analysed as described below.

To measure proteoglycan degradation, the slices were prelabelled by incubation for 6 h in fresh medium containing [^{35}S]sulphate (25–40 Ci/mg; Amersham) at 10 μCi/ml. The slices were washed well with at least four changes of warmed unlabelled medium over a period of 2 h, then cut in half. Six equivalent halves (each 7 mg wet wt.) were cultured per ml in the absence or presence of 0–500 pM-IL1 or -TNF (human recombinant, from Roche and Genzyme) with increasing concentrations of IGF 1 (human recombinant, from Ciba–Geigy) for up to 16 days. The used culture medium was changed every 2 days and stored separately at −20 °C for analysis. For the biochemical analysis of newly synthesized proteoglycans, 18 equivalent halves of cartilage were cultured with 3 ml of control medium alone, with IL1 (50 pM) or with TNF (500 pM) in the presence or absence of IGF 1 (100 ng/ml) for 72 h at 37 °C with one change of medium. [^{35}S]Sulphate (20 μCi/ml) was added for the last 3 h. The radiolabelled explants were washed and then frozen on a glass plate over solid CO_2.

Extraction of proteoglycans

The frozen cartilage pieces were sliced to 30 μm thickness with a cryostat and sequentially extracted twice with 4 M-guanidinium chloride in 0.05 M-sodium acetate buffer, pH 5.8, at 4 °C for 24 h. The pooled extracts, which contained 86 % of the radioactivity, were dialysed against 0.01 M-sodium acetate buffer, pH 6.8 at 4 °C, then freeze-dried. The following inhibitors were present in all the extraction/dialysis procedures: pepstatin (1 μg/ml) from the Peptide Institute, Osaka, Japan, and 1,10-phenanthroline (1 mM), iodoacetic acid (1 mM) and phenylmethanesulphonyl fluoride (1 mM), from Sigma. For analysis of total radioactivity, the explants were digested with papain (25 μg/ml; Sigma Type III) in 50 mM-sodium phosphate buffer, pH 6.5, containing 2 mM-*N*-acetylcysteine and 2 mM-EDTA at 60 °C for 2 h, or hydrolysed with alkali (1 M-$NaBH_4$ in 50 mM-NaOH at 45 °C for 48 h).

Gel chromatography

The average hydrodynamic size of [^{35}S]proteoglycans and glycosaminoglycans was determined on columns (100 cm × 6.6 mm) of Sepharose CL-2B and Sephacryl S-200. Portions of each extract containing the same amount of radioactivity were loaded, and eluted at 4 ml/h with 0.5 M-sodium acetate buffer, pH 7.0, at room temperature under dissociative conditions in the presence of 4 M-guanidinium chloride. Samples to be tested for the ability to aggregate were first mixed with high-M_r hyaluronate [2 % (w/w) of the sample glycosaminoglycan] at 4 °C for 4 h. Portions of the [^{35}S]glycosaminoglycan samples were digested with chondroitinase ABC (0.2 unit/mg of substrate) in 300 μl of 0.1 M-sodium acetate/0.1 M-Tris/HCl buffer, pH 7.4, at 40 °C for 5 h before chromatography on Sephacryl S-200. Recovery from the gel-filtration columns was 92–95 %.

Cellulose acetate electrophoresis

Samples of [^{35}S]glycosaminoglycans were spotted on to strips (50 mm × 200 mm; Gelman) of cellulose acetate and subjected to electrophoresis in 0.1 M-KH_2PO_4/0.1 M-HCl, pH 2, with a potential gradient of 5 V/cm for 3 h as described by Wessler (1971).

The sample strips were cut into 2 mm sections, and placed in vials containing 2.5 ml of scintillant solution and counted to determine radioactivity. A standard sample containing a mixture (1 mg/ml) of hyaluronic acid, chondroitin sulphate, keratan sulphate and heparin was run at the same time. After electrophoresis the strip

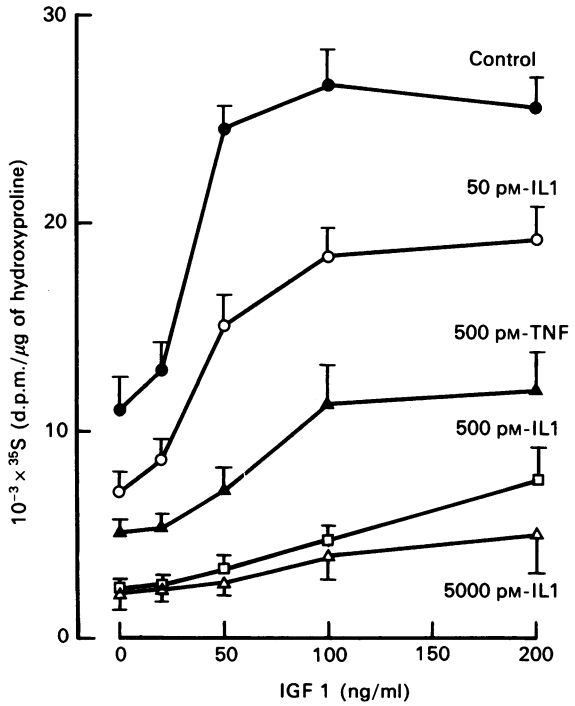

Fig. 1. Proteoglycan synthesis in cartilage explants cultured with increasing amounts of IGF 1

Cartilage was cultured for 72 h with increasing amounts of human recombinant IL1 (0–5000 pM) or TNF (0–500 pM) in the absence or presence of IGF 1 (0–200 ng/ml). Incorporation of [^{35}S]sulphate (3 h pulse) into total glycosaminoglycans is shown as the mean of six separate cultures. Bars indicate S.E.M.

was stained with a solution of Alcian Blue (0.02%) in 0.05 M-$MgCl_2$/0.05 M-sodium acetate buffer, pH 5.8, for 30 min at room temperature, then destained in the same buffer without Alcian Blue.

Analytical procedures

Glycosaminoglycan concentration was determined by reaction with 1,9-Dimethyl Methylene Blue (Farndale *et al.*, 1982). Shark chondroitin sulphate (5–50 μg) was used as a standard. Collagen was measured as hydroxyproline in neutralized acid hydrolysates of the medium or tissue (6 M-HCl, 105 °C, 20 h) as described by Tougaard (1973). [^{35}S]glycosaminoglycans were quantified by precipitation with cetylpyridinium chloride (1 % in water) at room temperature as described previously (Tyler, 1985*b*) or by the amount eluted as a void-volume peak from 10 ml Sephadex G-50 columns equilibrated with 50 mM-sodium acetate buffer, pH 6.0. Prostaglandin E_2 was measured by a specific radioimmunoassay (Steranti, St. Albans, Herts., U.K.) according to the manufacturers' instructions. Tracer [^{3}H]PGE_2 was purchased from Amersham.

RESULTS

Proteoglycan synthesis

Cartilage slices were cultured with either IL1 (0–5000 pM) or TNF (0–500 pM) with increasing concentrations of IGF 1 (0–200 ng/ml). Proteoglycan synthesis was measured after 72 h, as previous experiments (Benton & Tyler, 1988) showed that a maximal but reversible inhibitory effect of IL1 was achieved by this time. The data shown in Fig. 1 as the means of six cultures are expressed as a function of the hydroxyproline (representing collagen) content of the explants as this remains constant during the time of the experiment. IGF 1 induced a dose-dependent 2.5-fold increase in proteoglycan synthesis in the controls, with a half-maximal dose of 25 ng/ml. In the presence of 50 pM-IL1 a similar 2.6-fold dose-dependent increase was evident, each value being about 30% less than for the cartilage cultured without IL1. Even at 500 pM-IL1, IGF 1 was still able to induce a 3-fold increase in incorporation of [^{35}S]sulphate, although the overall synthesis at this dose of cytokine is only 15–30% of that in the controls. A much higher concentration of TNF is required to inhibit proteoglycan synthesis than with IL1. The proportional increase

Table 1. Prostaglandin E_2 production by cartilage cultured with IL1 and IGF 1

Cartilage was cultured for 72 h in medium alone, with IL1 (100 pM) or with IL1 (100 pM) + IGF 1 (100 ng/ml). Prostaglandin E_2 present in the culture medium was measured by a specific radioimmunoassay. Values are means ± S.E.M. ($n = 4$).

	Prostaglandin E_2 (ng/ml per 24 h)
No addition	0.5 ± 0.23
IL1 (100 pM)	2.55 ± 0.81
IL1 (100 pM) + IGF 1 (100 ng/ml)	2.26 ± 0.67

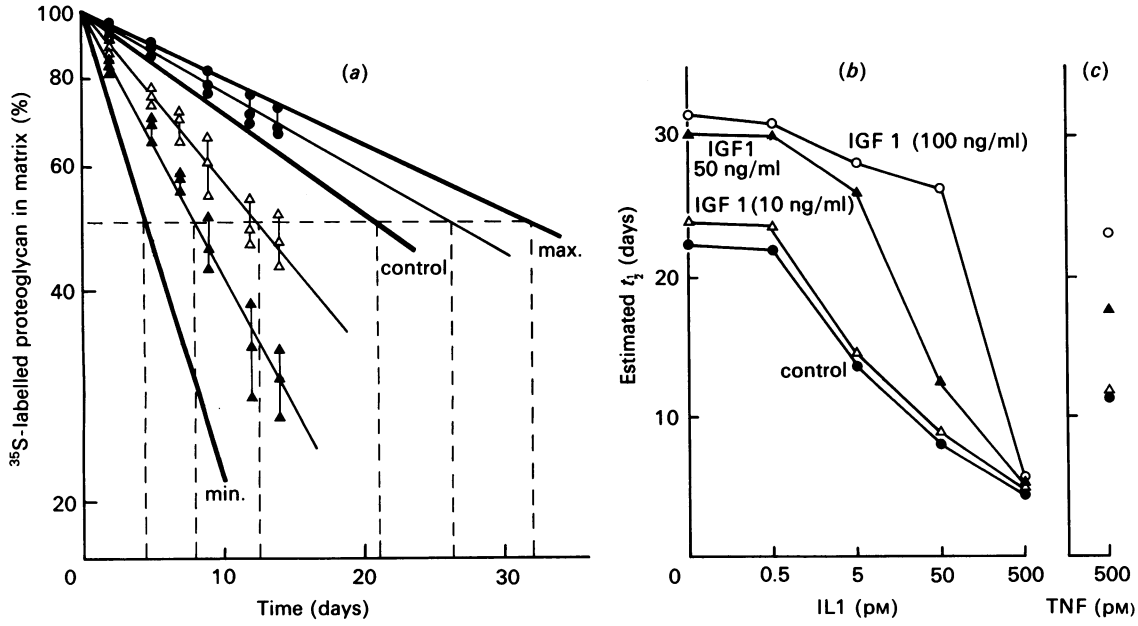

Fig. 2. Effects of IGF 1 on the basal and cytokine-stimulated degradation of cartilage proteoglycan

(*a*) The logarithm of the percentage of ^{35}S-labelled proteoglycan remaining in the matrix of cartilage explants is plotted as a function of time in culture. Cartilage proteoglycan was pre-labelled with [^{35}S]sulphate, and the explants were cultured in the presence of 50 pM-IL1 alone (▲) or containing in addition 50 (△) or 100 (●) ng of IGF 1/ml for 14 days. The points showing the amount of pre-labelled proteoglycan remaining in the explant are the mean values ($n = 6$) of three separate experiments. The 'control' curve is for cartilage cultured with no additions, and 'max.' and 'min.' are for cultures containing 100 ng of IGF 1/ml and 500 pM-IL1 respectively. (*b*) and (*c*), half-life ($t_{\frac{1}{2}}$) of pre-labelled proteoglycan in cultured explants. The time taken for 50% of the pre-labelled proteoglycan to be released from cultured explants ($t_{\frac{1}{2}}$) was determined from graphs as shown in Fig. 2(*a*). The $t_{\frac{1}{2}}$ is plotted as a function of (*b*) IL1 or (*c*) TNF concentration. The cultures contained 0 (●), 10 (△), 50 (▲) or 100 (○) ng of IGF 1/ml.

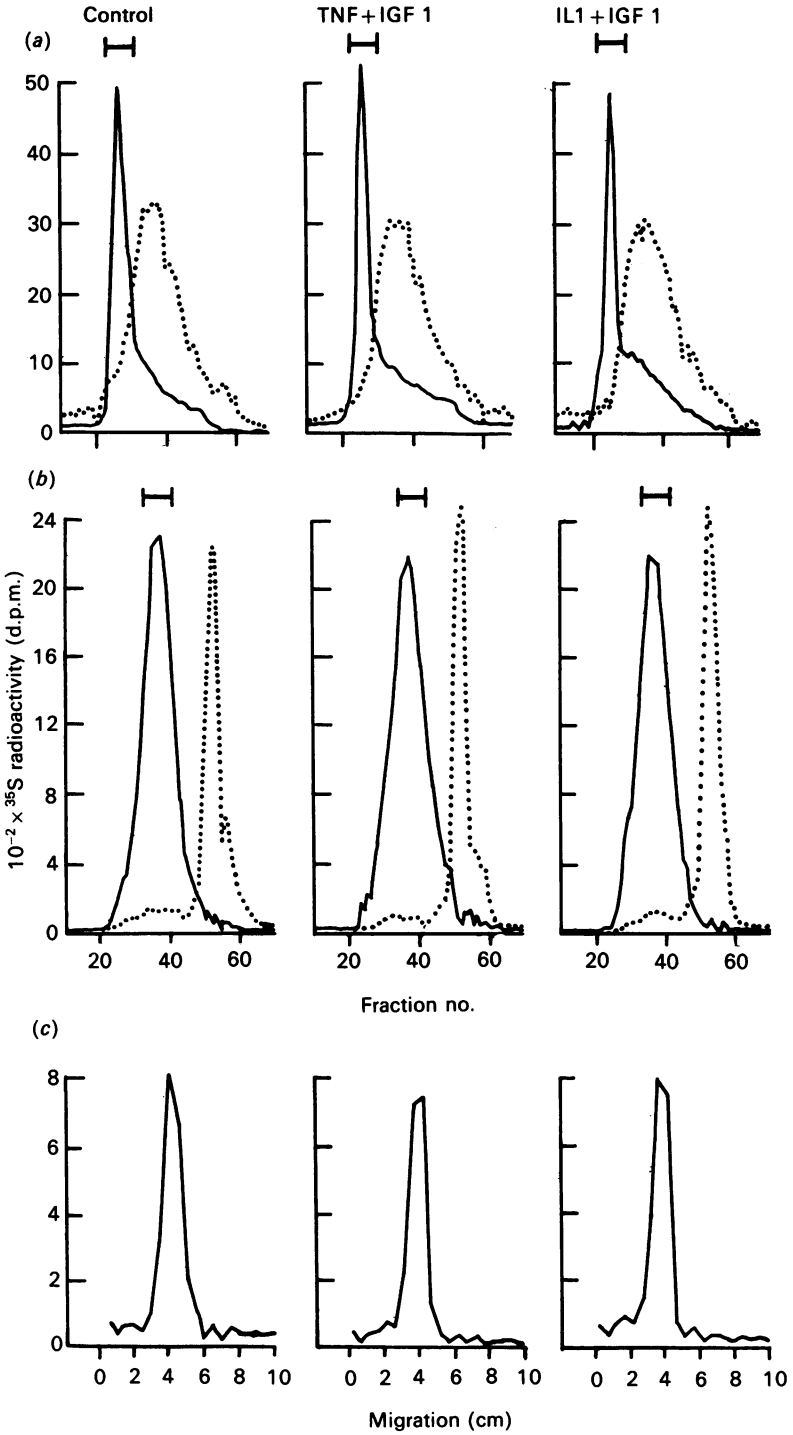

Fig. 3. Biochemical analysis of newly synthesized proteoglycans

For this, 18 equivalent halves of cartilage were cultured with 3 ml of control medium alone, with IL1 (50 pM) or with TNF (500 pM) in the absence or presence of IGF 1 (100 ng/ml) for 72 h at 37 °C with one change of medium. [^{35}S]Sulphate (20 μCi/ml) was added for the last 3 h. The [^{35}S]proteoglycans were extracted and analysed. (*a*) Gel chromatography on Sepharose CL-2B to show average hydrodynamic size (·····) of the proteoglycan and ability to aggregate with hyaluronate (——). (*b*) Gel chromatography on Sephacryl S-200 to show average hydrodynamic size of the [^{35}S]glycosaminoglycan chains (——) and susceptibility to chondroitinase ABC (·····). (*c*) Cellulose acetate electrophoresis of [^{35}S]glycosaminoglycan chains at pH 2 to indicate the degree of sulphation.

induced by IGF 1 in the TNF-treated cultures was the same as before. Values with 5 pM-IL1 or 5 pM- and 50 pM-TNF were not significantly different from the controls, and are not shown. These data demonstrate that IGF 1 can enhance proteoglycan synthesis in the presence of cytokines. No stimulation of proteoglycan synthesis was observed in explants cultured with fibroblast or epidermal growth factors (each 0–50 ng/ml) (results not shown).

Control cultures containing heat-treated IGF 1 gave the same results (not shown) as with IL1 alone, indicating that the effect was not due to the non-specific binding of IL1 to the IGF 1 protein. The response of the chondrocytes to IL1 was monitored by measuring the amounts of prostaglandin E_2 present in the medium. A similar increase was observed in cultures stimulated with IL1 alone or with IL1 and IGF 1 together (Table 1).

It was noted that the amount of proteoglycan released from cartilage cultured with IGF 1 always appeared to be less. The effect of IGF 1 on degradation alone can be assessed by prelabelling the proteoglycans with [^{35}S]sulphate. The data in Fig. 2(*a*) show the amount of pre-labelled proteoglycan remaining in cartilage cultured with 50 pM-IL1 alone (▲) or containing in addition 50 (△) or 100 (●) ng of IGF 1/ml over a period of 14 days. From such curves, the time taken for 50% of the proteoglycan to be released ($t_{\frac{1}{2}}$) is found to be 8, 12½ and 26 days respectively. These half-life determinations are not absolute values; they vary with age, species and location of the cartilage. The points shown are the means of three separate experiments, and indicate the considerable variation obtained on different days with similar tissue and culture conditions. However the relative effects of IGF 1 remained constant, and clearly indicate a marked decrease in proteoglycan release. The control curve shown is for cartilage cultured with no additions ($t_{\frac{1}{2}}$ 21 days), and the maximum and minimum obtained were for cartilage cultured with 100 ng of IGF 1/ml ($t_{\frac{1}{2}}$ 32 days) and 500 pM-IL1 ($t_{\frac{1}{2}}$ 4½ days). Similar experiments were performed with a range of concentrations of IL1 and TNF. For clarity, the individual graphs are not shown, but the estimated $t_{\frac{1}{2}}$ values are plotted in Fig. 2(*b*) as a function of IL1 concentration and in Fig. 2(*c*) for 500 pM-TNF. The values are for cartilage cultured with (●) 0, (△) 10, (▲) 50 or (○) 100 ng of IGF 1/ml. These results demonstrate a dramatic decrease in both the basal and cytokine-stimulated loss of proteoglycan from cartilage in the presence of IGF 1 (50–100 ng/ml), and are the first indication that this hormone can directly influence matrix degradation in cartilage.

Qualitative changes in the proteoglycan synthesized during replacement of a depleted matrix might well affect the stability and other properties of the cartilage and exacerbate deterioration of the joint. The nature of the large aggregating proteoglycan produced in cartilage exposed to IGF 1 and either IL1 or TNF was therefore determined. The average molecular size of proteoglycans extracted from the cultured explants and their ability to aggregate with hyaluronate was very similar, as determined by associative and dissociative gel chromatography (Fig. 3*a*). The proteoglycans in fractions indicated by the bar were pooled, and hydrolysed with alkali to generate free glycosaminoglycan chains. The range of size of the chains and chondroitin dermatan sulphate content was also the same (Fig. 3*b*). Glycosaminoglycans eluted in fractions indicated by the bars were pooled, processed and subjected to cellulose acetate electrophoresis. The electrophoretic mobility of the chains in 0.1 M-HCl is proportional to the sulphate content of the polysaccharide (Wessler, 1971). The mobility of each sample was very similar (Fig. 3*c*). The increased incorporation of [^{35}S]sulphate induced by IGF 1 in the presence of cytokines therefore reflects an increased production of proteoglycan with a similar fixed charge density to normal.

DISCUSSION

It has been shown that IGF 1 can enhance proteoglycan synthesis in cultured pig cartilage by 2–3-fold in the presence of IL1 and TNF, which partially overcame the inhibitory effect of these cytokines. The concentration of IGF 1 required to elicit a half-maximal stimulation (40 ng/ml) was the same whether or not the cytokines were present, and is between the doses of 5 and 250 ng/ml which were found to promote proteoglycan production in cultured bovine (McQuillan *et al.*, 1986*b*) and mouse cartilage (Schalkwijk *et al.*, 1988). The proteoglycan produced in the presence of IGF 1 and either IL1 or TNF had a similar size, ability to aggregate with hyaluronate and charge density to that normally produced by pig explants. Previous experiments (Benton & Tyler, 1988) have shown that IL1 inhibits cartilage proteoglycan synthesis by decreasing either transcription or translation of the core protein, with no significant alteration in the rate of secretion of the completed proteoglycan. Other work has shown that serum (mediated by IGF 1) increases core-protein production and the activity of several UDP-glycosyltransferases which polymerize the chondroitin sulphate chains (McQuillan *et al.*, 1986*a*).

It is not yet known whether IGF 1 is directly antagonizing the inhibitory effects of cytokines in the same target cell, or whether a distinct population of chondrocytes is responding separately to the different types of mediator. The increase in prostaglandin E_2 produced in response to IL1 was the same whether or not IGF 1 was present. IGF 1 is therefore not altering the binding of IL1 or the induction of immediate post-receptor events. Fibroblast and epidermal growth factors did not promote proteoglycan synthesis in cultured cartilage. This is consistent with other reports indicating that these growth factors alone mainly induce chondrocyte replication rather than matrix production (Hiraki *et al.*, 1988).

The ability of IGF 1 to influence the degradation of cartilage matrix is consistent with other reports showing that IGF 1 induced an inhibition of protein catabolism in myoblasts (Ballard *et al.*, 1986) and can inhibit RNA degradation (Zumstein & Stiles, 1987). This growth factor may therefore play an important role in regulating catabolic pathways as well as synthesis *in vivo*. In conclusion, these data clearly demonstrate that IGF 1 promotes the maintenance of chondroitin sulphate-rich aggregating proteoglycan and therefore the resilience of cartilage exposed to cytokines.

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