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Stimulation of proteoglycan biosynthesis by serum and insulin-like growth factor-I in cultured bovine articular cartilage

David J. McQUILLAN,*‡ Christopher J. HANDLEY,*§ Margaret A. CAMPBELL,* Shirley BOLIS,* Victoria E. MILWAY* and Adrian C. HERINGTON†

*Department of Biochemistry, Monash University, Clayton, Victoria 3168, Australia and †Medical Research Centre, Prince Henry's Hospital, Melbourne, Victoria 3004, Australia

The addition of foetal calf serum to explant cultures of adult bovine articular cartilage is known to stimulate proteoglycan synthesis in a dose-dependent manner. We have now shown the activity in serum responsible for this effect to be heat- and acid-stable, to be associated with a high-M_r complex in normal serum but converted to a low-M_r form under acid conditions. The activity has an apparent $M_r \sim 10000$ and isoelectric points similar to those reported for insulin-like growth factors (IGFs). Addition of a monoclonal antibody against insulin-like growth factor-I (IGF-I) prevented foetal calf serum from stimulating proteoglycan synthesis. Physiological concentrations of recombinant IGF-I or pharmacological levels of insulin when added to cartilage cultures mimicked the proteoglycan-stimulatory activity of serum. IGF-I appeared to act by increasing the rate of proteoglycan synthesis and did not change the nature of the proteoglycan synthesized nor the rate of proteoglycan catabolism by the tissue, suggesting that IGF-I may be important in the regulation of proteoglycan metabolism in adult articular cartilage. Furthermore, IGF-I can replace foetal calf serum in the culture medium, thereby allowing the use of a fully-defined medium which will maintain the synthesis and tissue levels of proteoglycan in adult articular cartilage explants for up to 5 days.

INTRODUCTION

Articular cartilage is predominantly composed of an insoluble fibrous collagen network immersed in a highly hydrated gel of high-M_r proteoglycan aggregate (for review, see Muir, 1980; Handley *et al.*, 1985). It is the presence of the proteoglycan molecule within the extracellular matrix that gives articular cartilage its unique ability to withstand compressive forces. Since the rate of turnover of proteoglycans within the extracellular matrix is relatively fast (Campbell *et al.*, 1984) compared with the collagen, the regulation of proteoglycan metabolism is therefore paramount to the correct physiological function of articular cartilage.

A variety of hormones and growth factors have been cited as stimulators of cartilage growth and chondrocyte multiplication, and the efficiency of each process appears to be dependent on both the culture system used and the assay conditions employed. The identification of circulating growth factors with the potential to modulate proteolgycan metabolism by articular cartilage is important to the understanding of the regulation of growth and maintenance of this tissue. Furthermore, these factors may play a role in the repair of articular cartilage after damage.

We have developed and characterized a culture system for bovine articular cartilage explants (Hascall *et al.*, 1983; McQuillan *et al.*, 1986*b*) in which the phenotype is maintained in both the presence and absence of serum. The ability to modulate the rate of proteoglycan synthesis makes this system ideal for the study of mechanisms controlling proteoglycan synthesis (McQuillan *et al.*, 1984, 1986*a*). We have now exploited this explant culture system to show that it is the insulin-like growth factors (IGFs) present in foetal calf serum that are responsible for the stimulation of proteoglycan synthesis. Furthermore, we have shown that substitution of foetal calf serum by recombinant IGF-I in the culture medium permits the type, rate of synthesis and tissue levels of proteoglycans in adult articular cartilage to be maintained for up to 5 days.

EXPERIMENTAL

Materials

Sephadex G-75 and G-50 were purchased from Pharmacia, and Ampholines (pH 3.5–10.0) were from LKB. Recombinant human IGF-I was obtained from AmGen Biologicals (Oak Park, CA, U.S.A.) through Amersham International. Bovine insulin was from the Commonwealth Serum Laboratories (Parkville, Victoria, Australia) and bovine growth hormone (NIH-GH-B-18) was provided by the National Pituitary Hormone Program of the NIADDK (Bethesda, MD, U.S.A.). A monoclonal antibody (SM 1.20B) to human IGF-I in the form of IgG purified from ascites fluid was kindly supplied by Drs. L. E. Underwood and J. J. Van Wyk, Department of Pediatrics, University of North Carolina (Van Wyk *et al.*, 1986). All other materials were obtained as described previously (Hascall *et al.*, 1983; McQuillan *et al.*, 1984, 1986*a*).

Cartilage cultures

Explant cultures of articular cartilage from the metacarpal–phalangeal joints of 1–2-year-old steers were

Abbreviation used: IGF(s), insulin-like growth factor(s).

‡ Present address: Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892, U.S.A.

§ To whom correspondence and reprint requests should be addressed.

prepared as described previously (Hascall *et al.*, 1983). Minced cartilage tissue was maintained in culture for up to 10 days in a modification of Dulbecco's modified Eagle's medium (Handley & Lowther, 1977) or the same medium supplemented with either foetal calf serum, serum fractions or hormones as indicated under 'Results'. Tissue was distributed into individual portions of 50–100 mg wet wt. for all experiments.

Labelling of proteoglycans with [^{35}S]sulphate

Cartilage cultures were preincubated in fresh medium for 1 h prior to incubation in the presence of 2 ml of medium containing 20–30 μCi of [^{35}S]sulphate/ml. Incubations were performed at 37 °C in a shaker bath in loosely capped culture vials for 1–2 h.

Extraction procedures

Cartilage cultures which had been incubated with [^{35}S]sulphate were extracted at 4 °C for 2–4 days with 2 ml of 4 M-guanidinium chloride/0.05 M-sodium acetate/acetic acid, pH 5.8, in the presence of proteinase inhibitors (1 mM-benzamidine hydrochloride, 100 mM-6-aminohexanoic acid, 10 mM-*N*-ethylmaleimide, 1 mM-phenylmethanesulphonyl fluoride, 10 mM-EDTA); more than 70% of ^{35}S-labelled proteoglycans were extracted by this treatment (Hascall & Kimura, 1982). The remaining ^{35}S-labelled macromolecules were extracted with 2 ml of 0.5 M-NaOH. In addition, cartilage explants were extracted directly with 2 ml of 0.5 M-NaOH to solubilize the total glycosaminoglycan pool in one step. The rate of [^{35}S]sulphate incorporation into macromolecules was determined by elution on Sephadex G-25 (PD-10) columns (Hascall *et al.*, 1983).

Analytical gel chromatography

^{35}S-labelled proteoglycans and glycosaminoglycans were analysed on columns of Sepharose CL-2B and CL-6B (0.8 cm × 100 cm) eluted with 4 M-guanidinium chloride/0.2 M-Na_2SO_4/0.5% (v/v) Triton X-100/0.1 M-sodium acetate/acetic acid, pH 6.0, at a flow rate of 6 ml/h. Fractions (1.4 ml) were collected and assayed for radioactivity.

Preparative gel chromatography of serum

Under neutral pH conditions. Foetal calf serum was dialysed extensively against phosphate-buffered saline (0.4 M-NaCl, 3 mM-KCl, 3 mM-Na_2HPO_4, 1 mM-KH_2PO_4, pH 7.4) at 4 °C. The dialysis residue was centrifuged at 60000 *g* for 30 min to remove any precipitate. Portions (5–10 ml) of the residue were eluted in a column (2.5 cm × 80 cm) of Sephadex G-75 equilibrated in phosphate-buffered saline. Fractions (~ 10 ml) were collected and assayed for protein content (Bradford, 1976). Every two fractions were pooled, dialysed against water, lyophilized, resuspended in a small amount of 1 M-acetic acid and made up to an appropriate volume with culture medium. Each fraction was then adjusted to pH 7.4 with concentrated NaOH and sterilized by passage through a 0.45 μm membrane.

Under acid pH conditions. Foetal calf serum was dialysed extensively against 1% (v/v) formic acid, pH 2.4 at 4 °C. The dialysis residue was treated in the same manner as described above for neutral pH chromatography except that the column of Sephadex G-75 was equilibrated and eluted with 1% (v/v) formic acid, pH 2.4.

Gel chromatography on Sephadex G-50. Fractions of foetal calf serum eluting on Sephadex G-75 under acid conditions between K_{av} 0.25 and 0.75 were pooled and concentrated by ultrafiltration using an Amicon membrane with an exclusion limit of 500 Da. A portion of the retentate was applied to a Sephadex G-50 column (1.8 cm × 90 cm) equilibrated with 1% (v/v) formic acid and pre-calibrated with standard proteins. Eluted fractions (~ 4.5 ml) were assayed for protein content and the ability to stimulate proteoglycan synthesis in cultures of bovine articular cartilage.

Isoelectric focusing

Preparative flat-bed electrofocusing, over the pH range 3.5–10.0, was performed in a gel of Sephadex G-75 as described by Radola (1975). The sample to be applied was first dialysed against phosphate-buffered saline, pH 7.4, at 4 °C. The sample was applied to the centre of the gel bed and electrofocusing was performed at 10 °C for 16 h. The gel bed was divided into 30 equal fractions and the gel in each fraction was eluted with a portion of the running buffer. The pH and protein content of each eluate was determined and pooled as described under 'Results'. Samples were dialysed against phosphate-buffered saline to remove ampholytes, and then assayed for stimulatory activity in the bovine articular cartilage culture system.

Measurement of proteoglycan turnover

Articular cartilage from a single steer was maintained in medium containing 20% (v/v) foetal calf serum for 6 days and then incubated for 6 h with 300 μCi of [^{35}S]sulphate in 10 ml of medium containing 20% (v/v) foetal calf serum (Campbell *et al.*, 1984). The tissue was then washed three times in medium alone prior to distribution into individual vials (~ 100 mg of tissue/vial) containing medium appropriate to each experiment. The medium from each culture was collected every 24 h and replaced with 4 ml of fresh medium. After 5 days in culture the tissue was extracted with 4 M-guanidinium chloride for 2 days, followed by 0.5 M-NaOH for 24 h at room temperature. The culture medium, guanidinium chloride and NaOH extracts were analysed for ^{35}S-labelled proteoglycans by chromatography on Sephadex G-25 (PD-10) columns, and from these data the percentage of ^{35}S-labelled proteoglycans remaining in the matrix was determined (Campbell *et al.*, 1984).

RESULTS

Temperature- and pH-stability of serum activity

Preliminary experiments were carried out to investigate the heat and pH-stability of the activity in serum responsible for the stimulation of proteoglycan synthesis by articular cartilage explants. Samples from the same batch of serum were incubated under sterile conditions for 5–30 min at temperatures of 50–100 °C or at pH 1, 2, 3 at 4 °C overnight. The samples were either clarified by centrifugation at 50000 *g* for 30 min or neutralized before being reconstituted with culture medium at a final concentration of 20% (v/v). Cultures of articular cartilage which had been maintained for 5 days in

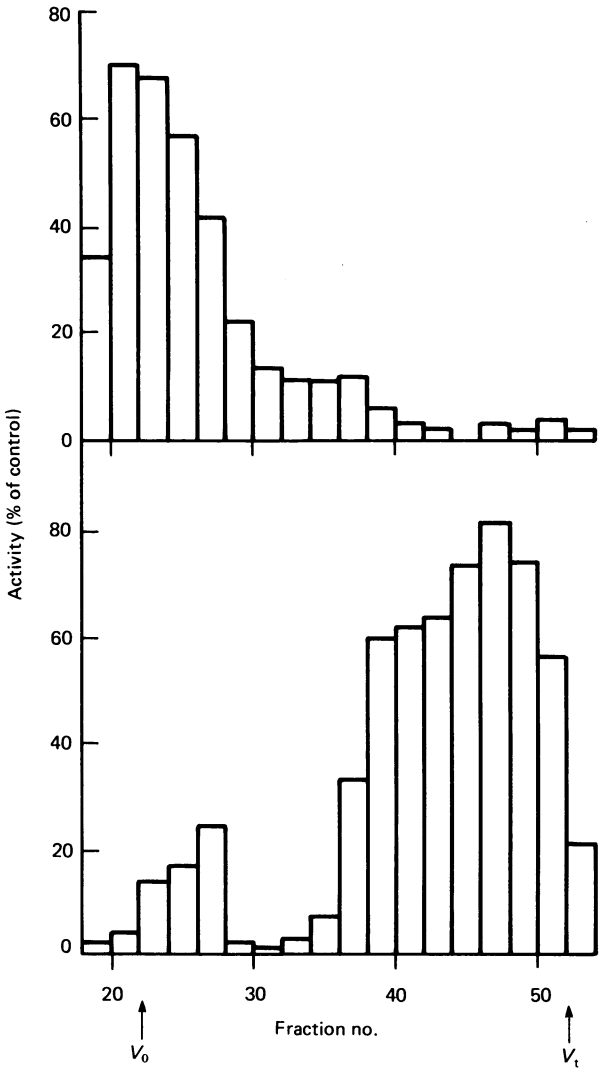

Fig. 1. Sephadex G-75 chromatography of foetal calf serum under neutral and acid conditions

Foetal calf serum (50 ml) was dialysed extensively into either phosphate-buffered saline, pH 7.4, or 1% (v/v) formic acid, pH 2.4, and 10 ml portions of the dialysed serum were applied to a column of Sephadex G-75 equilibrated in the appropriate solvent. Equivalent fractions from each column run were pooled, dialysed against water, lyophilized and made up to 20 ml with culture medium. Culture medium was made to 20% (v/v) with the reconstituted fractions and their activity assessed by the stimulation of [^{35}S]sulphate incorporation into proteoglycans by bovine articular cartilage explant cultures. Activity is expressed as a percentage of the stimulation observed in control cultures maintained in medium with 20% (v/v) foetal calf serum over cultures maintained in serum alone. The activity of each fraction under neutral (upper panel) and acid (lower panel) conditions is shown.

medium alone were incubated for a further 4 days in medium containing the heat- or pH-treated serum. The rate of [^{35}S]sulphate incorporation into proteoglycans was then measured. No activity was lost when serum was heated at 50 °C or 60 °C for 30 min, but in samples heated at 80 °C for 30 min or 100 °C for 5 min the stimulatory activity was decreased to 58% of that observed with native serum. When heated at 100 °C for increasing periods of time, there was a steady decrease in the stimulatory activity of serum; after heating at 100 °C for 30 min 21% of the serum activity remained. At pH 1, but not pH 2 or 3, an appreciable decrease (by 40%) in activity was observed.

Gel chromatography of foetal calf serum

To test whether the proteoglycan-stimulatory activity present in foetal calf serum exists in acid-dissociable complexes, portions of foetal calf serum, previously dialysed against the appropriate solvent, where subjected to gel filtration on Sephadex G-75 equilibrated in phosphate-buffered saline, pH 7.4 (neutral conditions) or 1% (v/v) formic acid (acid conditions), as described previously (Franklin *et al.*, 1979). Samples of the neutral and acid-treated serum were taken prior to gel filtration and showed no loss of proteoglycan stimulatory activity

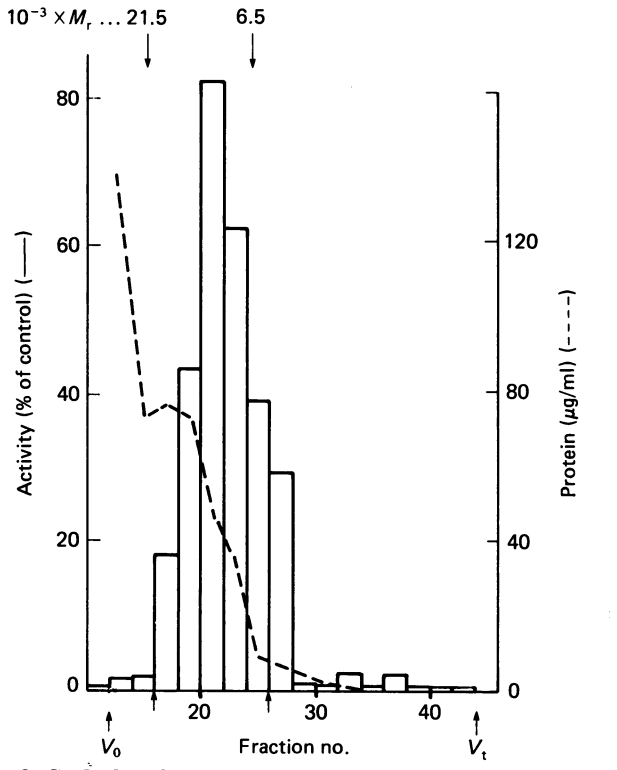

Fig. 2. Sephadex G-50 chromatography of the active fractions obtained from foetal calf serum

A sample of foetal calf serum (20 ml) was dialysed against 1% (v/v) formic acid and 5 ml portions were applied to Sephadex G-75 eluted under acid conditions. Equivalent fractions eluting from each column between $K_{av.}$ 0.25 and 0.75 were pooled, dialysed against phosphate-buffered saline, pH 7.4, and concentrated to 4 ml by ultrafiltration. A sample (2 ml; 5.1 mg of protein/ml) was applied to a column of Sephadex G-50 equilibrated with 1% (v/v) formic acid. Every two fractions were pooled and dialysed against phosphate-buffered saline, pH 7.4, prior to sterilization. The fractions were added to culture medium at a concentration of 19% (v/v) and the proteoglycan-stimulatory activity was assessed (bars) as described in the legend to Fig. 1. The column was calibrated with Blue Dextran (V_0), sodium [^{35}S]sulphate (V_t) and protein standards (indicated by arrows). Each fraction was assayed for protein content (broken line).

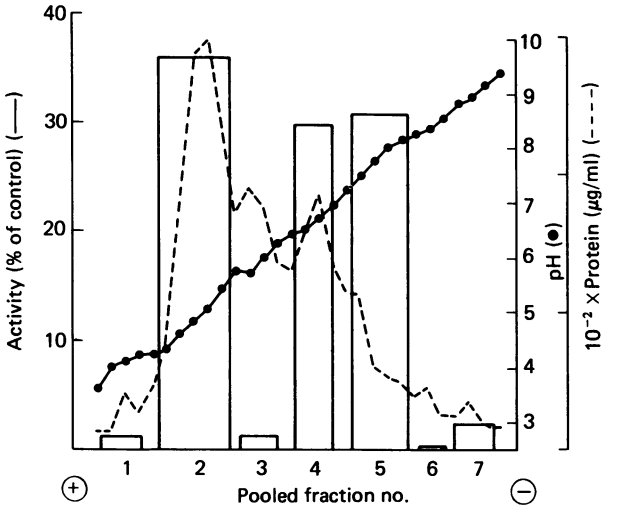

Fig. 3. Isoelectric focusing of the active fractions obtained from foetal calf serum

A 2 ml portion of the pooled and concentrated fractions, obtained from the fraction of foetal calf serum on Sephadex G-75, as described in the legend to Fig. 2, was subjected to isoelectric focusing in a flat-bed of Sephadex G-75. Focusing was performed in the presence of ampholytes to yield a pH gradient of 3.5–9.5 (●). Fractions were assayed for protein content and pooled as indicated (1–7). Pooled fractions were dialysed against phosphate-buffered saline, pH 7.4, prior to addition to culture medium at a concentration of 16% (v/v). Activity (bars) of each fraction was determined as described in the legend to Fig. 1. Each fraction was assayed in triplicate; the S.D. was less than ±6% in all cases.

compared with untreated foetal calf serum (results not shown).

Gel filtration of foetal calf serum under neutral conditions revealed a peak of activity near the void volume of the column (Fig. 1, upper panel). Filtration of the same batch of foetal calf serum in 1% (v/v) formic acid showed a marked shift in the peak of activity to a lower-M_r region of the column (Fig. 1, lower panel). Under acid conditions, the peak of activity eluted at a $K_{av.}$ of about 0.76. Although the bulk of activity had shifted to a low-M_r position under acid conditions, a small peak of activity remained near the void volume. This activity may represent undissociated carrier protein–IGF complexes or a high-M_r component known to possess non-suppressible insulin-like activity (Poffenbarger, 1975; Franklin *et al.*, 1979) which has not been previously reported to exhibit proteoglycan-stimulatory activity in cartilage. In other experiments, this acid-stable high-M_r activity was not always apparent and never constituted more than 10% of the total activity recovered.

Hydrodynamic size of the active serum factors

A portion (2 ml; ~ 5.1 mg of protein/ml) of the pooled fractons eluting from Sephadex G-75 under acid conditions with $K_{av.}$ 0.25–0.75 was re-chromatographed on a Sephadex G-50 column in 1% (v/v) formic acid, pH 2.4. The elution profile obtained (Fig. 2) shows an included region of activity between $K_{av.}$ 0.20 and 0.53 with the peak of activity eluting at $K_{av.}$ 0.37 (M_r ~ 10000), consistent with the M_r of free IGFs (~ 7500).

Isoelectric point of serum factors

Evidence that the proteoglycan-stimulatory activity in foetal calf serum was largely attributable to IGFs was provided by determination of the pI of the active fraction from serum. A portion (2 ml) of the active fraction from Sephadex G-75 chromatography eluted under acid conditions was subjected to isoelectric focusing across the pH range 3.5–10.0 (Fig. 3). Each pooled fraction was dialysed to remove ampholytes, and then assayed for activity. The profile (Fig. 3) shows three active fractions, two of which are adjacent, corresponding to isoelectric points of 4.3–5.5, 6.5–7.0, and 7.3–8.3. These pH ranges correlate well with isoelectric points for IGFs in human serum (Van Wyk *et al.*, 1980; Kuffer & Herington, 1984).

Effects of foetal calf serum treated with monoclonal antibody to IGF-1 on proteoglycan synthesis by articular cartilage

Articular cartilage was maintained in culture for 5 days in medium alone and then incubated for a further 3 days in medium alone, medium containing the monoclonal antibody (SM 1.20B) against IGF-1, medium containing 10% (v/v) foetal calf serum, or medium containing 10% foetal calf serum and the same monoclonal antibody to IGF-1. The monoclonal antibody was added to culture medium to give a final dilution of antibody of 1:480 (32 μg/ml). The tissue was then

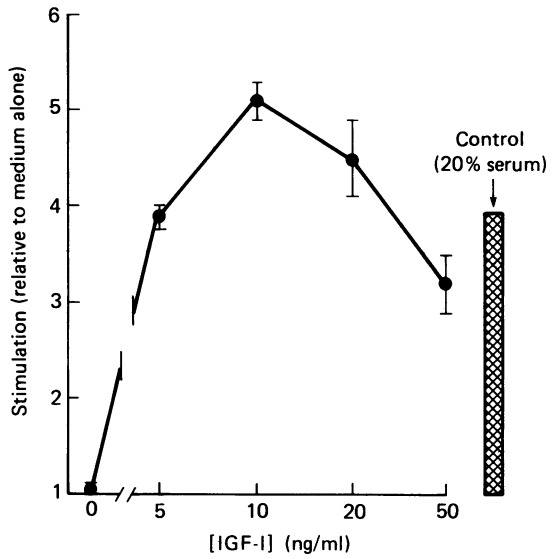

Fig. 4. Concentration-dependence of stimulated proteoglycan synthesis by IGF-I

Bovine articular cartilage was maintained in explant culture for 5 days in the presence of medium supplemented with IGF-I concentrations ranging from 5 to 50 ng/ml. On day 5, the rate of proteoglycan synthesis was determined by incubation in the presence of [^{35}S]sulphate for 2 h, followed by extraction and quantification of the ^{35}S-labelled glycosaminoglycans. The rate of proteoglycan synthesis is shown relative to that observed for cultures maintained in medium alone. The stimulation of proteoglycan synthesis for control cultures maintained in the presence of 20% (v/v) foetal calf serum is also shown (hatched bar). Each result is the mean of two determinations, with the range of each set of duplicates indicated by the error bars.

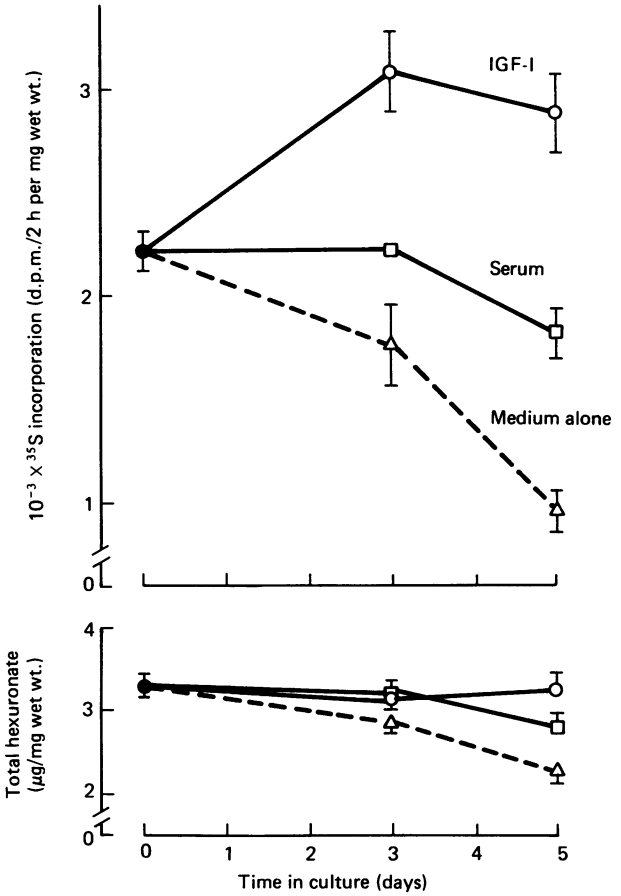

Fig. 5. Effects of IGF-I on proteoglycan synthesis and hexuronate content of bovine articular cartilage

Cultures of articular cartilage were maintained in culture in medium containing 20% (v/v) foetal calf serum (□), 0.01% (w/v) bovine serum albumin plus 20 ng of IGF-I/ml (○), or 0.01% (w/v) albumin (△) for up to 5 days. On the days indicated, [^{35}S]sulphate incorporation (upper panel) and hexuronic acid contents (lower panel) were determined. Each result is the mean of two determinations, with the range of each set of duplicates shown by the error bars.

incubated with [^{35}S]sulphate for 2 h and the rate of incorporation of this precursor into proteoglycan determined. Articular cartilage switched to medium containing 10% foetal calf serum incorporated approximately twice the amount of isotope into proteoglycans (2160 ± 63 d.p.m./mg wet wt.; mean ± range for duplicate determinations) compared with tissue maintained in medium alone (1125 ± 96 d.p.m/mg wet wt.). Tissue switched to medium containing antibody to IGF-I and 10% foetal calf serum showed no stimulation of proteoglycan synthesis (1149 ± 72 d.p.m./mg wet wt.). Tissue incubated in medium alone treated with the antibody showed a decrease of 18% in proteoglycan synthesis (930 ± 76 d.p.m./mg wet wt.). The reported specificity of the monoclonal antibody (SM 1.20B) against IGF-1 (Van Wyk *et al.*, 1986) suggests that at the dilution used in these experiments, the antibody would also bind to any IGF-II present in foetal calf serum. Nevertheless, these data provide further evidence that the proteoglycan stimulatory activity in foetal calf serum is derived from IGFs. A decrease in proteoglycan synthesis was observed in tissue incubated in medium alone plus antibody, which may indicate the production of endogenous IGFs by the cartilage explants.

Effect of recombinant IGF-I on proteoglycan biosynthesis by articular cartilage

Explant cultures of bovine articular cartilage were maintained for 5 days in the presence of 1–50 ng of recombinant human IGF-I/ml in medium containing 0.01% (v/v) bovine serum albumin to prevent non-specific absorption of the active protein. Following 5 days exposure to this medium, cultures were pulse-labelled with [^{35}S]-sulphate for 2 h and the rate of incorporation of this precursor into proteoglycan determined. Fig. 4 shows the relative rate of proteoglycan synthesis compared with cultures maintained in medium containing either albumin alone or albumin plus 20% (v/v) foetal calf serum. Maximal stimulation was observed at 20 ng of IGF-I/ml, after which the stimulatory effect of this factor decreased.

On the days indicated in Fig. 5, both the rate of proteoglycan synthesis (upper panel) and the uronic acid content of the tissue (lower panel) were determined. These data show a maintenance in the rate of

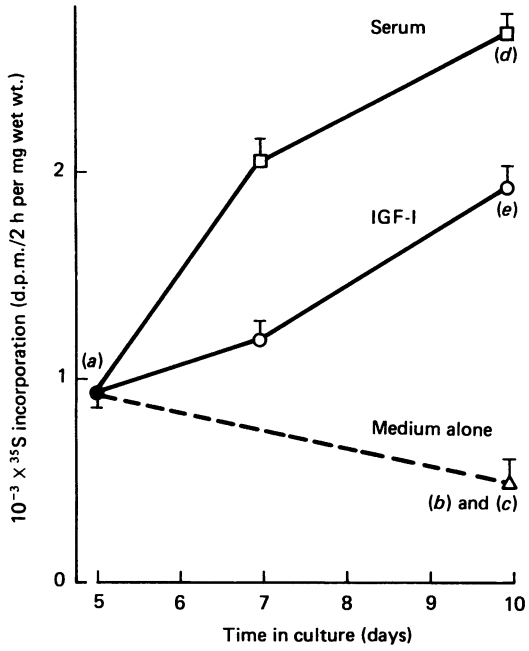

Fig. 6. Effects of IGF-I on proteoglycan synthesis by bovine articular cartilage maintained in the absence of serum for 5 days

Bovine articular cartilage was maintained in explant culture in the presence of medium alone for 5 days (●). Cultures were maintained for a further 5 days in the presence of 20% (v/v) foetal calf serum (□), 0.01% (w/v) bovine serum albumin plus 20 ng of IGF-I/ml (○), or 0.01% (w/v) albumin (△). On the days indicated, the rate of [^{35}S]sulphate incorporation into proteoglycan was determined. Each result is the mean of two determinations, with the range of each set of duplicates shown by the error bars. The letters indicate the samples used for the analyses in Fig. 7. Total values (sum of 4 M-guanidinium chloride and NaOH extracts of each sample) are given. Cultures were also maintained in the absence of 0.01% (w/v) albumin; however, omission of this protein did not alter the measured rates.

proteoglycan synthesis by tissue maintained in the presence of serum (□) whereas tissue exposed to 20 ng of IGF-1/ml (○) exhibited a 1.5-fold increase in the rate of synthesis by day 3 compared with the day 0 control. This elevated rate of synthesis was maintained up to day 5 of culture. Tissue cultured throughout with 0.01% (w/v) albumin (△) showed a steady decline in the rate of synthesis to reach 45% of the day 0 control value by day 5. Cultures maintained either in the presence of foetal calf serum (□) or IGF-I (○) for 5 days showed no change in the amount of proteoglycan in the tissue, suggesting that an equilibrium between proteoglycan synthesis and degradation was achieved. Cultures maintained in the absence of serum (△) showed a significant decrease in uronic acid content to 76% of day 0 control cultures, indicating that the rate of proteoglycan catabolism exceeded the rate of proteoglycan anabolism.

In a separate experiment, cultures were maintained in the presence of medium alone for 5 days and were then exposed for up to 5 days either with 20% foetal calf serum (Fig. 6, □) or 20 ng of IGF-I/ml (Fig. 6, ○). On days 7 and 10 the rate of proteoglycan synthesis was determined; compared with day 5 control cultures, the rate of proteoglycan synthesis on day 10 was stimulated 2.7- and 2.0-fold by serum and IGF-I respectively. In the absence of serum, the rate of proteoglycan synthesis decreased to 53% of the day 5 control cultures. The stimulation in the rate of proteoglycan synthesis induced by IGF-I was 70% of that induced by 20% foetal calf serum. For tissue cultured in the presence of hydroxyurea, an inhibitor of DNA synthesis, IGF-I stimulated the rate of proteoglycan synthesis to 80% of that observed in cultures maintained in medium containing serum and hydroxyurea, confirming our previous observation (McQuillan *et al.*, 1986*b*) that DNA synthesis was not a necessary prerequisite for a stimulation of proteoglycan synthesis to occur (results not shown).

The hydrodynamic size of the proteoglycans synthesized by the explant cultures in the presence of IGF-I was investigated. The elution profile of the day 0 control (Fig. 7*a*) shows the presence of two distinct species characteristic of this tissue (Hascall *et al.*, 1983): a large chondroitin sulphate- and keratan sulphate-containing proteoglycan ($K_{av.}$ 0.22) which constitutes greater than 85% of the newly synthesized sulphated macromolecules, and a smaller chondroitin sulphate proteoglycan ($K_{av.}$ 0.71). After 5 days in culture, irrespective of the culture conditions, there was a slight increase in the size of the larger species ($K_{av.}$ 0.20) with the smaller species comprising 5–15% of the total (Figs. 7*b*–7*e*). Alkali extracts of cultures treated in a manner similar to that described for Fig. 7 were eluted on a column of Sepharose CL-6B in order to compare the hydrodynamic size of the constituent glycosaminoglycan chains. The size of the chains remained essentially constant in the presence of serum or IGF-I compared with cultures maintained in medium alone (results not shown).

Effect of growth hormone and insulin on proteoglycan synthesis

Several hormones and growth factors have been implicated in the metabolism of connective tissues, particularly during growth and repair. Bovine growth hormone and insulin were tested and compared with the effects of IGF-I and foetal calf serum on proteoglycan synthesis by adult articular cartilage. Bovine growth

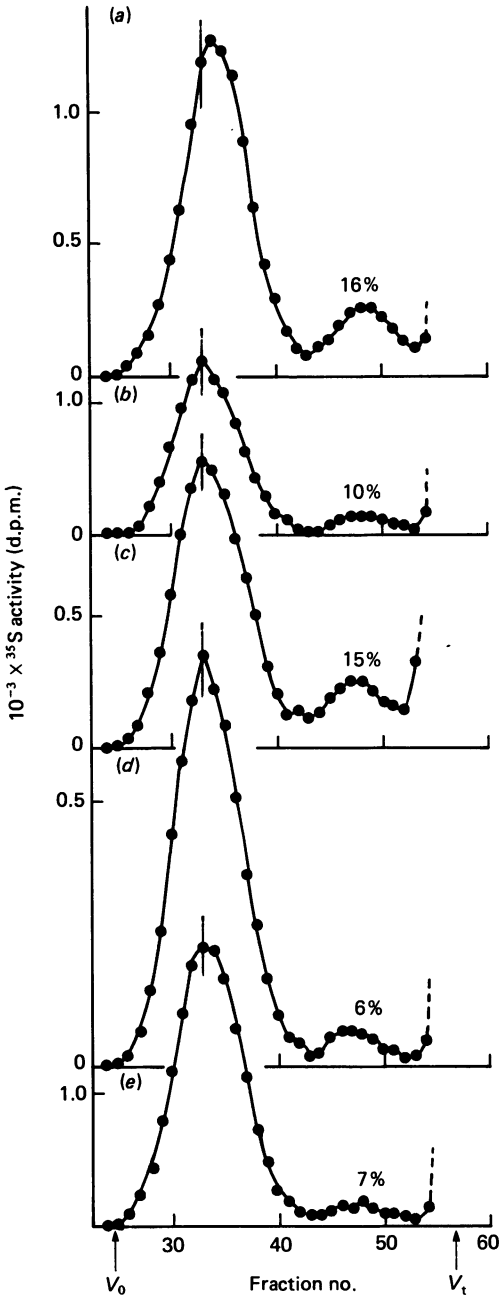

Fig. 7. Elution from Sepharose CL-2B of proteoglycans synthesized by cartilage explants cultured in the presence of IGF-I and foetal calf serum

Portions of the 4 M-guanidinium chloride extracts indicated by (*a*) – (*e*) in Fig. 6 were applied to a column of Sepharose CL-2B eluted with 4 M-guanidinium chloride (V_0, fraction 26; V_t, fraction 58). Profiles are shown for labelled proteoglycans from cartilage: (*a*) maintained for 5 days in medium alone; (*b*) maintained for 10 days in medium containing 0.01% (w/v) bovine serum albumin; (*c*) maintained for 10 days in medium alone; (*d*) switched after 5 days in medium alone to medium containing 20% (v/v) foetal calf serum; (*e*) switched after 5 days in medium alone to medium containing 0.01% (w/v) albumin plus 20 ng of IGF-I/ml. The percentage values indicate the proportion of total macromolecular ^{35}S label in the smaller proteoglycan species. A large peak of unincorporated isotope eluting at V_t in each case is not shown.

Table 1. Effects of hormones and growth factors on proteoglycan synthesis by bovine articular cartilage in culture

Articular cartilage was maintained in culture for 5 days in medium alone. The tissue was then maintained for a further 4 days in medium containing hormones or growth factors, and the rate of proteoglycan synthesis was measured. Values are given as means ± range of duplicates. Values in parentheses are expressed as a percentage of control cultures; the rate of proteoglycan synthesis of cultures maintained in medium alone was designated 0% and for cultures maintained in foetal calf serum 100%.

Culture medium	Concentration (ng/ml)	[^{35}S]Sulphate incorporation (d.p.m./2 h per mg wet wt.)
Expt. 1		
Medium alone	–	546 ± 39 (0)
Growth hormone	10	563 ± 87 (1)
	10^2	563 ± 72 (1)
	10^3	529 ± 44 (−1)
IGF-I	20	1511 ± 105(57)
20% foetal calf serum	–	2239 ± 112(100)
Expt. 2		
Medium alone	–	928 ± 85 (0)
Insulin	10	1161 ± 143(7)
	10^2	1429 ± 54 (15)
	10^3	1797 ± 156(26)
	10^4	2031 ± 110(33)
20% foetal calf serum	–	4269 ± 175(100)

hormone (NIH-GH-B-18) was tested for its effect on the rate of proteoglycan synthesis by adult bovine articular cartilage *in vitro*. No stimulation was apparent at any of the concentrations tested (10–1000 ng/ml) covering the high physiological to relatively high pharmacological range (Table 1).

Insulin significantly stimulated proteoglycan synthesis by cartilage only at pharmacological concentrations, with a maximal response of 33% above control levels observed at a concentration of 10 μg of insulin/ml. In other experiments employing a similar protocol, fibroblast growth factor, epidermal growth factor, and relaxin were tested, but no stimulation in proteoglycan synthesis was observed (results not shown).

Effect of recombinant IGF-I on proteoglycan catabolism

Maintenance of tissue proteoglycan levels comparable with those measured in freshly dissected articular cartilage was obtained by incubating cartilage cultures in the presence of IGF-I (Fig. 5, lower panel). To ascertain whether this was entirely attributable to maintenance of elevated rates of proteoglycan synthesis or to changes in the rate of proteoglycan catabolism, the effect of recombinant IGF-I on proteoglycan catabolism by cartilage explants was investigated. On the basis of the ^{35}S-labelled proteoglycans appearing in the respective media on each day (Fig. 8), the calculated half-life of proteoglycans in cultures treated with IGF-I ($t_{\frac{1}{2}}$ 10.4 days) was comparable with that of control cultures ($t_{\frac{1}{2}}$ 10.0 days). Cycloheximide inhibited the loss of the labelled proteoglycan from the tissue ($t_{\frac{1}{2}}$ 21.0 days),

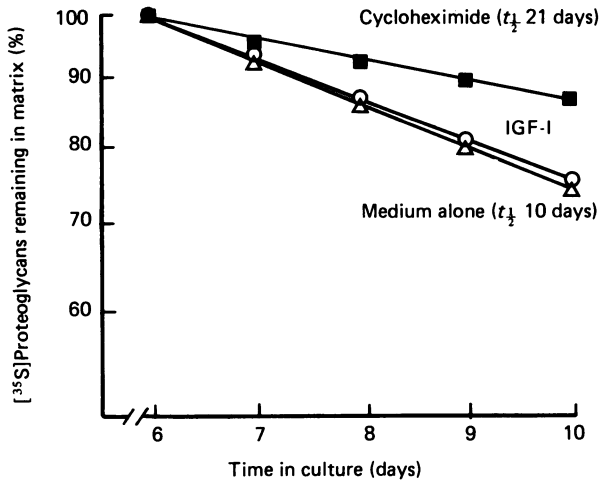

Fig. 8. Effect of IGF-I on proteoglycan catabolism by bovine articular cartilage

The logarithm of the percentage of [^{35}S]proteoglycans remaining in the matrix of articular cartilage incubated in medium containing either 0.5 mM-cycloheximide (■), 0.01% (w/v) bovine serum albumin (△) or 0.01% (w/v) bovine serum albumin (△) or 0.01% (w/v) albumin plus 20 ng of IGF-1/ml (○) is shown. Bovine articular cartilage was maintained in medium containing 20% (v/v) foetal calf serum, then labelled with [^{35}S]sulphate for 6 h. The tissue was then washed, returned to culture in the appropriate medium and the amount of [^{35}S]proteoglycans appearing in the medium on each day was determined. At the end of the experiment, the tissue was extracted with 4 M-guanidinium chloride followed by NaOH and the amount of [^{35}S]proteoglycans remaining in the matrix was determined. From these data the percentage of [^{35}S]proteoglycans remaining in the matrix on each day was calculated. From the decay curves, the half-lives were calculated. Duplicate cultures were used for each determination.

indicating that cells actively synthesizing proteins were required for proteoglycan catabolism to occur. These data suggest that it is an IGF-mediated maintenance of a high rate of proteoglycan synthesis that determines the levels of proteoglycan in the tissue.

In a similar experiment, bovine growth hormone (100 ng/ml of medium) was also shown to have no effect on the rate of loss of labelled proteoglycan from the matrix of the cultures ($t_{\frac{1}{2}}$ 9.0 days) compared with control tissue ($t_{\frac{1}{2}}$ 8.7 days).

DISCUSSION

It has been shown that the factor(s) present in foetal calf serum, and responsible for the maintenance of proteoglycan synthesis by articular cartilage, were relatively heat- and acid-stable and were of $M_r \leqslant 10000$, although in serum were bound in a biologically active, acid-dissociable, high-M_r complex. These observations strongly suggest that the factors belong to the IGF family (Smith, 1984). Furthermore, the proteoglycan-stimulatory activity could be resolved by isoelectric focusing into at least two distinct components with pIs comparable with those of the IGF/somatomedin family. The probable identity of these factors with the IGFs (IGF-I in particular) was further indicated by their inactivation by an anti-IGF-I monoclonal antibody and the repro-

duction of their actions by the addition of physiological concentrations of highly purified human IGF-I. It has recently been shown (Honegger & Humbel, 1986) that bovine and human IGF-I are identical.

These data are consistent with the postulate that IGFs may be crucial for the homeostasis of the extracellular matrix of articular cartilage *in vivo*. Indeed, it has been reported by a number of workers that chondrocytes of bovine cartilage possess receptors for IGF-I (Trippel *et al.*, 1983; Watanabe *et al.*, 1985). Furthermore, the ability of high pharmacological concentrations of insulin to stimulate proteoglycan synthesis supports the role of IGFs in the regulation of the metabolism of articular cartilage. Hajek & Solursh (1975) reported a similar response to insulin for glycosaminoglycan synthesis by chick embryo chondrocytes. The substantial sequence homology between IGF-I and insulin (Humbel & Rinderknecht, 1979) and the ability of high concentrations ($\geqslant 1$ μg/ml) of insulin to cross-react with IGF-I receptors in many tissues (Froesch, 1983), may account for these observations. Growth hormone had no direct effect on the synthesis of proteoglycans by adult bovine articular cartilage in this culture system. This finding is in agreement with most observations that the action of this hormone on articular cartilage is mediated through the IGFs (for review, see Spencer, 1983; Herington *et al.*, 1983).

Previous studies from our laboratory (Hascall *et al.*, 1983; McQuillan *et al.*, 1984, 1986*a*,*b*) have suggested that the stimulation of proteoglycan synthesis by serum is a co-ordinated process in the chondrocyte and is manifested at the level of DNA-dependent RNA synthesis. If, as suggested by this work, IGFs in foetal calf serum are responsible for this activity then this would suggest a potential mechanism of action of these growth factors. However, this is in contrast to other workers (Kemp *et al.*, 1984) who have postulated a direct action of somatomedins at the level of RNA-dependent protein synthesis. Although IGF-I had a marked effect on proteoglycan synthesis by articular cartilage, this growth factor did not appear to affect the rate of loss of proteoglycans from the matrix. The absence of an effect of IGF-I on proteoglycan catabolism contrasts with the IGF-induced inhibition of protein degradation in a variety of other cell types (Ballard *et al.*, 1980). This suggests that the mechanism involved in the regulation of proteoglycan catabolism is independent of proteoglycan synthesis. Previous work from our laboratory (Campbell *et al.*, 1984) indicated that the presence of foetal calf serum in the culture medium results in a suppression of proteoglycan catabolism compared with cultures maintained in medium alone, suggesting the presence of inhibitors or factors in serum capable of modulating the loss of proteoglycan from the matrix of cartilage.

The results presented in this study also show that the IGFs may be used to provide a defined medium for the study of cartilage metabolism *in vitro*. By replacing foetal calf serum in the culture medium with IGF-I, adult articular cartilage can be maintained in a culture system where the phenotype and composition of the extracellular matrix of the tissue is conserved as observed *in vivo*. Furthermore, this work suggests that IGFs may have the potential to enhance the repair of the extracellular matrix of articular cartilage after damage, as seen in arthritis and degenerative joint disease.

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