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Modulation of aggrecan and link-protein synthesis in articular cartilage

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The addition of serum or insulin-like growth factor-I (IGF-I) to the medium of explant cultures of bovine articular cartilage is known to stimulate the synthesis of aggrecan in a dose-dependent manner. The half-life of the pool of proteoglycan core protein was measured in adult articular cartilage cultured for 6 days in the presence and absence of 20 ng of IGF-I/ml and shown to be 24 min under both sets of conditions. The half-life of the mRNA pool coding for aggrecan was also determined and shown to be approx. 4 h in cartilage maintained in culture with or without IGF-I. The pool size of mRNA coding for aggrecan core protein increased 5–6-fold in cartilage explants maintained in culture in medium containing 20% (v/v) fetal-calf serum; however, in tissue maintained with medium containing IGF-I there was no increase in the cellular levels of this mRNA. This suggests that aggrecan synthesis is stimulated by IGF-I at the level of translation of mRNA coding for the core protein of this proteoglycan and that other growth factors are present in serum that stimulate aggrecan synthesis at the level of transcription of the core-protein gene. Inclusion of serum or IGF-I in the medium of cartilage explant cultures induced increases in the amounts of mRNA coding for type II collagen and link protein, whereas only serum enhanced the amount of mRNA for the core protein of decorin.

INTRODUCTION

Articular cartilage is a specialized tissue that has the ability to withstand both compressive forces and shear stress. The predominant macromolecular components of the extracellular matrix of articular cartilage are type II collagen and the proteoglycan aggregate. It is the presence of the highly hydrated proteoglycan aggregate which gives articular cartilage its unique biomechanical property of resisting compression. The proteoglycan aggregate consists of aggrecan macromolecules (large, aggregating, cartilage-specific proteoglycan) complexed to hyaluronan. This association of aggrecan and hyaluronan is further stabilized by the presence of link protein (Hardingham, 1979). Other proteins and glycoconjugates such as types IX, X and XI collagen and small proteoglycans (decorin and biglycan) are also present in the extracellular matrix of cartilage and these molecules also contribute to the organization of this tissue (Handley & Ng, 1992).

The control of the biosynthesis of the various macromolecular components of cartilage is complex, and an increasing number of circulating hormones and growth factors have been implicated in the regulation of cartilage metabolism. At present, the insulin-like growth factors (IGFs) stand out as one group of growth factors which play a major role in the regulation of the metabolism of articular cartilage. Insulin-like growth factor-I (IGF-I) has been shown to enhance the synthesis by explant cultures of articular cartilage of aggrecan (McQuillan *et al.*, 1986*c*; Luyten *et al.*, 1988; Tesch *et al.*, 1991), as well as link protein and hyaluronan (Curtis *et al.*, 1992).

In previous kinetic studies it was shown that the serum-induced stimulation of proteoglycan synthesis by explant cultures of adult articular cartilage was due to the influence of growth factors present in serum acting at the level of DNA-dependent RNA synthesis (McQuillan *et al.*, 1986*b*). In the present study, both kinetic and hybridization experiments are described which further investigate the molecular level at which fetal-calf serum and IGF-I act to enhance the synthesis of various matrix macromolecules, including aggrecan, link protein and type II collagen by explant cultures of articular cartilage.

EXPERIMENTAL

Materials

Recombinant human IGF-I was kindly supplied by Dr. A. Skottner (Kabi Pharmacia, Stockholm, Sweden). The plasmids pRC1 and pRL3 carrying cDNA inserts which are specific for aggrecan and link protein respectively, were kindly supplied by Dr. Y. Yamada (Doege *et al.*, 1986, 1987). cDNA probes were obtained for the α1(II) chain of type II collagen from Dr. F. Ramirez (Su *et al.*, 1989) and for human decorin (DS-PG-II) from Dr. L. Fisher (Fisher *et al.*, 1989). Hybond-N nylon membrane and L-[3-^{3}H]serine (30 Ci/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.), [α-^{32}P]dCTP (3000 Ci/mmol), [U-^{3}H]uridine (9.8 Ci/mmol) and $H_2{}^{35}SO_4$ (carrier free) from Dupont (Wilmington, DE, U.S.A.), an oligolabelling kit from Pharmacia (Uppsala, Sweden) and collagenase (prepared from *Clostridium histolyticum*, 0.23 unit/mg) from Boehringer-Mannheim (Mannheim, Germany). All other materials were obtained as described previously (Hascall *et al.*, 1983; McQuillan *et al.*, 1984, 1986*a–c*).

Measurement of proteoglycan synthesis

Explant cultures of articular cartilage from a single metacarpalphalangeal joint of a 1–2-year-old steer were prepared as previously described (Hascall *et al.*, 1983). Tissue was maintained in batch culture (2–3 g of tissue in 30 ml of medium) for 5 days in either medium alone, medium containing 20% (v/v) fetal-calf serum, medium containing 0.01% (w/v) BSA and 20 ng of IGF-I/ml, or medium containing 0.01% (w/v) BSA. The medium used was Dulbecco's modified Eagle's medium supplemented with Eagle's non-essential amino acids and organic buffers (Handley & Lowther, 1977). The culture medium was changed every 24 h. After 5 days in culture, tissue was distributed into

Abbreviations used: IGFs, insulin-like growth factors (e.g. IGF-I); SSPE, 0.18 M-sodium chloride, 0.01 M-sodium phosphate, 1.0 mM-EDTA, pH 7.7.
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vials so that each vial contained between 100–150 mg of tissue in 4 ml of medium.

Proteoglycan synthesis was measured by the incorporation of [^{35}S]sulphate into macromolecules. For each experiment, a sufficient amount of the respective medium containing 40 μCi of $H_2{}^{35}SO_4$/ml was prepared so that each culture was incubated in medium of identical specific radioactivity. Before incubation with the radiolabelled precursor, each culture was preincubated in 2 ml of the respective fresh medium for 1 h. Cultures were then incubated in 2 ml of medium alone containing $H_2{}^{35}SO_4$ with or without 0.4 mM-cycloheximide or 10 μg of actinomycin D/ml. Incubations were performed in a shaking water bath for periods ranging from 20 min to 24 h at 37 °C.

The incubations were terminated by the addition of 5.0 M-NaOH to the medium and tissue to give a final concentration of 0.5 M-NaOH. The cultures were then left for 48 h at room temperature before samples (0.25 ml) of extracts were subjected to size-exclusion chromatography on Sephadex G-25 (PD-10) columns to determine the rate of [^{35}S]sulphate incorporation into macromolecules (Hascall *et al.*, 1983).

Measurement of link-protein synthesis

Cartilage cultures, containing 300 mg of tissue in 6 ml of medium, were preincubated for 1 h in 2 ml of fresh medium. The medium was then removed and replaced with 3 ml of medium without added non-essential amino acids but containing 20 μCi of [^{3}H]serine/ml and incubated for 5 h at 37 °C (Curtis *et al.*, 1992). The tissue was then extracted for 48 h at 4 °C with 4 M-guanidine hydrochloride containing 0.05 M-sodium acetate, pH 5.8, and proteinase inhibitors (Oegema *et al.*, 1979). Each guanidine hydrochloride extract was dialysed to associative conditions against 0.05 M-sodium acetate, pH 6.0, and the retentate was adjusted to a density of 1.6 g/ml by addition of solid CsCl and subjected to centrifugation at 40000 rev./min using a 70.1 Ti rotor in a Beckman L8-80 ultracentrifuge for 40 h at 10 °C. The lower one-third (A1) of the gradient was removed and dialysed against 4 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 6.0. Each sample was then concentrated to approx. 1 ml by vacuum dialysis before being applied to a column of Sepharose CL-4B (1.4 cm × 64 cm) eluted with 4 M-guanidine hydrochloride containing 0.05 M-sodium acetate, 0.1 M-sodium sulphate, 0.01% (v/v) Triton X-100, pH 6.0 (Kimura *et al.*, 1980). The column was eluted at a flow rate of 6.0 ml/h and fractions (1.6 ml) collected and analysed for radioactivity. Link protein eluted from the column as a single peak with a $K_{av.}$ of 0.64 and the amount of radioactivity appearing in this peak was used as a measure of link-protein synthesis. All the ^{3}H-labelled link protein was recovered in the bottom one-third of the CsCl gradient (Curtis *et al.*, 1992).

Isolation and analysis of total RNA from cartilage

Cartilage was dissected from 10–12 metacarpalphalangeal joints, pooled, and distributed in 3–4-g amounts in flasks containing 30 ml of medium. The cultures were maintained for 5 days in medium supplemented with either 0.01% (w/v) BSA and 20 ng of IGF-I/ml, 20% (v/v) fetal-calf serum, or 0.01% (w/v) BSA. At the end of the culture period, the tissue was washed with medium alone and then digested with 0.25% (w/v) trypsin in unsupplemented medium for 20 min followed by 0.25% (w/v) collagenase in unsupplemented medium for 4–5 h. The digestions were carried out at 37 °C with gentle stirring. The digest was filtered through three layers of sterile gauze and the cells collected by centrifugation. The cell pellet was washed three times in phosphate-buffered saline, pH 7.4, before being resuspended in 10 ml of 7.5 M-guanidine hydrochloride containing 50 mM-sodium citrate, 1 mM-dithiothreitol and 0.1% (w/v) *N*-dodecanoyl-*N*-methylglycine, pH 7.4. The suspension was homogenized by 10 passages through a sterile syringe with a 19-gauge needle, followed by 10 passages through a syringe fitted with a 25-gauge needle. Total RNA was isolated from the homogenate by centrifugation on CsCl gradients (Glisin *et al.*, 1974) followed by phenol/chloroform extraction (Leboy *et al.*, 1988). The purity and quantity of the resulting RNA was determined spectrophotometrically.

For dot-blot analysis, total RNA was dissolved in sterile water containing 50% (v/v) formamide, the samples were heated to 65 °C for 15 min, then quenched on ice and applied to a Hybond-N membrane. Approx. 50 ng of purified insert cDNA or whole plasmid cDNA containing the equivalent of 50 ng of purified insert were labelled with [^{32}P]dCTP by random oligolabelling and were hybridized to the filter-bound RNA overnight at 42 °C in 50% (v/v) formamide under standard conditions (Sambrook *et al.*, 1989). After hybridization the cDNA-probed filter was washed twice for 15 min in 5 × SSPE (0.18 M-sodium chloride, 0.01 M-sodium phosphate, 1.0 mM-EDTA, pH 7.7) at 42 °C, then with 1 × SSPE/0.1% (v/v) SDS for 30 min and once at room temperature for 15 min in 0.1 × SSPE/0.1% (v/v) SDS. Autoradiographs of the RNA dot-blots were analysed with a laser densitometer connected to an LKB integrator. Several exposures of dot-blot autoradiographs were scanned to ensure that quantification was performed in the linear-response range of the film.

To examine the quality of mRNA present in the RNA preparation, samples of the RNA were subjected to electrophoresis on 0.7% agarose–formaldehyde gels (Sambrook *et al.*, 1989). Some of the gels were stained with ethidium bromide and showed a typical pattern of large and small ribosomal RNAs with a diffuse mRNA band. Other gels were soaked in 50 mM-NaOH/10 mM-NaCl for 45 min followed by neutralization with 0.1 M-Tris/HCl, pH 7.5, for a further 45 min. Then the RNA in the gels was capillary blotted on to Hybond-N membrane in 20 × SSPE at room temperature overnight, as described by Sambrook *et al.* (1989). The membranes containing the RNA were then hybridized to cDNA probes for aggrecan, link protein and the α1 chain of type II collagen. Autoradiographs of the blots showed the cDNA probes hybridized to mRNA species of the size established in previous work (Doege *et al.*, 1986, 1987; Su *et al.*, 1989).

Kinetic experiments were undertaken to establish the metabolic stability of RNA since the time taken for the enzymic isolation of chondrocytes from cartilage was between 4 and 5 h. Confluent cultures of bovine chondrocytes in 25 cm^2 flasks were incubated with 2 ml of medium containing 20% (v/v) fetal-calf serum and 5 μCi of [^{3}H]uridine/ml for 1 h, the cultures were then washed three times with medium not containing radiolabelled uridine and replaced in culture in the medium without radiolabelled precursor. Samples of the chondrocyte cultures were digested with 0.25% (w/v) trypsin in phosphate-buffered saline for 5 min immediately after incubation with [^{3}H]uridine or 2, 4 and 6 h into the chase period. The number of cells present in each trypsin digest was determined and the cells pelleted by centrifugation before extraction with 2 ml of 7.5 M-guanidine hydrochloride containing 50 mM-sodium citrate, 1 mM-dithiothreitol and 0.1% (w/v) *N*-dodecanoyl-*N*-methylglycine, pH 7.4. The suspension was homogenized by passing through 19- and 25-gauge syringe needles as described above. The amount of ^{3}H-labelled macromolecules was determined by size-exclusion chromatography on Sephadex G-25 (PD-10) columns equilibrated and eluted with 0.5 M-NaCl containing 20 mM-Tris/HCl, 1 mM-EDTA, 0.1% (w/v) *N*-dodecanoyl-*N*-methylglycine, pH 7.4. The amount of mRNA present in the excluded fractions from Sephadex G-25

chromatography was determined by affinity chromatography using oligo(dT)-cellulose. Oligo(dT)-cellulose (10 mg dry weight) equilibrated in 0.5 M-NaCl containing 20 mM-Tris/HCl, 1 mM-EDTA, 0.12% (w/v) *N*-dodecanoyl-*N*-methylglycine, pH 7.4, was added to the excluded fraction (total vol. 2 ml) from a Sephadex G-25 column and the resulting suspension was gently mixed for 60 min at room temperature. The cellulose was then washed four times by centrifugation using the same buffer. The poly(A)$^+$ RNA was eluted from the affinity medium by four washes in 1 ml aliquots of 10 mM-Tris/HCl containing 1 mM-EDTA and 0.05% (w/v) SDS, pH 7.4. It was shown that the amount of ^{3}H radioactivity associated with either RNA eluting at the excluded volume of the Sephadex G-25 column or present in the poly(A)$^+$-selected RNA was constant over the 6 h chase period, indicating that the cellular pool of total RNA and mRNA remained constant during the time taken for the enzymic isolation of chondrocytes from explant cultures of articular cartilage.

RESULTS

Effect of IGF-I on the half-life of aggrecan-core-protein synthesis

Previous work from our laboratory showed that the addition of IGF-I to the culture medium of explant cultures of bovine articular cartilage results in the stimulation of proteoglycan synthesis (Curtis *et al.*, 1992). This stimulation of proteoglycan synthesis may come about by increased rates of transcription or translation of the core protein of aggrecan. An experiment was carried out to investigate whether addition of IGF-I to the culture medium resulted in a change in the time taken for processing of the aggrecan core protein. Cultures were prepared from bovine metacarpalphalangeal articular cartilage and maintained in medium supplemented with either 0.01% (w/v) BSA plus 20 ng of IGF-I/ml, or 0.01% (w/v) BSA alone for 6 days. On day 6, cultures were incubated with medium containing 0.4 mM-cycloheximide for up to 120 min. The rate of proteoglycan synthesis was determined by incubating the cultures with [^{35}S]sulphate for the last 20 min of cycloheximide treatment. The initial rate of proteoglycan synthesis was determined by incubating some cultures with [^{35}S]sulphate for 20 min in the absence of cycloheximide.

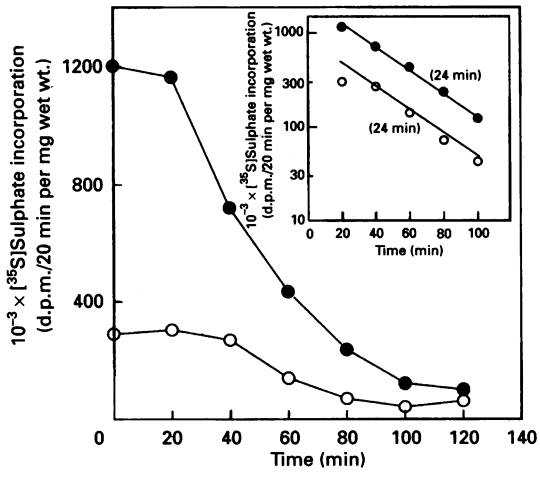

Fig. 1. Effect of IGF-I on the pool size of aggrecan-core-protein precursor in bovine articular cartilage

Cultures of bovine articular cartilage were maintained for 6 days in the presence of 0.01% (w/v) BSA (○) or 0.01% (w/v) BSA plus 20 ng of IGF-I/ml (●). On day 6, individual cultures were exposed to 0.4 mM-cycloheximide for up to 120 min. The rate of aggrecan synthesis was determined by incubating duplicate cultures with [^{35}S]sulphate for the last 20 min of treatment. The half-life for each curve was determined from a semi-logarithmic plot (inset).

Fig. 1 shows that in articular cartilage maintained for 6 days in the presence of IGF-I, the initial rate of synthesis of proteoglycan was about 4-fold greater than that in tissue maintained in the absence of IGF-I for the same period of time. This figure also shows that the synthesis of proteoglycans in the presence of cycloheximide followed an exponential decay. Despite the initial differences in the rate of proteoglycan synthesis under different culture conditions, the decay in the rate of synthesis induced by cycloheximide was similar and followed first-order kinetics as described by Kimura *et al.* (1981). The half-life of the core protein was 24 min for tissue maintained in the presence or absence of IGF-I. This indicates that the time taken for the processing of aggrecan core protein to form mature proteoglycan is constant. Since the rate of proteoglycan synthesis was increased in the presence of IGF-I and the half-life of the core protein remained constant, there must be a larger pool of core protein present in cultures maintained with IGF-I compared with cultures maintained in its absence.

Effect of IGF-I on the half-life of the mRNA for aggrecan-core-protein synthesis in bovine articular cartilage

An experiment was undertaken to investigate the effect of IGF-I on the processing time of mRNA coding for the core protein of aggrecan in order to determine whether this growth factor regulates the transcription of the aggrecan gene. Explant cultures of articular cartilage were prepared from bovine articular cartilage and maintained for 6 days in medium supplemented with either 0.01% (w/v) BSA and 20 ng of IGF-I/ml, or 0.01% (w/v) BSA alone. On day 6, cultures were incubated with 10 μg of actinomycin D/ml in unsupplemented medium for up to 24 h. The rate of proteoglycan synthesis was determined by incubating cultures with [^{35}S]sulphate for the last hour of exposure of the culture to actinomycin D. The initial rate of proteoglycan synthesis was determined by incubating some cultures with [^{35}S]sulphate for 1 h in the absence of actinomycin D.

The initial rate of proteoglycan synthesis in cartilage maintained for 6 days in medium containing IGF-I was about 5.6-fold greater than that of tissue maintained in medium alone (Fig. 2). Despite this initial difference in the rate of proteoglycan synthesis under different culture conditions, the decrease in the rate of synthesis induced by actinomycin D was similar. The half-life of the pool of aggrecan-core-protein mRNA was 4.4 h for cultures maintained in the presence of IGF-I, and 4.0 h for cultures maintained in medium not containing IGF-I. This exponential decay represents the depletion of the available pool of mRNA coding for core protein as well as the depletion of the pool of core protein itself. These results suggest that IGF-I does not prolong the life of the available pool of core-protein mRNA. Therefore, it is unlikely that the initial rate of proteoglycan synthesis in cultures maintained with IGF-I is determined solely by the availability of the pool of mRNA encoding the proteoglycan core protein. This suggests that IGF-I stimulates proteoglycan synthesis by regulating the efficiency of translation. This observation is in contrast to the effect of fetal-calf serum on aggrecan synthesis by explant cultures of articular cartilage, where in the presence of fetal-calf serum the half-life of aggrecan-core-protein mRNA was determined to be 8.5 h, compared with 3.8 h in tissue cultured in unsupplemented medium (McQuillan *et al.*, 1986*b*). These results suggest that the increase in aggrecan

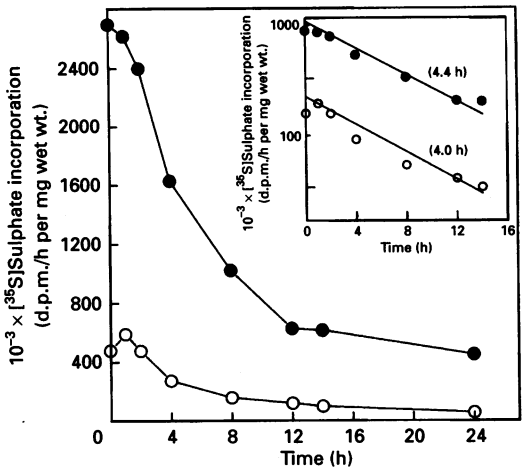

Fig. 2. Effect of IGF-I on the half-life of aggrecan-core-protein mRNA in bovine articular cartilage

Cultures of bovine articular cartilage were maintained for 6 days in the presence of 0.01% (w/v) BSA (○) or 0.01% (w/v) BSA plus 20 ng of IGF-I/ml (●). On day 6 cultures were exposed to 10 μg of actinomycin D/ml for up to 24 h. The rate of aggrecan synthesis was determined by incubating duplicate cultures with [^{35}S]sulphate for the last 1 h of treatment. The half-life for each curve was determined from a semi-logarithmic plot (insert).

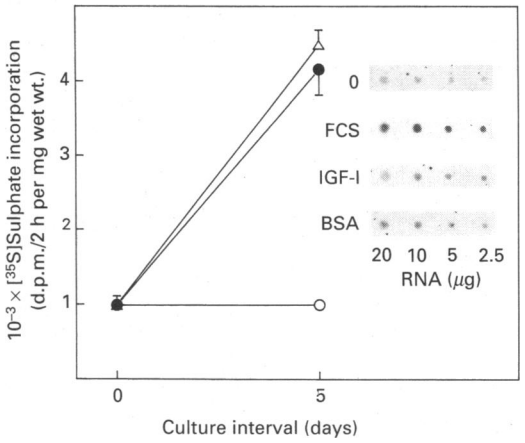

Fig. 3. Effect of serum and IGF-I on aggrecan synthesis and the cellular mRNA pool of aggrecan core protein in bovine articular cartilage explants

The rate of aggrecan synthesis was determined in explant cultures of articular cartilage on day 0 and after 5 days of culture in medium supplemented with either 0.01% BSA (○), 20% (v/v) fetal-calf serum (△), or 0.01% (w/v) BSA and 20 ng of IGF-I/ml (●). Each point is the mean of two determinations and the range of each set of duplicates is indicated by the error bars. RNA was also isolated from the explant cultures on day 0 and day 5 as described in the Experimental section and aliquots of total RNA corresponding to 20, 10, 5, and 2.5 μg were bound to nylon membranes and hybridized to a ^{32}P-labelled cDNA clone specific for the core protein of aggrecan. After hybridization and washing, the membranes were subjected to autoradiography (inset).

synthesis induced by fetal-calf serum is owing to an increase in the rate of transcription of the aggrecan-core-protein gene thereby increasing the cellular pool of aggrecan-core-protein mRNA.

Effect of fetal-calf serum and IGF-I on the mRNA pools of aggrecan core protein and other matrix macromolecules

To confirm the above observation that the stimulation of aggrecan synthesis induced by serum or IGF-I was the result of increased transcription or translation, the level of aggrecan-core-protein mRNA was measured in cytoplasmic RNA dot-blots by hybridization using a cDNA probe to aggrecan core protein. Explant cultures of articular cartilage were set up and maintained in culture for 5 days in medium containing either 20% (v/v) fetal-calf serum, 0.01% (w/v) BSA plus 20 ng of IGF-I/ml, or 0.01% (w/v) BSA alone. On days 0 and 5 some of the tissue was incubated with [^{35}S]sulphate for 2 h to ascertain the rate of aggrecan synthesis. On the same days mRNA was extracted from samples of the cartilage explants as outlined in the Experimental section and the relative amounts of aggrecan-core-protein mRNA were determined by dot-blot hybridization. Fig. 3 shows that there was an increase in the rate of aggrecan synthesis in articular cartilage maintained in culture with either fetal-calf serum or IGF-I. The cellular levels of aggrecan-core-protein mRNA were increased 5–6-fold in cultures maintained in the presence of serum (Fig. 3 and Table 1). In contrast, while IGF-I induced a similar stimulation of aggrecan synthesis, there was no significant increase in the cellular levels of mRNA encoding aggrecan core protein.

The effects of serum and IGF-I on the synthesis of link protein by cartilage explants were also investigated. Explant cultures were prepared and maintained in culture for 5 days in medium containing either 20% (v/v) fetal-calf serum, 0.01% (w/v) BSA plus 20 ng of IGF-I/ml, or 0.01% (w/v) BSA alone. On days 0 and 5 of culture, the rate of link-protein synthesis was measured in some of the tissue by the incorporation of [^{3}H]serine into the protein. The relative amount of link-protein mRNA was determined in the same cartilage cultures described in Fig. 3. As shown in Fig. 4, the synthesis of link protein is stimulated by both serum and IGF-I. On day 0 link-protein mRNA was visible; however, the level was not measurable by laser densito-

Table 1. Effect of serum and IGF-I on the cellular pools of mRNA of matrix macromolecules

Total RNA was extracted from explant cultures of articular cartilage on day 0 and after 5 days in culture in medium supplemented with either 0.01% (w/v) BSA, 20% (v/v) fetal-calf serum (FCS) or 0.01% (w/v) BSA plus 20 ng of IGF-I/ml, was bound to nylon membranes and hybridized to ^{32}P-labelled cDNA probes for aggrecan, link protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), type II collagen and decorin. After hybridization and washing, the membranes were subjected to autoradiography followed by densitometry. The values obtained by densitometry were expressed relative to day 0 values except in the case of link protein. Abbreviation: ND, not determined.

Day of culture	Culture conditions	Amount of mRNA (arbitrary units)				
		Aggrecan	Link protein	GAPDH	Type II collagen	Decorin
Day 0	Medium alone	1.00	ND	1.00	1.00	1.00
Day 5	Medium + 0.01% BSA	1.60	1.00	1.40	0.04	1.60
Day 5	Medium + 20% FCS	5.30	10.00	2.00	0.63	16.70
Day 5	Medium + IGF-I	1.60	4.00	1.60	0.12	2.00

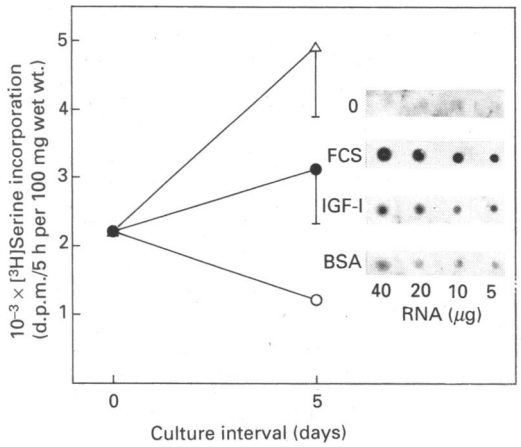

Fig. 4. Effect of serum and IGF-I on synthesis of link protein and the cellular mRNA pool of link protein in bovine articular cartilage explants

The rate of link-protein synthesis was determined in explant cultures of articular cartilage on day 0 and after 5 days of culture in medium supplemented with either 0.01% (w/v) BSA (○), 20% (v/v) fetal-calf serum (△) or 0.01% (w/v) BSA plus 20 ng of IGF-I/ml (●). Each point is the mean of two determinations, with the range of duplicates indicated by the error bars. Total RNA was isolated from the cultures and aliquots of total RNA solution corresponding to 40, 20, 10, and 5 μg were bound to nylon membranes and hybridized to a ^{32}P-labelled cDNA clone specific for link protein. After hybridization and washing, the membranes were subjected to autoradiography (inset).

metric analysis because of the high background value. In the presence of serum, the level of link-protein mRNA was approx. 10-fold greater than that present in tissue cultured in medium containing 0.01% BSA for the same period of time. In the presence of IGF-I, there was an approx. 4-fold increase in levels of link-protein mRNA (Fig. 4; Table 1).

Using samples of RNA prepared in the experiment described in Fig. 3, the cellular levels of mRNAs encoding glyceraldehyde-3-phosphate dehydrogenase (Piechaczyk *et al.*, 1984), type II collagen, and decorin were also determined by hybridization (Table 1). The pool size of mRNA encoding glyceraldehyde-3-phosphate dehydrogenase was determined as a control, since this enzyme is not subject to hormonal regulation (Fort *et al.*, 1985). Table 1 shows that cellular levels of glyceraldehyde-3-phosphate dehydrogenase RNA did not differ significantly in cartilage maintained in culture in the presence or absence of fetal-calf serum or IGF-I. The cellular levels of collagen mRNA declined over the 5-day culture period; however, in cultures maintained in medium containing fetal-calf serum or IGF-I this was less marked (Table 1). The amount of mRNA coding for the core protein of decorin increased approx. 16-fold in cartilage cultured for 5 days in medium containing fetal-calf serum. In comparison, for cartilage cultured in the presence of IGF-I for 5 days, no increase in the amount of mRNA for this core protein was observed.

DISCUSSION

Our results show that serum-mediated stimulation of proteoglycan synthesis was associated with a 5–6-fold enhancement of the amount of mRNA encoding the core protein of aggrecan. This confirms our previous interpretation of work using inhibitors of protein and RNA synthesis, which suggested that the stimulation of aggrecan synthesis by serum is owing to the influence of growth factors which act at the level of transcription of the aggrecan-core-protein gene (McQuillan *et al.*, 1986*b*).

We and others have shown that a factor present in serum and synovial fluid that will stimulate aggrecan synthesis in articular cartilage is IGF-I (McQuillan *et al.*, 1986*c*; Schalkwijk *et al.*, 1989). Kinetic studies using cycloheximide and actinomycin D revealed that the half-lives of the aggrecan core protein or the mRNA encoding the core protein of this proteoglycan were the same in explant cultures of articular cartilage maintained in medium with or without IGF-I. We have shown that both cycloheximide and actinomycin D have little effect on the activities of some of the glycosyltransferases involved in the synthesis of chondroitin sulphate in explant cultures of bovine articular cartilage, when the activities of these enzymes were measured directly (McQuillan *et al.*, 1984, 1986*a*). Using benzyl β-D-xyloside as an exogenous initiator of chondroitin sulphate synthesis, it was shown in explant cultures of bovine articular cartilage exposed to either cycloheximide or actinomycin D there was a decrease in the rate of synthesis of chondroitin sulphate (McQuillan *et al.*, 1984, 1986*a*). The decrease in the rate of chondroitin sulphate synthesis occurred 12 h after addition of actinomycin D to the cultures, and in the case of cycloheximide the decrease in the rate of chondroitin sulphate synthesis was evident 20 min after addition of the inhibitor. These observations are consistent with the possibility that the intracellular levels of aggrecan core protein may be influencing organization of these glycosyltransferase enzymes and hence their activity (McQuillan *et al.*, 1984, 1986*a*). The half-lives of aggrecan core protein pool in bovine cartilage explants maintained in culture for different times and under different conditions have been shown to be similar and in the order of 24–32 min (McQuillan *et al.*, 1984). If cycloheximide was indirectly influencing the activity of the glycosyltransferases by decreasing the intracellular levels of aggrecan core protein, the effect of this inhibitor on the activity of these enzymes would be comparable in all explant cultures of bovine articular cartilage regardless of time or conditions of culture.

When the amount of mRNA coding for aggrecan core protein was determined in cultures maintained in medium with or without IGF-1 no difference was observed. These results indicate that IGF-I probably regulates the synthesis of aggrecan at the level of translation. This may involve the more efficient utilization of the existing aggrecan-core-protein-mRNA transcripts by mechanism such as increased association of the core protein mRNA with polyribosomes and/or increased polypeptide-chain initiation or elongation (Thomas *et al.*, 1982). It is known that insulin acts on chondrocytes by stimulating the dephosphorylation of eukaryotic initiation factor 2α which is necessary for the initiation of further rounds of protein synthesis (Towle *et al.*, 1984). Insulin has also been shown to stimulate the synthesis of eukaryotic elongation factor 2 (Levenson *et al.*, 1989). Since the cell receptors of both insulin and IGF-I exhibit tyrosine kinase activity, it is possible that IGF-I acts to promote aggrecan synthesis by the same molecular mechanism. Alternatively, modifications to the secondary structure of individual mRNA species may promote translational efficiency. For instance, it has been suggested that insulin may stimulate translation of certain mRNAs by activating initiation factors which modify the secondary structure of the 5′ non-coding regions of the mRNAs (Manzella *et al.*, 1991).

These observations indicate the complexity of the regulatory mechanisms involved in the synthesis of aggrecan, in that the synthesis of this proteoglycan may be modulated both at the level of transcription or translation. The modulation of aggrecan synthesis by serum or IGF-I does not appear to result in major changes in the glycosylation of the core protein of aggrecan, indicating that the activity of the enzymes involved in the post-translational events associated with synthesis of this macro-

molecule may well be modulated by the same growth factors in a synergistic manner (Hascall *et al.*, 1983; McQuillan *et al.*, 1986*c*; Tesch *et al.*, 1991).

Previous work has shown that collagen synthesis by explant cultures of articular cartilage maintained in medium alone or medium containing serum fell by 80% and 40% respectively (Hascall *et al.*, 1983). These changes in the rate of collagen synthesis are consistent with changes in the pool sizes of mRNA coding for type II collagen reported in this paper, for cartilage explants maintained with and without serum in the medium. The inclusion of IGF-I in the medium of cartilage explants did not result in the maintenance of type II collagen mRNA levels compared with fetal-calf serum. This work indicates that collagen synthesis is regulated predominantly by the amount of available mRNA, although from our experiments it is not possible to determine whether the changes in mRNA levels arise from alterations in the rate of collagen gene transcription or in the stability of the mRNA transcript.

As well as increasing the synthesis of aggrecan we have shown that both serum and IGF-I also stimulate the synthesis of the other two components of the proteoglycan complex, namely link protein and hyaluronan (Curtis *et al.*, 1992). This suggests that the chondrocyte synthesizes the macromolecules that make up the proteoglycan in a co-ordinated manner, thereby retaining optimum amounts of each component of this functionally important complex. It appears that both serum and IGF-I regulate the synthesis of link protein at the level of transcription thus increasing the amount of link-protein mRNA available for translation.

It was evident that IGF-I had little effect on the pool of mRNA coding for decorin, whereas the presence of serum in the culture medium of the cartilage explants resulted in a 16-fold increase in the pool of decorin-core-protein mRNA. The latter figure is surprising since studies from our laboratory showed that the addition of serum to the culture medium of cartilage explants did not result in the stimulation of synthesis of the two small matrix proteoglycans present in the extracellular matrix of articular cartilage (McQuillan *et al.*, 1986*c*; Tesch *et al.*, 1991).

The observed increase in the pool of mRNA coding for aggrecan core protein in cartilage cultures maintained in medium with serum indicates that other growth factor(s) are present in fetal-calf serum that are capable of stimulating aggrecan synthesis by increasing the rate of transcription of the aggrecan-gene core protein. Furthermore, the apparent inability of IGF-I to maintain collagen synthesis at a similar level to that observed for serum suggests that other factor(s) are present in serum that are responsible for the maintenance of collagen synthesis.

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