Serum factors, growth factors and UDP-sugar metabolism in bovine articular cartilage chondrocytes

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1. The effect of different batches of fetal bovine serum and of growth factors on [³⁵S]sulphate incorporation into glycosaminoglycans and on UDP-sugar pools in explant cultures of bovine articular cartilage was investigated. 2. [³⁵S]Sulphate incorporation was variably stimulated between 1.2- and 3.5-fold by four different batches of serum. The UDP-glucuronate pool size expanded 4.3–6.5-fold in the presence of serum, even in those cultures in which little stimulation of [³⁵S]sulphate incorporation occurred. The UDP-*N*-acetylhexosamine and UDP-hexose pools expanded by about 1.5- and 2.0-fold respectively in the presence of serum. UDP-xylose was not detected. 3. Equilibrium-labelling and pulse-chase experiments with D-[1-³H]glucose indicated that the rate of flux through the UDP-sugar pools was unaffected by serum. UDP-hexose, UDP-*N*-acetylhexosamine and UDP-glucuronate have approximate half-lives (*t*_{1/2}) of 7, 12 and 3–4 min respectively. At equilibrium, the ³H specific activities of UDP-hexose and UDP-*N*-acetylhexosamine were very similar but that for the UDP-glucuronate pool was much higher, especially in serum-supplemented cultures. The results suggest that UDP-glucuronate synthesis occurs via a pathway which is independent of the main UDP-hexose pathway. 4. Supplementing cultures with heat-treated serum had no effect on the serum-induced

expansion of UDP-sugar pools but stimulation of [³⁵S]sulphate incorporation into glycosaminoglycans was 50 % lower than for native serum. Acid-treated serum promoted a 2-fold expansion of the UDP-glucuronate and UDP-*N*-acetylhexosamine pool over that obtained with native serum but was 20 % less effective in stimulating [³⁵S]sulphate incorporation than the latter. Prior dialysis of serum had no effect on its modulatory action on either [³⁵S]sulphate incorporation or on the size of UDP-sugar pools. 5. Insulin-like growth factor 1 (IGF-1), transforming growth factor β -1 (TGF β -1), platelet-derived growth factor (PDGF) (BB homodimer) and epidermal growth factor (EGF) all stimulated [³⁵S]sulphate incorporation into glycosaminoglycans as expected. The UDP-glucuronate pool expanded by 1.5- and 2.0-fold in the presence of IGF-1 and TGF β -1 respectively, and by about 1.8-fold in the presence of PDGF or EGF. None of the factors investigated, or combinations of IGF-1 and TGF β -1 or IGF-1 and EGF, stimulated expansion of the UDP-glucuronate pool to the same extent as native serum. 6. Overall the results indicate that the size of the UDP-glucuronate pool is controlled by an additional serum factor which is different from those which stimulate [³⁵S]sulphate incorporation into glycosaminoglycans in articular cartilage.

INTRODUCTION

The extracellular matrix of articular cartilage consists of a collagen network interspersed with proteoglycan aggregates. It is these large aggregating proteoglycans (aggrecan) which confer on cartilage its abilities to withstand wear and compressive pressure. Each aggrecan molecule (*M*_r approx. 2×10^6) is composed of a protein core (*M*_r 220000) to which the glycosaminoglycan chains, chondroitin sulphate and keratan sulphate, are covalently attached. The chains are unbranched polymers composed of repeating disaccharide units. In chondroitin sulphate, this repeating structure is *N*-acetylgalactosamine and glucuronic acid and in keratan sulphate, *N*-acetylglucosamine and galactose. The monosaccharide components are derived from the corresponding UDP-sugar nucleotides which are synthesized in the cytosol and transported into the Golgi apparatus for glycosaminoglycan chain polymerization [1–3]. As proteoglycans are constantly lost through degradation their synthesis must be continuous to maintain the structure of the cartilage matrix. A variety of serum components [4,5] and in particular the growth factors, insulin-like growth factor 1 (IGF-1) [6,7] and transforming growth factor β (TGF β) [8], are known to stimulate

glycosaminoglycan synthesis in isolated chondrocytes and cartilage explant cultures. It has been suggested that these growth factors may also modulate proteoglycan synthesis *in vivo*. The enhanced rates of proteoglycan synthesis produced by IGF-1 and TGF β are associated with increases in the core protein mRNA pools [8,9]. However, little is known about the effects of these growth factors on other parts of the proteoglycan biosynthetic pathway. The synthesis of UDP-sugars from glycolytic precursors has been characterized [10], but it is not known how this relates to the rate of glycosaminoglycan synthesis.

We have investigated whether UDP-sugar levels and rates of turnover in articular cartilage correlate with changes in glycosaminoglycan synthesis, and whether both processes are controlled by common factors.

MATERIALS AND METHODS

Tissue culture

Articular cartilage was excised from the metacarpal-phalangeal joints of adult steers (age 20–24 months), washed twice with Dulbecco's modified Eagle's medium (DMEM) containing 2 % (w/v) penicillin/streptomycin/fungizone (Gibco BRL) and

Abbreviations used: FBS, fetal bovine serum; IGF-1, insulin-like growth factor; TGF β , transforming growth factor β ; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; rh, recombinant human.

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chopped into small pieces. Cartilage explants of approx. 150 mg wet weight were incubated in flasks containing 4 ml of culture medium [DMEM containing 1% (w/v) penicillin/streptomycin/fungizone, 25 mM D-glucose, 4 mM glutamine, 1 mM pyruvate, 15 mM Hepes, 10 mM Tes, 10 mM Bes (pH 7.6)] supplemented with 20% fetal bovine serum (FBS) (ICN-Flow or Northumbria Biochemicals) or serum-free medium containing 0.1% BSA (Sigma Chemical Co. Ltd.). In some experiments, FBS from batch 9130042 (see Table 1) was either dialysed at 4 °C against 10 vol. of PBS, pH 7.4, or heated to 80 °C for 30 min, or treated with 6 M HCl to reduce the pH from 8.0 to 1.0 at 4 °C for 24 h before neutralization using 10 M NaOH. Growth factors were added to serum-free medium to give concentrations of 0–50 ng/ml recombinant human (rh) IGF-I (ICN-Flow), 0–10 ng/ml TGF β -1 [obtained from human platelets (Sigma)], 0–50 ng/ml recombinant human epidermal growth factor (rhEGF) (a gift from Dr. E. Kolettas, Charing Cross and Westminster Medical School, London, U.K.), 0–20 ng/ml recombinant human platelet-derived growth factor (PDGF) BB homodimer (Peninsula Laboratories Europe Ltd.) or 0–100 ng/ml insulin [obtained from bovine pancreas (Sigma)]. Cartilage explants were incubated for up to 7 days in a humidified atmosphere of 5% CO₂/95% air at 37 °C. The culture medium was changed daily.

Glycosaminoglycan synthesis

On days 0, 1, 5 and 7 of culture, the medium was removed and replaced with 2 ml of the appropriate medium containing 25 μ Ci/ml Na₂³⁵SO₄, and incubation continued for a further 5 h. ³⁵S-labelled glycosaminoglycans were extracted from the cartilage explants with 1 ml of 0.5 M NaOH at room temperature for 24 h. ³⁵S incorporation into glycosaminoglycans was determined by separating them from unincorporated radioactivity using Sephadex G25 (PD10) (Pharmacia) chromatography [11].

Lactate production

Lactate production in explant cultures was measured [12] after terminating the incubation with perchloric acid [13].

UDP-sugar extraction and analysis

After 0, 1, 5 and 7 days of culture, cartilage explants were placed on ice, culture medium removed, the tissue washed twice with 2 ml of water (4 °C) and UDP-sugars extracted by boiling in 2 ml of water for 15 min [14]. Supernatants, containing the UDP-sugars, were stored at –20 °C and subsequently thawed and filtered through a 0.2 μ m-pore-size filter immediately before h.p.l.c. analysis. In some experiments UDP-sugars were labelled by incubating explant cultures with medium containing D-[1-³H]glucose (50 μ Ci/ml) for various periods before boiling.

UDP-sugars were analysed using either a Spectrophysics 8700 h.p.l.c. system connected to a LC871 detector and 4370 integrator (see Table 1, Figures 1 and 2), or a Waters 501 dual-pump system linked to an NEC Powermate 1 computer integrator and Waters 484 spectrophotometer (see Tables 2 and 3 and Figures 3–5). UDP-sugars were detected at 254 nm after separation using a Partisphere 5 SAX anion-exchange column (Whatman) and a linear gradient of 10–300 mM KCl/KH₂PO₄, pH 4.0, over 35 min with a flow rate of 1 ml/min [14]. In [1-³H]glucose-labelling experiments, fractions (0.5 ml) were collected and counted for ³H

radioactivity [15]. Retention times of the UDP-sugars were: UDP-*N*-acetylglucosamine, 14.5 min; UDP-*N*-acetylgalactosamine, 15.0 min; UDP-glucose and UDP-galactose, 16.5 min; and UDP-glucuronate, 32.0 min. UDP-sugar concentrations were determined by comparison with the peak areas of known standards.

RESULTS

The effect of serum batch on UDP-sugar pool size and [³⁵S]sulphate incorporation into glycosaminoglycans

Articular cartilage explants were cultured for 5 days either without FBS, or supplemented with 20% (v/v) serum. UDP-sugar pools and [³⁵S]sulphate incorporation into glycosaminoglycans were then measured. The effects of four different batches of FBS were compared (Table 1). The UDP-glucuronate pool was consistently increased by 4.3–6.5-fold in the presence of different sera. The UDP-*N*-acetylhexosamine and UDP-hexose pools only expanded by about 1.5- and 2.0-fold respectively, with serum supplementation, while the effects on [³⁵S]sulphate incorporation were quite variable. Two batches of serum increased the incorporation of [³⁵S]sulphate into glycosaminoglycans by 3.1–3.5-fold, while with two other batches the stimulation was only 1.2–1.9-fold over non-supplemented cultures. This is likely to reflect differences in the stimulation of proteoglycan synthesis, since it seems unlikely that variations in the inorganic sulphate concentration of different serum batches could account for differences in ³⁵S incorporation of this magnitude. The UDP-glucuronate pool was expanded by 4.3 and 4.5 times in cartilage explants incubated with the two sera which had low stimulatory activity for [³⁵S]sulphate incorporation. This suggests that [³⁵S] proteoglycan synthesis and the UDP-glucuronate pool size are modulated by different serum factors. Alternatively, the mechanisms involved in expanding the pool are more sensitive to lower concentrations of a serum factor than those controlling [³⁵S]proteoglycan synthesis.

Serum stimulation of UDP-sugar pool size and [³⁵S]glycosaminoglycan synthesis in different cartilage preparations

Serum batch 9130042 was used for each experiment reported below, but different cartilage explant cultures were, of necessity, established on each occasion. Table 2 summarizes data on the serum stimulation of UDP-sugar pools and [³⁵S]sulphate incorporation for five different cartilage preparations. When cartilage explants maintained for 5–7 days in the presence of 20% FBS were compared with controls maintained in medium alone, [³⁵S]sulphate incorporation into glycosaminoglycans was increased between 3.1- and 6.2-fold in the supplemented cultures. Likewise serum always stimulated an expansion of the UDP-glucuronate pool (2.4–9.2-fold), even though the basal level (non-supplemented cultures) varied considerably between different cartilage preparations (0.18–0.74 pmol/mg wet cartilage wt). The basal levels for the UDP-*N*-acetylhexosamine and UDP-hexose pools also varied and although these were generally increased (means, 1.4- and 2.2-fold respectively), this did not occur in some cartilage preparations (Table 2).

Flux through the UDP-sugar pools in bovine articular chondrocytes

Explant cultures were maintained in medium with or without 20% (v/v) FBS present for 7 days, after which time they were labelled for various periods from 15 min to 4 h with [1-³H]glucose.

Table 1 The effect of serum batch on UDP-sugar pool size and on [³⁵S]sulphate incorporation into glycosaminoglycans in bovine articular cartilage explant cultures

Articular cartilage explants were cultured for 5 days in either medium alone or in medium supplemented with four different batches of FBS (20% v/v). UDP-sugars and [³⁵S]sulphate incorporation were measured on day 5 on duplicate cultures. The UDP-sugar ratios are for the mean values shown for serum-treated cultures.

Serum batch	UDP-sugar pool size (pmol/mg wet wt.)				[³⁵ S]Sulphate incorporation (d.p.m./mg wet wt. per 5 h)
	UDP-GlcNAc	UDP-GalNAc	UDP-Glc/Gal	UDP-GlcU	
No serum	1.5	0.87	2.68	0.24	1939 ± 175
9130122	2.10	1.23	6.36	1.56	5954 ± 322
9130042	2.11	1.44	5.33	1.32	6876 ± 168
9130092	1.77	0.97	5.10	1.04	2360 ± 510
9130072	2.26	1.72	5.57	1.09	3609 ± 299
Mean ± S.E.M.	2.06 ± 0.10	1.34 ± 0.11	5.59 ± 0.27	1.25 ± 0.21	4999
Serum stimulation (fold)	1.4	1.5	2.1	5.2	2.6
UDP-sugar ratios	1.00	0.65	2.71	0.61	

Table 2 Serum stimulation of UDP-sugar pool size and [³⁵S]sulphate incorporation into glycosaminoglycans in different cartilage preparations

Different articular cartilage explant preparations were used for each experiment. Each was maintained for 5 (expt. 1) or 7 days (expts. 2–5) in medium alone (a) or in medium supplemented with 20% FBS, batch 9130042, (b), after which UDP-sugar pools and [³⁵S]sulphate incorporation into glycosaminoglycans were measured. [³⁵S]Sulphate incorporation is expressed as the increase (fold) in ³⁵S incorporated (d.p.m.) into glycosaminoglycans in serum-supplemented cultures compared with that in serum-free cultures. The table also shows the identity of each cartilage preparation for experiments reported in this paper.

Experiment	Table or Figure	UDP-sugar pool size (pmol/mg wet wt.)						Stimulation of [³⁵ S]sulphate incorporation (fold)
		UDP-HexNAc		UDP-Hex		UDP-GlcU		
		(a)	(b)	(a)	(b)	(a)	(b)	
1	Table 1	2.4	3.6	2.7	5.3	0.24	1.32	3.6
2	Figure 1	2.5	2.5	2.9	4.7	0.18	0.92	5.3
3	Figure 2	6.0	6.0	6.6	5.9	0.65	1.58	5.0
4	Figures 3/4	1.4	4.8	3.0	13.5	0.74	2.36	3.1
5	Figure 5	1.4	2.8	3.2	8.7	0.32	2.95	6.2
Mean increase (fold)		—	1.4	—	2.2	—	5.1	4.6

Table 3 Rates of flux of the UDP-sugar pools in cartilage explants cultured in the presence or absence of FBS

(a) Rates of UDP-[³H]sugar synthesis were assessed by labelling explants on day 7 of culture with [1-³H]glucose for 0.25, 0.5, 1.0, 2.0 and 4 h, after which the levels and specific activities of the UDP-N-acetylhexosamines (UDP-HexNAc), UDP-glucose/galactose (UDP-Glc/Gal) and UDP-glucuronate (UDP-GlcU) pools were measured. The half-time ($t_{1/2}$) taken to equilibrate each pool to a constant ³H specific activity was calculated. (b) Rates of UDP-sugar turnover ($t_{1/2}$) were measured by labelling cartilage explants to constant specific activity by incubating with [1-³H]glucose for 2 h. Explants were then washed with several changes of fresh medium and the ³H radioactivity remaining measured after 0, 10, 20, 40 and 60 min.

(a) Equilibration of UDP-sugar pools with [1- ³ H]glucose		³ H specific activity at equilibrium (d.p.m./pmol)	Half-time ($t_{1/2}$) to equilibrium (min)
UDP-sugar	Culture conditions ± FBS		
UDP-HexNAc	—	0.96	7.5
	+	1.03	6.0
UDP-Glc/Gal	—	0.91	7.5
	+	0.93	4.5
UDP-GlcU	—	1.97	3.8
	+	4.80	3.0
(b) Disappearance of radioactivity from [1- ³ H]glucose-equilibrated UDP-sugar pools		Half-life ($t_{1/2}$) of UDP-[³ H]sugar (min)	
UDP-sugar	Culture conditions ± FBS		
UDP-HexNAc	—	12.25	
	+	12.50	
UDP-Glc/Gal	—	6.25	
	+	6.75	
UDP-GlcU	—	21.5	
	+	11.0

Figure 1 Effects of heat treatment of serum on $[^{35}\text{S}]$ sulphate incorporation into glycosaminoglycans and on UDP-sugar pools

Explants were cultured for 7 days in medium containing either 20% native FBS (○), 20% FBS heated to 80 °C for 30 min (●), or in medium without FBS (□). $[^{35}\text{S}]$ Sulphate incorporation (a), UDP-glucuronate (b), UDP-N-acetylhexosamines (c) and UDP-hexose (d), were measured on days 0, 1, 5 and 7 of culture. Results are the means for three cultures $\pm$ S.E.M. Where no bars are shown the S.E.M. is too small to show over the data symbol.

UDP-sugars were analysed at each point and the half time ($t_{1/2}$) taken to reach a constant ^3H specific activity was calculated from a semi-logarithmic plot of the latter against labelling time, as described previously for cultures of Swarm chondrocytes [15].

The rate of incorporation of $[^3\text{H}]$ glucose into each of the three UDP-sugar pools is little affected by the presence or absence of serum in the culture, as shown by the $t_{1/2}$ values (Table 3). The UDP-N-acetylhexosamine and UDP-hexose pools reached a constant ^3H specific activity within 1 h and 30 min respectively, irrespective of the presence or absence of serum, and these equilibrium activities were numerically similar for both pools (Table 3a). The UDP-glucuronate pool equilibrated very rapidly in both serum-supplemented and non-supplemented cultures and attained constant specific activity within a 30 min labelling period. Surprisingly its ^3H specific activity was higher than those of the UDP-N-acetylhexosamine and UDP-hexose pools, especially in serum-stimulated cultures (Table 3a).

The rate of utilization of UDP-sugars was measured by

labelling them with $[^3\text{H}]$ glucose to a constant specific activity over a 2 h period and analysing their radioactivity during a subsequent chase of 0, 10, 20, 40 and 60 min (Table 3b). The turnover rate of the UDP-hexose pool ($t_{1/2}$ 6–7 min) was similar to that for its synthesis and was unaffected by serum. UDP-N-acetylhexosamine has a longer half-life (12–13 min) than that derived from equilibrium rates and again this is not affected by serum. Ideally the half-time to equilibrium (Table 3a) should be the same as the half-life for turnover (Table 3b) of these two UDP-sugars. However, the half-lives derived from the turnover experiments are likely to be a more accurate indicator of flux through the pools as they are derived from a greater number of data points over a short period.

In contrast, the half-life for the UDP-glucuronate pool from label-chase experiments will be a less accurate indicator of pool flux than data from equilibration times. This pool is likely to be topped up with ^3H -labelled precursor (UDP- $[^3\text{H}]$ glucose) during the chase. In the case of the UDP-hexose and UDP-N-acetyl-

Figure 2 Effects of acid treatment of serum on $[^{35}\text{S}]$ sulphate incorporation into glycosaminoglycans and on UDP-sugar pools

Explants were cultured for 7 days in medium containing either 20% native FBS (○) or 20% acid-treated FBS (●), or medium alone (□). $[^{35}\text{S}]$ sulphate incorporation (a), UDP-glucuronate (b), UDP-N-acetylhexosamines (c) and UDP-hexose (d), were measured on days 0, 1, 5 and 7 of culture. Results are the means for three cultures $\pm$ S.E.M. (* $P < 0.020$).

hexosamine pools, the precursor, $[^3\text{H}]$ glucose, can be washed out of the explants effectively after labelling. This may explain the wide discrepancy between the $t_{1/2}$ values for UDP-glucuronate in the equilibration and turnover experiments (Table 3). Moreover, the apparent difference in the turnover time of UDP-glucuronate in the presence ($t_{1/2}$ 11 min) and absence ($t_{1/2}$ 22 min) of serum may reflect topping-up effects rather than differences in turnover rate. Since the UDP-glucuronate pool is expanded 5-fold in the presence of serum, continued input of UDP- $[^3\text{H}]$ glucose into it during the chase would have less effect on its level of radioactivity than input into the much smaller pool in serum-free medium. Thus for UDP-glucuronate the $t_{1/2}$ for equilibration from $[^3\text{H}]$ glucose is likely to be a more accurate indicator of flux through the pool than the $t_{1/2}$ for turnover.

In summary, taking account of both sets of data (Table 3a,b),

the best estimates of half-lives for UDP-hexose, UDP-N-acetylhexosamine and UDP-glucuronate are about 7, 12 and 3–4 min respectively, and these undergo little or no change in the presence of serum.

Distinguishing serum factors which expand UDP-sugar pools from those which stimulate $[^{35}\text{S}]$ sulphate incorporation into glycosaminoglycans

The extra requirement for UDP-hexose and UDP-N-acetylhexosamine in cultures where $[^{35}\text{S}]$ proteoglycan synthesis is stimulated by a serum factor(s) is met by limited expansion of the precursor pools without any apparent change in the rate of flux through them. A similar but much larger expansion of the UDP-glucuronate pool accommodates the increased demand for that

sugar. Next we addressed the question as to whether the same serum factor(s) stimulates both proteoglycan synthesis and UDP-sugar pool expansion. FBS was treated by dialysis, heat, or acidification before using it to maintain cartilage explants in culture. UDP-sugar pools and [35 S]sulphate incorporation into glycosaminoglycans were then measured in newly prepared cartilage explants (day 0) and after 1, 5 and 7 days culture in medium containing 20% (v/v) native FBS, treated FBS, or no FBS.

Heat treatment of serum had no effect on the size of any of the UDP-sugar pools compared with controls cultured with native serum, but it reduced the stimulation of [35 S]sulphate incorporation into glycosaminoglycans by 50% (Figure 1). Thus UDP-sugar pool sizes are modulated by a heat-stable serum factor while a significant proportion of the stimulus for proteoglycan synthesis is heat labile. The rise in [35 S]proteoglycan synthesis on day 1 in explants maintained in medium alone has been observed previously and is due to culturing in a 95% air/5% CO₂ atmosphere since it does not occur under anaerobic conditions [14].

Prior acid treatment of FBS intensified its stimulatory effects on the expansion of UDP-sugar pools (Figure 2). By day 7 the UDP-glucuronate and UDP-*N*-acetylhexosamine pools had expanded by approx. 2-fold over those in explant cultures maintained in normal serum. UDP-hexoses increased, but to a lesser extent. In contrast [35 S]sulphate incorporation into glycosaminoglycans was 20% lower in cultures maintained in acid-treated serum than in those maintained in native serum (Figure 2).

Incubation of cartilage explants in medium supplemented with 20% (v/v) dialysed FBS had no effect on either UDP-sugar pools or glycosaminoglycan synthesis when compared with control cultures supplemented with native serum (results not shown). The dialysis membrane had a relative molecular mass cut-off of 12000–14000, so this shows that the serum factors which modulate UDP-sugar pool size and glycosaminoglycan synthesis are either macromolecular or complexed with other serum components.

UDP-xylose was rarely detected in either fresh articular cartilage or in explants cultured under any of the above conditions.

Effects of growth factors on UDP-sugar pools and [35 S]sulphate incorporation into glycosaminoglycans

Reduced stimulation of [35 S]sulphate incorporation in bovine articular cartilage explants by heat-treated or acid-treated serum is in accord with previous reports that IGF-1 stimulates proteoglycan synthesis in the chondrocytes [5,16,17]. IGF-1 is heat labile at 80 °C [5] and exists in serum as a large (150000-*M_r*) acid-dissociable complex [18]. However, expansion of the UDP-sugar pools is stimulated by a serum factor(s) which is heat stable and potentiated by acid treatment. TGF β -1 is found in serum, is heat stable and is activated by low pH [19]. Thus we investigated the effects of IGF-1 and TGF β -1 in the explant cultures. The actions of PDGF, EGF and insulin were also investigated. Cultures were maintained in either medium alone, medium supplemented with various concentrations of growth factors or in medium plus 20% (v/v) FBS, for 7 days.

A maximum 3-fold stimulation of [35 S]sulphate incorporation into glycosaminoglycans occurred with either 50 ng/ml IGF-1 or 20% FBS, in agreement with previous reports (Figure 3). IGF-1 induced only a 1.5-fold increase in either the UDP-glucuronate or UDP-*N*-acetylhexosamine pool sizes, while 20% FBS stimu-

Figure 3 Effects of IGF-1 on [35 S]sulphate incorporation into glycosaminoglycans and on UDP-sugar pools

Explants were cultured in serum-free medium containing 0–50 ng/ml IGF-1 or in medium containing 20% FBS. [35 S]sulphate incorporation (a), UDP-glucuronate (b), UDP-*N*-acetylhexosamines (c) and UDP-hexose (d) were measured on day 7 of culture. Results are the means for three cultures $\pm$ S.E.M. (**P* = < 0.010 when compared with controls lacking IGF-1 or FBS).

Table 4 Lactate production in IGF-1- and FBS-treated cartilage explant cultures

Cartilage explants were cultured as described in Figure 3 and rates of lactate production measured on day 7 of culture. Results are the means for three cultures $\pm$ S.E.M. (**P* = < 0.010, when compared with controls incubated in medium alone).

IGF-1 concentration (ng/ml)	Lactate production (nmol/mg wet cartilage wt. per h)
0	1.91 $\pm$ 0.13
1	1.66 $\pm$ 0.22*
10	1.68 $\pm$ 0.26*
20	1.85 $\pm$ 0.10
50	1.95 $\pm$ 0.26
20% FCS	2.74 $\pm$ 0.15*

lated a 3-fold expansion. However, the UDP-hexose pool expanded 3–4-fold in the presence of either IGF-1 or FBS (Figure 3). This was accompanied by an apparent increase in the rate of glycolysis in FBS-supplemented cultures but not in IGF-1-supplemented cultures, as indicated by changes in lactate production (Table 4). Increased uptake of pyruvate from the culture medium and its reduction to lactate could contribute to the changes in the FBS-treated cultures. Since energy metabolism in the bovine cartilage explants is anaerobic [14], the failure of

Figure 4 Effects of TGF β -1 on [^{35}S]sulphate incorporation into glycosaminoglycans and on UDP-sugar pools

Explants were cultured in serum-free medium containing 0–10 ng/ml TGF β -1 or in medium containing 20% FBS. [^{35}S]Sulphate incorporation (a), UDP-glucuronate (b), UDP-N-acetylhexosamine (c) and UDP-hexose (d) were measured on day 7 of culture. Results are the means for three cultures $\pm$ S.E.M. (* P = < 0.010 when compared with controls lacking IGF-1 or FBS).

IGF-1 to stimulate glycolysis shows that the basal level of ATP and precursor (glucose 1-phosphate) is sufficient to accommodate up to 3-fold expansion of the UDP-hexose pool.

TGF β -1 has been identified as a modulator of proteoglycan synthesis in bovine articular cartilage [8]. At 3 ng/ml it induced a 4-fold increase in [^{35}S]sulphate incorporation into glycosaminoglycans while 20% FBS stimulated a 3-fold increase (Figure 4). It also promoted an approx. 2-fold expansion of each of the UDP-sugar pools, but in every case this was less than the expansion stimulated by 20% FBS (Figure 4). Thus, while TGF β is likely to be one of the factors in serum which promotes an increase in the UDP-sugar pools, it does not by itself precisely mimic the effects of whole serum.

To investigate whether other growth factors could modulate the size of UDP-sugar pools and/or stimulate [^{35}S]sulphate incorporation into glycosaminoglycans, cartilage explants were cultured in either medium alone, or in medium supplemented with PDGF (BB-homodimer, 0–20 ng/ml), EGF (0–50 ng/ml), insulin (0–100 ng/ml) or 20% FBS. The responses to PDGF (20 ng/ml, Figure 5) and EGF (50 ng/ml, results not shown) were essentially the same. [^{35}S]Sulphate incorporation into glycosaminoglycans was stimulated by approx. 3-fold, but this was

Figure 5 Effects of PDGF on [^{35}S]sulphate incorporation into glycosaminoglycans and on UDP-sugar pools

Explants were cultured for 7 days in serum-free medium containing 0–20 ng/ml PDGF or in medium containing 20% FBS. [^{35}S]Sulphate incorporation (a), UDP-glucuronate (b), UDP-N-acetylhexosamine (c) and UDP-hexose (d) were measured on day 7 of culture. Results are means for three cultures $\pm$ S.E.M.

only about half the stimulation achieved with 20% FBS (Figure 5). The UDP-glucuronate pool expanded by 1.8-fold in the presence of either growth factor, much less than the expansion which occurred in serum. The UDP-N-acetylhexosamine and UDP-hexose pools were expanded by about 2-fold and to a size similar to that present in FBS-supplemented cultures.

Insulin had no effect on either the size of the UDP-sugar pools or on [^{35}S]sulphate incorporation (results not shown).

In other experiments, cartilage explants were cultured with combinations of either IGF-1 (50 ng/ml) and TGF β -1 (5 ng/ml) or with IGF-1 (50 ng/ml) and EGF (50 ng/ml). No additive effects on either [^{35}S]sulphate incorporation or UDP-sugar pool sizes were observed with IGF-1 and TGF β . However, the combination of IGF-1 and EGF stimulated [^{35}S]sulphate incorporation into glycosaminoglycans to greater levels than either growth factor alone, and by 34% more than the level present in cultures supplemented with 20% FBS for 7 days. The sizes of the UDP-sugar pools in cultures treated with a combination of IGF-1 and EGF were, however, not additive when compared with those induced by the individual factors.

DISCUSSION

Serum factors stimulate proteoglycan synthesis in bovine articular chondrocytes by increasing the amount and stability of core protein mRNA and the activity of glycosyl transferases. IGF-1 and TGF β -1 have been identified as stimulatory serum components [4–8]. More recently, PDGF has also been identified as a stimulatory factor [20]. The degree of response to PDGF was

dependent on the age of the animal from which the cultures were established, and this may be the case for other growth factor effects on articular cartilage. Elevated levels of proteoglycan synthesis demand an increased supply of UDP-sugars for polymerizing the glycosaminoglycan chains of these molecules. This could be met by increasing the flux through appropriate UDP-sugar pools without increasing their size. This occurs in serum or insulin-stimulated cell cultures of Swarm chondrosarcoma chondrocytes [15]. Alternatively, an increased supply of UDP-sugars could be provided by expanding UDP-sugar pool sizes without any change in the rate of flux through the pool. The present study indicates that this latter mechanism operates in chondrocytes in serum-stimulated explant cultures of bovine articular cartilage. The UDP-*N*-acetylhexosamine and UDP-hexose pools expand by about 1.5- and 2.0-fold, while the UDP-glucuronate pool increases by about 5-fold. There is no firm evidence for an increased rate of flux through the expanded pools. The increased UDP-sugar pools are not due to increased numbers of cells as serum does not stimulate cell division in these explant cultures [4].

Both IGF-1 and TGF β stimulated increases in proteoglycan synthesis in the explant cultures comparable with levels achieved with 20% FBS, which is in agreement with previous reports [6–9]. However, neither factor alone, nor combinations of them, promoted the approx. 5-fold expansion of the UDP-glucuronate pool which occurred consistently in serum-supplemented cultures. PDGF and EGF, which expand the UDP-glucuronate pool in fibroblasts [21], stimulated a 1.8-fold enlargement in the explants. Insulin had no effect on the pool in bovine chondrocytes. Thus the size of this pool must be controlled, at least in part, by a serum factor(s) in addition to those known to stimulate proteoglycan synthesis. The observation that the level of stimulation of proteoglycan synthesis depends on serum batch, while expansion of the UDP-glucuronate pool does not, provides further evidence for this. Moreover, the UDP-glucuronate pool size increased in response to a serum factor(s) which is heat-stable and activated by acid treatment, whereas stimulation of proteoglycan synthesis was reduced after heat- or acid-treatment of serum.

UDP-glucuronate is synthesized by oxidation of UDP-glucose and is itself decarboxylated to form UDP-xylose [10]. Experimentally, the latter is a feedback inhibitor of UDP-glucose dehydrogenase [22] although, due to its very low concentration in chondrocytes, we have questioned whether this is of significance *in vivo* [15]. UDP-xylose was barely detectable in bovine articular cartilage and could not be quantified, a situation similar to that found for Swarm chondrosarcoma chondrocytes [15]. Nevertheless, the large and consistent expansion of the UDP-glucuronate pool in serum-stimulated bovine explant cultures indicates that its biosynthesis can certainly be modulated by extrinsic factors. UDP-glucuronate pool size in the explants was unaffected by oxygen tension in the range 6–24% [14].

Two features of UDP-glucuronate synthesis in bovine articular cartilage are especially noteworthy. First, equilibration of the UDP-glucuronate pool from the precursor [$1\text{-}^3\text{H}$]p-glucose to a constant level of radioactivity is more rapid ($t_{\frac{1}{2}}$ 3–4 min), in either serum-free, or serum-stimulated conditions, than for UDP-glucose ($t_{\frac{1}{2}}$ approx. 7 min). This differs from Swarm chondrocyte cell cultures [15] where the equilibration time for both pools was the same ($t_{\frac{1}{2}}$ approx. 12 min). Secondly, in bovine explant cultures, the ^3H specific activity of the UDP- ^3H glucuronate pool at equilibrium is higher, particularly in serum-stimulated cultures, than that of the UDP- ^3H glucose pool. Again this differs from the Swarm chondrocytes where both UDP-sugar pools had the same ^3H specific activity [15]. The results suggest that there may

be a dedicated pathway in bovine chondrocytes, glucose, glucose 1-phosphate, UDP-glucose, UDP-glucuronate, operating as a separate entity from a similar pathway leading to the formation of the main UDP-glucose pool. Presumably the two pathways are compartmentalized separately. There is evidence for two separate cytosolic pools of glucose 6-phosphate in rat hepatocytes, one linked to glycogenolysis, the other to gluconeogenesis [23]. Moreover, hepatic UDP-glucuronate destined for the glucuronidation of xenobiotics is derived predominantly from glucose 6-phosphate generated from glycogenolysis rather than from exogenous glucose [24]. It seems unlikely that this is a major route for synthesis of UDP-glucuronate in bovine chondrocytes because of the rapid equilibration of the sugar nucleotide pool with exogenous [^3H]glucose.

The rapid equilibration and high ^3H specific activity of the UDP-glucuronate pool in bovine chondrocytes would be compatible with its separate UDP-glucose precursor pool being relatively small and dependent on an increased input from exogenous glucose following stimulation with the serum factor(s). The main, or general, UDP-hexose pool is large compared with the UDP-glucuronate pool (Table 1) and its lower ^3H specific activity (Table 3) suggests that it may be diluted with non-radioactive glucose precursors produced in the bovine chondrocytes. The pentose phosphate shunt decarboxylates glucose at C-1 and, potentially, could produce non-radioactive glucose in the non-oxidative part of its pathway. The pentose phosphate pathway is operative in chondrocytes [13], including those of bovine articular cartilage explant cultures (D. Goh and R. M. Mason, unpublished work).

The way in which serum factors promote expansion of the UDP-glucuronate pool and, to a lesser extent, the UDP-hexose and UDP-*N*-acetylhexosamine pools in bovine chondrocytes is unknown. Serum, insulin, PDGF and EGF have been shown to promote uridine uptake, concomitant with an increase in UDP-glucuronate levels in cultured fibroblasts [21]. IGF-1 promotes an increase in glucose uptake by a variety of cells [19], but we were unable to show an increase in the glycolytic rate in bovine explant cultures supplemented with the growth factor. Similarly, TGF β -1 is known to stimulate glucose uptake in fibroblasts [25]. In addition to increasing the uptake of precursors for UDP-glucuronate synthesis, serum stimulatory factors may induce increases in the expression, or activation, of specific enzymes such as UDP-glucose dehydrogenase. This enzyme appears to be inducible in other situations [26].

Osborn et al. [27] reported that EGF (10 ng/ml) and insulin (5 $\mu\text{g}/\text{ml}$) stimulated a 7-fold and 8-fold increase in [^{35}S]-glycosaminoglycan synthesis in bovine articular cartilage. The maximum increase with EGF (50 ng/ml) in our experiments was about 3-fold and no stimulation was observed with the concentrations of insulin used (0–100 ng). EGF is unlikely to be an important component of the serum in stimulating increased proteoglycan synthesis. As its relative molecular mass is 6100 [28] it would have been lost on dialysis and dialysed serum promoted the same increase in proteoglycan synthesis as non-dialysed serum. Nevertheless, the action of EGF on articular chondrocytes is of interest. Its stimulation of proteoglycan synthesis was additive to that of IGF-1, suggesting action on the cell via a different pathway. EGF receptors have been demonstrated on cultured rabbit costal cartilage chondrocytes [29].

The effects of serum on the proteoglycan biosynthetic pathway in articular cartilage are complex and include actions on gene transcription, mRNA stability and increasing the availability of biochemical precursors such as the relevant UDP-sugars. No single serum component accounts for all these activities. Even where a specific component promotes a specific effect, the

significance of its role *in vivo* may be difficult to interpret. For example, the actions of IGF-1 may be regulated by binding to specific carrier proteins in the serum [18,30,31], the bound form being less effective in stimulating proteoglycan synthesis in bovine cartilage cultures than the free form [32]. The concentration of IGF-1 (approx. 0.5 µg/ml) in the serum used in experiments *in vitro* is approx. 500-fold greater than is found in cartilage itself [33]. Moreover, while serum IGF-1 is present mainly as a complex it is not known whether this can penetrate the cartilage matrix to reach cell receptors or whether it dissociates in the environment of the matrix to release free IGF [32].

The results of the present *in vitro* investigation show that FBS contains a factor(s) which has a very pronounced effect on expanding the UDP-glucuronate pool. They suggest that a dedicated pathway leading to the formation of this precursor can be induced by an extrinsic factor which is different in its properties to growth factors which are currently known to stimulate proteoglycan synthesis in chondrocytes. The importance of this in the *in vivo* situation, however, remains to be determined.

We are grateful to the Arthritis and Rheumatism Council (U.K.) for financial support and to the Royal Society for a grant for h.p.l.c. equipment.

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