The effects of trypsin treatment on proteoglycan biosynthesis by bovine articular cartilage

Jennifer S. BARTHOLOMEW, Christopher J. HANDLEY and Denis A. LOWTHER
Department of Biochemistry, Monash University, Clayton, Victoria 3168, Australia

(Received 11 September 1984/Accepted 13 December 1984)

The effects of mild or severe trypsin treatment of bovine articular-cartilage slices in tissue culture were studied by monitoring the incorporation of [35 S]sulphate into proteoglycans. Moderate trypsin treatment caused a subsequent marked inhibition of proteoglycan biosynthesis, which was reversible with time. Analysis on Sepharose CL-2B of the proteoglycan species synthesized showed that, directly after trypsin treatment, there was a 30% increase in the synthesis of the low- M_r proteoglycan (K_{av} 0.71), and the total decrease in proteoglycan biosynthesis was reflected in a decrease in the synthesis of the high- M_r proteoglycan species (K_{av} 0.31). The small proteoglycan was partially characterized and shown to be a true biosynthetic product and not a breakdown product. Trypsin treatment (20 μ g/ml per 100 mg of tissue) of cartilage slices also resulted in an increase in the glycosaminoglycan chain size of the large proteoglycan, but not of the small proteoglycan.

Proteoglycans are a major component of the extracellular matrix of cartilage, and by virtue of their size and highly polyanionic nature they are capable of exerting large hydrodynamic pressures within the collagen network, providing cartilage with the ability to withstand compressive forces. Cartilage proteoglycans comprise a central core-protein to which are covalently attached chondroitin sulphate and keratan sulphate glycosaminoglycan chains via glycosidic linkages (Heinegård & Axelsson, 1977). Most proteoglycans isolated from cartilage are polydisperse species (Heinegård, 1977) with an average M_r of 1×10^6 – 2×10^6 (Hascall & Sajdera, 1970), existing as high- M_r aggregates with hyaluronic acid (Hardingham & Muir, 1973; Hascall & Heinegård, 1974*a,b*; Hardingham *et al.*, 1976), several proteoglycan monomers interacting with hyaluronic acid via specific hyaluronic acid-binding sites in the core protein.

Heinegård & Hascall (1979) have reported the isolation of non-aggregating proteoglycans and, more recently, small non-aggregating proteoglycans of low buoyant density have been reported in articular cartilage (Heinegård *et al.*, 1981) and from chick embryonic cartilage cultures (Kimata *et al.*, 1978). The chondrocyte is actively involved in the regulation of matrix proteoglycan biosynthesis in order to maintain a steady-state amount of proteoglycan under the influence of environmental, hormonal, mechanical and other stimuli.

Pathological changes observed in articular cartilage in both degenerative and inflammatory arthritis are thought to be primarily attributed to increased enzymic activity within the cartilage. Studies utilizing mild depletion of the matrix proteoglycan by enzymic treatment (Bosman, 1968; Milroy & Poole, 1974) resulted in a stimulation of chondrocyte biosynthesis.

The following investigation was designed to study the effects of trypsin treatment of articular cartilage in culture on the synthesis of proteoglycans by the chondrocytes.

Experimental

Materials

Benzamidine hydrochloride, phenylmethanesulphonyl fluoride, 6-aminohexanoic acid, soya-bean trypsin inhibitor (type I-S), trypsin (EC 3.4.21.4; bovine pancreas, type III) and collagenase (*Clostridium histolyticum*, type VII; EC 3.4.24.3) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Chondroitinase ABC was kindly given by Dr. H. C. Robinson, Department of Biochemistry, Monash University. Sepharose CL-2B, CL-6B, Sephadex G-50 and DEAE-Sephacel were purchased from Pharmacia, Uppsala, Sweden. CsCl (analytical grade) was from Nova Chemicals, Melbourne, Australia. Tissue-culture medium was a modification of Dulbecco's Modi-

fied Eagle's Medium containing 1 g of D-glucose/l and supplemented with organic buffers and amino acids as described by Handley & Lowther (1977). Adult bovine serum was prepared from fresh blood by removing the serum after clotting for 1 h at 25°C, centrifugation at 10000g for 30 min and sterilized by passage through a 0.45 µm microporous filter. Hyaluronic acid (Healon) was purchased from Pharmacia. [³⁵S]Sulphate (sp. radioactivity 5 Ci/µg) was supplied by Amersham International, Amersham, Bucks., U.K., and L-[4,5-³H(n)]-leucine (sp. radioactivity 5 Ci/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A.

Tissue-culture conditions

Bovine articular cartilage was removed aseptically from the metacarpal-phalangeal joints of 1–2-year-old steers, directly after slaughter. Tissue (3 g per joint) was washed three times in medium, diced into 3 mm² slices, weighed and then maintained in medium containing 20% (v/v) adult bovine serum for 5 days before enzyme treatment. On day 6 in culture (hereafter called day 1) tissue was distributed into 100 mg portions into sterile plastic vials containing 4 ml of medium. Unless otherwise stated, all medium contained 20% adult bovine serum.

Enzyme treatment of tissue

Duplicate tissue samples (100 mg) were washed three times in serum-free medium and finally resuspended in 2 ml of serum-free medium. All trypsin solutions were sterilized by passing through a 0.45 µm microporous filter. Trypsin was added to duplicate samples in 20 µl of medium to give final concentrations of 5 µg/ml, 20 µg/ml or 1 mg/ml. Tissue was exposed to the enzyme for 16 h, the medium was removed and the tissue was washed three times in medium containing 20% adult serum, until no trypsin activity could be detected. The tissue was finally resuspended in 4 ml of medium containing serum. Tissue was then maintained in culture for periods up to 9 days, and proteoglycan biosynthesis estimated at various time intervals by measuring the incorporation of [³⁵S]sulphate into glycosaminoglycans.

Measurement of proteoglycan biosynthesis by the incorporation of [³⁵S]sulphate

Tissue (100 mg) was preincubated in fresh medium for 1 h, and the medium was then replaced with 2 ml of medium containing 12–22 µCi of [³⁵S]sulphate/ml. Incubations were terminated after 2 h by removing the medium and extracting the tissue with 2 ml of 4 M-guanidine hydrochloride containing 2 mM-phenylmethanesulphonyl fluoride, 1 mg of soya-bean trypsin inhibitor/ml, 2 mM-N-ethylmaleimide and 5 mM-benzamidine hydro-

chloride. The proteoglycans extracted by guanidine hydrochloride were normally analysed by gel chromatography on Sepharose CL-2B columns. Since 4 M-guanidine hydrochloride extracts a maximum of 50–60% of the total cartilage proteoglycans, an attempt was made to increase the extraction by treating the tissue with bacterial collagenase. Since it could be argued that trypsin treatment could alter the extractability of the tissue, it was necessary to demonstrate that the remaining 40% of proteoglycans comprised a similar population to those initially extracted by guanidine hydrochloride. After radiolabelling, the tissue samples were rapidly freeze-thawed three times on ethanol/solid CO₂, thawed and digested for 6 h with collagenase (5 units) in the presence of 2 mM-phenylmethanesulphonyl fluoride, 2 mM-N-ethylmaleimide and 1 mg of soya-bean trypsin inhibitor/ml. The tissue was then extracted in guanidine hydrochloride as described above. Tissue samples were further extracted in 0.5 M-NaOH for 24 h. [³⁵S]Sulphate-labelled proteoglycans were isolated from the extracts by gel filtration on Sephadex G-25 columns (0.5 cm × 5.5 cm) as previously described (Stevens *et al.*, 1981). Samples (1 ml) of the eluate were analysed for radioactivity in a Philips liquid-scintillation counter, by adding 8 ml of a scintillation 'cocktail' [toluene/Triton X-100 (1:1, v/v) containing 8 g of 2,5-diphenyloxazole/litre]. All experimental points were obtained from duplicate or triplicate cultures, and each experiment was repeated three times.

Dual-labelling of proteoglycans with [³⁵S]sulphate and [³H]leucine

The amino acid composition of the high-*M_r* aggregating proteoglycan and the low-*M_r* non-aggregating proteoglycan have been demonstrated to be quite distinct (Heinegård *et al.*, 1981), the small proteoglycan having a considerably higher content of leucine. A dual-label experiment was therefore designed to distinguish these two proteoglycans.

The incorporation of both [³⁵S]sulphate and [³H]leucine into proteoglycans after trypsin treatment was determined. Treated or control tissue (1 g) was incubated for 3 h at 37°C in 5 ml of medium containing [³H]leucine (140 µCi/ml) and [³⁵S]sulphate (100 µCi/ml). The incubation was terminated by extraction in 4 M-guanidine hydrochloride containing proteinase inhibitors for 48 h. The extract was applied to a Sephadex G-50 column (0.8 cm × 30 cm) and eluted with 7 M-urea/0.2 M-NaCl, pH 6.0, to remove unincorporated radioactivity. Fractions containing [³H]leucine and [³⁵S]sulphate in macromolecular form were pooled and applied to a DEAE-Sephacel column (10 ml bed volume) equilibrated in 7 M-urea/0.15 M-

NaCl/0.05M-sodium acetate, pH 6.0. The column was washed with 3 bed volumes of the same buffer and then eluted with 2 bed volumes of buffer containing 2M-NaCl. Isolated proteoglycans (Chang *et al.*, 1983) were dialysed into 4M-guanidine hydrochloride/0.05M-sodium acetate, pH 6.0, and chromatographed on a Sepharose CL-2B column under dissociative conditions as described below. Fractions were analysed for [35 S]sulphate and [3 H]leucine to establish the ratio of these radioisotopes incorporated into each proteoglycan species.

Column chromatography

[35 S]Sulphate-labelled proteoglycans were analysed on a Sepharose CL-2B column (1.5cm $\times$ 90cm), eluted at 7ml/h either in 4M-guanidine hydrochloride/0.1M-Na₂SO₄/0.05M-sodium acetate buffer, pH 6.0 (dissociative conditions), or in 0.5M-sodium acetate/0.02% bovine serum albumin, pH 7.0 (associative conditions), for aggregation studies. Normally 1 ml samples of each guanidine hydrochloride extract were applied to the column and 1.5 ml fractions collected. For aggregation studies, proteoglycan samples were eluted first on a column under dissociative conditions, fractions were pooled, and guanidine hydrochloride was removed by an Amicon ultrafiltration cell (PM 10 membrane). The concentrate was then applied to a Sepharose CL-2B column (1.5cm $\times$ 90cm) eluted with 0.5M-sodium acetate in the presence of both 0.8mg of unlabelled aggregate isolated from the Swarm rat chondrosarcoma (Faltz *et al.*, 1979) and 10 μ g of high-*M*_r hyaluronic acid.

Glycosaminoglycan chains were prepared by alkaline borohydride treatment (Hopwood & Robinson, 1973). Pooled fractions from a Sepharose CL-2B column eluted under dissociative conditions were dialysed into 0.1M-sodium acetate, pH 7.0, and diluted with an equal volume of 1M-NaOH/2M-NaBH₄ and incubated at 37°C for 36h. Samples were neutralized with acetic acid and then applied to a Sepharose CL-6B column (0.8cm $\times$ 90cm) eluted at 4ml/h in 4M-guanidine hydrochloride buffer, pH 6.0. Fractions (1 ml) were analysed for radioactivity.

Degradation of [35 S]sulphate-labelled cartilage by trypsin

Bovine cartilage was incubated for 6h with [35 S]sulphate (20 μ Ci/ml) on day 1. The tissue was washed three times with serum-free medium and then treated with 1mg of trypsin/ml for 16h in culture. The amount of hexuronic acid released into the medium was monitored (Bitter & Muir, 1962) and the amount of [35 S]sulphate-labelled proteoglycan released into the medium was

measured by gel filtration on Sephadex G-25. The degradation products released by trypsin treatment were analysed by both CsCl-density-gradient centrifugation and Sepharose CL-2B gel chromatography under dissociative conditions.

CsCl-density-gradient centrifugation

Proteoglycan samples from dissociative Sepharose CL-2B gel chromatography were adjusted to a density of 1.40g/ml with solid CsCl in the presence of 4M-guanidine hydrochloride/0.05M-sodium acetate buffer, pH 6.0, and centrifuged at 35000 rev./min in a Beckman type 65 rotor for 60h at 15°C. Fractions (10 $\times$ 1 ml) were removed manually by syringe, and the density of each fraction was determined by weighing. Each fraction was then assayed for radioactivity.

Results

Effect of trypsin treatment on proteoglycan biosynthesis

Duplicate cultures (100mg) obtained from a single joint were pulse-labelled for 2h with [35 S]sulphate at time intervals between 1 and 7 days after various trypsin treatments as described in the Experimental section. The incorporation of [35 S]sulphate into proteoglycans for control cultures was shown to be relatively constant over the time period 1–7 days (Fig. 1); similar results were obtained when [3 H]glucosamine was used as an alternative precursor (results not shown). Fig. 1 illustrates the effect of trypsin treatment on

Fig. 1. Effect of trypsin treatment of articular cartilage on proteoglycan biosynthesis with time in culture

Incorporation of [35 S]sulphate is expressed in pmol/h per mg of tissue; each point represents the mean of three samples, with s.e.m. less than 0.4 in each case. ●, Untreated control; ○, cartilage treated with 5 μ g of trypsin/ml; ■, cartilage treated with 20 μ g of trypsin/ml; △, cartilage treated with 1mg of trypsin/ml.

proteoglycan biosynthesis by articular cartilage. Clearly, trypsin treatment caused a marked inhibition, which persisted with time in culture when the enzyme concentration was high, but at low enzyme concentration the inhibition was short-lived and subsequent stimulation of biosynthesis was observed. Cultures treated with 5 μ g of enzyme/ml showed an initial 21% decrease in proteoglycan biosynthesis 1 day after treatment, but there was a rapid recovery, and stimulation above the rates observed for the control cultures 2 days after treatment. Tissue treated with 20 μ g and 1 mg of trypsin/ml showed a severe depression (up to 70%, depending on the batch of tissue) of proteoglycan biosynthesis up to 2 days after removal of the enzyme from the culture. Subsequent recovery of biosynthetic rates to control values then depended on the concentration of trypsin used.

Partial characterization of the proteoglycan species synthesized during the recovery phase was performed by Sepharose CL-2B gel chromatography. Guanidine hydrochloride extracts of control cultures showed two distinct proteoglycan populations, of K_{av} 0.31 and 0.71 respectively (Fig. 2). The higher- M_r species corresponded to the major proteoglycan found in cartilage, whereas the smaller species represented 2–10% of the total proteoglycan synthesized (Table 1), depending on the length of time in culture. In control cultures a greater proportion of the smaller species was normally synthesized with increasing length of time in culture. Analysis on a Sepharose CL-2B column of guanidine hydrochloride extracts of tissue treated with trypsin indicated a change in the relative proportions of the two proteoglycan populations normally synthesized. Extracts of tissue treated with 5 μ g of trypsin/ml showed a small increase in the proportion of [35 S]sulphate incorporated into the low- M_r proteoglycan (Table 1) compared with control cultures. However, treatment with trypsin at 20 μ g/ml (Fig. 2) led to a considerable increase in the rate of synthesis of the

small proteoglycan; 2 days after treatment there was a 30–40% increase in the rate of synthesis of the small proteoglycan compared with controls. The small proteoglycan represented 36% of the total proteoglycan synthesized by the trypsin-treated tissue, whereas in control tissue the proportion of small proteoglycan compared with

Fig. 2. Chromatography on Sepharose CL-2B of proteoglycans extracted by guanidine hydrochloride from trypsin (20 μ g/ml)-treated and control cartilage, synthesized 48 h after treatment

—, Control, guanidine hydrochloride extract (38 500 d.p.m.); ----, trypsin-treated, guanidine hydrochloride extract (15 700 d.p.m.) V_0 , Void volume; V_t , total volume.

Table 1. Synthesis of high- and low- M_r proteoglycans by articular cartilage in culture after trypsin treatment
Trypsin-treated cartilage was labelled for 2 h with [35 S]sulphate, and 4M-guanidine hydrochloride extracts of the tissue were chromatographed on a Sepharose CL-2B column under dissociative conditions. The relative peak areas of each proteoglycan (PG) were calculated as a percentage of the total radioactivity.

Time in culture after treatment (days)	Control		Trypsin (5 μ g/ml)		Trypsin (20 μ g/ml)	
	Large PG (%)	Small PG (%)	Large PG (%)	Small PG (%)	Large PG (%)	Small PG (%)
1	97.7	2.3	96.1	3.9	50.7	49.3
2	96.0	8.0	89.5	10.5	—	—
3	89.4	10.6	80.7	19.5	55.7	44.3
4	89.7	10.3	78.7	17.3	—	—
6	89.7	10.3	78.7	17.3	57.1	42.9
9	82.9	17.1	74.7	25.3	63.2	36.8

large proteoglycan synthesized was only 2%. The initial increase in the rate of synthesis of the small proteoglycan was observed to decrease slightly with recovery of the tissue, but remained higher than controls (Table 1). Severe trypsin treatment (1 mg/ml) resulted in very low rates of proteoglycan biosynthesis; the small proteoglycan represented 90% of the total proteoglycan synthesized on each of the 3 days after treatment, but by 4 days 22% of the total proteoglycan synthesized was of the high-*M_r* proteoglycan subunit form.

The ability of the newly synthesized high-*M_r* proteoglycans, after trypsin treatment of the cartilage, to aggregate with hyaluronic acid was unchanged compared with control samples, in which at least 60% of the total proteoglycan was aggregating (results not shown). The small proteoglycan was not able to aggregate in either control or treated samples, in agreement with previous reports (Heinegård *et al.*, 1981; Campbell *et al.*, 1984).

It was clear that exposure of cartilage to trypsin resulted in a decreased synthesis of proteoglycan, which was in turn reflected by a decrease in the amount of proteoglycan extracted with guanidine hydrochloride. However, collagenase treatment of normal and trypsin-treated cartilage after radio-labelling of the tissue resulted in an increase in the guanidine hydrochloride-extractable proteoglycan and a concomitant decrease in the NaOH-extractable proteoglycans (Table 2). Gel chromatography on Sepharose CL-2B of these extracts showed that the increase in guanidine hydrochloride-extractable material consisted of 95% of the high-*M_r* proteoglycan (*K_{av}*, 0.31) and only 5% of the low-*M_r* proteoglycan (results not shown).

Estimation of glycosaminoglycan chain size of newly synthesized proteoglycans

The chondroitin sulphate chains of the newly synthesized large proteoglycan after enzyme treat-

ment had a *K_{av}* of 0.38, compared with control samples, which were eluted with a peak *K_{av}* of 0.61 on a Sepharose CL-6B column (Fig. 3). This corresponded to an estimated apparent *M_r* of 30000 (Wasteson, 1971) for glycosaminoglycans synthesized after trypsin treatment of the tissue, compared with *M_r* 16000 for control cultures. However, no alteration in the glycosaminoglycan chain length (Heinegård *et al.*, 1981) was seen in the newly synthesized low-*M_r* proteoglycan after trypsin exposure.

Dual-labelling of proteoglycans with [³⁵S]sulphate and [³H]leucine

The incorporation of both [³⁵S]sulphate and [³H]leucine into the high- and low-*M_r* proteo-

Fig. 3. Chromatography on Sepharose CL-6B of glycosaminoglycan chains prepared by alkaline borohydride treatment of newly synthesized high-*M_r* proteoglycans from (A) trypsin-treated and (B) control cartilage *V₀*, Void volume; *V₁*, total volume.

Table 2. Effect of collagenase treatment of cartilage on the extractability of proteoglycans

Cartilage (100mg) was treated with trypsin (20 µg/ml) for 18 h and returned to culture for 1 day. Control tissue was untreated but maintained in serum-free medium and washed as for trypsin-treated tissue. Tissue was then pulse-labelled for 2 h with [³⁵S]sulphate and treated with collagenase in 50 mM-Tris/HCl/10 mM-CaCl₂, pH 7.6, as described in the text. All samples were extracted with 4M-guanidine hydrochloride, followed by 0.5M-NaOH. Results are means for triplicate samples, expressed as d.p.m./mg, and then expressed as a percentage of the total extractable proteoglycan for each sample.

Extract	Trypsin-treated cartilage				Untreated cartilage			
	- Collagenase		+ Collagenase		- Collagenase		+ Collagenase	
	(mean d.p.m./mg)	(%)	(mean d.p.m./mg)	(%)	(mean d.p.m./mg)	(%)	(mean d.p.m./mg)	(%)
Guanidine hydrochloride	358	41.2	701	75.7	1410	50.1	2255	77.2
NaOH	510	58.8	225	24.3	1404	49.9	669	22.8

glycans was determined. Trypsin-treated and untreated tissue were labelled with both radioisotopes, and samples of the guanidine hydrochloride extracts were chromatographed on a Sepharose CL-2B column. Fractions were analysed for both [^{35}S]sulphate and [^3H]leucine (Fig. 4). Clearly the ratio of $^3\text{H}/^{35}\text{S}$ was markedly different for the two proteoglycans, the small proteoglycan having a higher content of [^3H]leucine (ratio 2.10) than the large proteoglycan (ratio 0.15). The ratios were the same for treated and control cultures.

Characterization of the degradation products of articular cartilage after trypsin treatment

Exposure of [^{35}S]sulphate-labelled cartilage to trypsin resulted in the release of 96% of newly labelled proteoglycans into the medium (Table 3). This was paralleled by the release of hexuronic acid into the medium. Samples of the culture medium after enzyme treatment of the tissue were analysed by Sepharose CL-2B gel chromatography (Fig. 5). A peak eluted at K_{av} 0.84 was observed, which, when compared with control samples, which were eluted with a peak K_{av} of 0.39, indicated that there was severe degradation of the proteoglycans released from the cartilage. Analysis of the guanidine hydrochloride extract of trypsin-

digested tissue showed that the proteoglycans remaining in the tissue were also highly degraded, being eluted from Sepharose CL-2B with a peak K_{av} of 0.72. Aggregation studies of these proteoglycans with hyaluronic acid revealed that neither proteoglycans released nor those remaining in the tissue after treatment were able to aggregate, suggesting that exposure to trypsin had resulted in the loss of the hyaluronic acid-binding region of the core protein. The size of the glycosaminoglycan chains as estimated on a Sepharose CL-6B column

Table 3. Release of [^{35}S]sulphate-labelled proteoglycan from cartilage by trypsin

Prelabelled tissue was treated with trypsin (1 mg/ml). [^{35}S]Sulphate-labelled proteoglycans were monitored in both the medium and tissue extracts in parallel with hexuronic acid. Results are expressed as a percentage of the total incorporated radioactivity. Hexuronic acid is expressed as $\mu\text{g}/100$ mg of tissue.

Extract	Trypsin-treated		Control	
	%	Hexuronic acid	%	Hexuronic acid
Medium	96.0	419.3	5.9	9.0
Guanidine HCl	2.7	12.3	52.4	581.0
NaOH	1.3	—	41.7	—

Fig. 4. Sepharose CL-2B chromatography of proteoglycans dual-labelled with [^{35}S]sulphate and [^3H]leucine (a) Guanidine hydrochloride extract of untreated tissue (46000 d.p.m. of [^{35}S]sulphate). (b) Guanidine hydrochloride extract of trypsin-treated tissue (23500 d.p.m. of [^{35}S]sulphate) labelled 2 days after treatment.

Fig. 5. Chromatography on Sepharose CL-2B of proteoglycans released into the culture medium after trypsin treatment

Cartilage slices were labelled for 6 h with [^{35}S]sulphate (20 $\mu\text{Ci}/\text{ml}$) before treatment. The tissue was then treated with trypsin (1 mg/ml) for 16 h. The degradation products released into the medium were then analysed on a Sepharose CL-2B column as described in the text. A, Medium from untreated cartilage (98900 d.p.m.); B, medium from trypsin-treated cartilage (75000 d.p.m.).

Fig. 6. CsCl-density-gradient centrifugation of proteoglycans

Proteoglycan samples were centrifuged at 90000g for 60 h at 15°C in 4M-guanidine hydrochloride/0.05M-sodium acetate buffer, pH 6.0. Solid CsCl was added to give the appropriate starting density. (a) Centrifugation of low- M_r newly synthesized proteoglycan. Tissue was extracted in guanidine hydrochloride, 24 h after trypsin treatment (20 μ g/ml). The extract was applied to a Sepharose CL-2B column, and fractions 65–75 (1800 d.p.m.) were subjected to density-gradient centrifugation. The starting density was 1.5 g/ml, and the gradient was fractionated into ten 1 ml fractions. (b) Centrifugation of newly synthesized high- M_r proteoglycans. Tissue was extracted 24 h after trypsin treatment (20 μ g/ml), the extract applied to a Sepharose CL-2B column and fractions 40–55 (1500 d.p.m.) were subjected to density-gradient centrifugation at a starting density of 1.48 g/ml, and fractionated into 1 ml fractions. (c) Centrifugation

after β -elimination of the proteoglycans, appeared unchanged in both medium samples and guanidine hydrochloride extracts of the tissue (results not shown).

CsCl-density-gradient centrifugation

Since trypsin treatment of cartilage resulted in a degradation product of the same apparent M_r as the newly synthesized small proteoglycan, it was essential to demonstrate that the newly synthesized low- M_r proteoglycan after treatment was a biosynthetic species, and not merely a breakdown product resulting from prolonged activity of trypsin. This was most easily achieved by virtue of the low buoyant density of the small proteoglycan species.

Fractions 40–55 and 60–75 from a Sepharose CL-2B dissociative column of an extract from trypsin-treated cultures were pooled separately, concentrated in an Amicon ultrafiltration cell (PM 10 membrane) and applied to a CsCl density gradient in 4M-guanidine hydrochloride. Fig. 6(a) demonstrates the low buoyant density of the low- M_r proteoglycan species synthesized after trypsin treatment compared with the large proteoglycan, which is clearly of higher buoyant density (fraction 1; Fig. 6b). A sample of the culture medium (7000 d.p.m.) was also centrifuged on a CsCl density gradient, and the results are shown in Fig. 6(c). The proteoglycan fragments released into the culture medium are of high buoyant density. These results give further evidence to support the conclusion that the newly synthesized low- M_r proteoglycan is a synthetic product and not a breakdown product of the large proteoglycan.

Discussion

Exposure of cartilage to trypsin resulted in the release of degraded proteoglycans into the culture medium, and the small proportion of proteoglycans remaining in the tissue were also in a degraded form, having lost their ability to aggregate with hyaluronic acid. When subjected to CsCl-density-gradient centrifugation, the proteoglycans released into the medium, of peak K_{av} 0.71, were found to be of high buoyant density. Previous reports (Heinegård & Hascall, 1974; Mathews, 1971) have indicated that trypsin will degrade isolated proteoglycan from bovine tracheal cartilage to peptides containing 1–8 glycosaminoglycan chains,

of proteoglycans (6600 d.p.m.) released into the culture medium after trypsin digestion (1 mg/ml) of prelabelled cartilage. The starting density was 1.5 g/ml, and the gradient was fractionated into ten 1 ml fractions. ●, Radioactivity; ○, density.

which are linked as doublets to the core protein. The sizes of the fragments seen in the present study were consistent with these observations.

An interesting observation was the inhibitory effect on the rate of proteoglycan biosynthesis, which was demonstrated when high doses of trypsin were added to the culture medium. The extent of the inhibition of biosynthesis depended on the initial amount of enzyme; trypsin concentrations of 20 µg/ml or 1 mg/ml resulted in a 45% decrease in biosynthesis. Recovery to control values was reached 3 days after treatment with 20 µg of trypsin/ml, but a much slower recovery was observed for the higher enzyme concentration. Nevertheless the inhibition was reversible with time. Mild trypsin treatment (5 µg/ml) of the tissue, however, did result in a stimulation of biosynthesis above control values, which is consistent with the observations by other workers, where cartilage was subjected to brief exposure with papain (Bosman, 1968) or trypsin (Milroy & Poole, 1974; Hascall *et al.*, 1983), and depletion of the matrix proteoglycans was rapidly followed by a marked stimulation of proteoglycan biosynthesis. However, our results suggest that longer exposure to a higher concentration of trypsin resulted in a failure to replace lost proteoglycan and resulted in a lasting inhibition of synthesis. This suggests either that inhibition is proportional to the extent of cellular damage, or that it is mediated by a secondary factor resulting from severe matrix damage. Histological and autoradiographic studies using [³⁵S]sulphate (results not shown) exclude the possibility that the inhibition was due to cell death. Studies (Bartholomew *et al.*, 1984) on the effect of rabbit leucocyte elastase on articular cartilage have also shown an inhibitory effect on proteoglycan synthesis, accompanied by a general decrease in protein synthesis. These results suggest that enzymic treatment of articular cartilage results not only in a depression of proteoglycan biosynthesis, but in a general alteration of the regulation of metabolism by the chondrocyte.

Studies on the proteoglycans synthesized after proteolytic treatment showed dramatic alterations in the type of proteoglycans synthesized. In control cultures, two distinct proteoglycans were normally seen; approx. 90% were of large hydrodynamic size and high buoyant density; the remainder were of a smaller size and low buoyant density. The proportion of small proteoglycan varied from 2 to 10%, increasing with time in culture. The small proteoglycan had similar properties to the low- M_r proteoglycans isolated from bovine nasal cartilage (Heinegård *et al.*, 1981), from articular cartilage (Swann *et al.*, 1979) and from embryonic-chick cartilage cultures (Kimata *et al.*, 1978). Previous studies on the metabolism of the small proteoglycan from

normal bovine cartilage (Campbell *et al.*, 1984) have confirmed that the small proteoglycan is a separate biosynthetic product and not a breakdown product of the large species. Dual-labelling studies using both [³⁵S]sulphate and [³H]leucine confirmed the unique identity of the small proteoglycan as synthesized after trypsin treatment of the tissue. After moderate trypsin treatment (20 µg/ml) an increase in the proportion of the small proteoglycan synthesized was observed, representing at least a 30% increase in the rate of synthesis of the small proteoglycan. The proportion of the small proteoglycan, however, did decrease with increased recovery of the tissue, suggesting that the situation was reversible. Since low concentrations of trypsin resulted in only a slight increase in the rate of synthesis of small proteoglycan, whereas a high concentration (1 mg/ml) resulted in the predominant biosynthesis of the small proteoglycan initially, it would suggest that the degree of damage dictates the type of proteoglycan synthesized. A possible explanation for these observations, after damage of the tissue, is that the rate of biosynthesis of the small proteoglycan is more rapid or occurs via an alternative pathway to that for the large proteoglycan, and this may represent an attempt by the chondrocyte to replace rapidly a depleted matrix. Alternatively, the small non-aggregating proteoglycan may serve a specific function in organizing the fibrillar matrix of cartilage, requiring it to be synthesized before the larger, aggregating, proteoglycan.

Since guanidine hydrochloride extracts 50–60% of the total proteoglycan from cartilage, it may be argued that trypsin treatment results in an alteration in the extractability of the proteoglycan and may account for the observed phenomena. However, bacterial collagenase treatment of the tissue enabled extraction of up to 75% of the total tissue proteoglycan, and analysis by gel chromatography demonstrated that this population of proteoglycans comprised 95% in the high- M_r species. We therefore conclude that the observed increase in the synthesis of the small proteoglycan after trypsin exposure is not due to an alteration of the tissue matrix resulting in a change in extractability.

An increase in the glycosaminoglycan chain size of the newly synthesized high- M_r proteoglycans was demonstrated after trypsin treatment. However, no such change in chain size was seen for the low- M_r proteoglycan. This suggests that trypsin treatment may be limiting the amount of core protein available for the synthesis of the large proteoglycan. This is consistent with the studies by Mitchell & Hardingham (1982), who suggested that limitation of the core protein results in the synthesis of longer glycosaminoglycan chains. Since no change in the glycosaminoglycan chain size was detected for the small proteoglycan, this suggests that the biosynthe-

tic pathway or the control of this pathway is independent from that of the high- M_r species.

References

- Bartholomew, J. S., Lowther, D. A. & Handley, C. J. (1984) *Arthritis Rheum.* **27**, 905–912
- Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 320–334
- Bosman, H. B. (1968) *Proc. R. Soc. London Ser. B* **169**, 399–425
- Campbell, M. A., Handley, C. J., Hascall, V. C., Campbell, R. A. & Lowther, D. A. (1984) *Arch. Biochem. Biophys.* **234**, 275–289
- Chang, Y., Yanagashita, M., Hascall, V. C. & Wight, T. N. (1983) *J. Biol. Chem.* **258**, 5679–5688
- Faltz, L. L., Reddi, A. H., Hascall, G. K., Martin, D., Pita, J. C. & Hascall, V. C. (1979) *J. Biol. Chem.* **254**, 1375–1380
- Handley, C. J. & Lowther, D. A. (1977) *Biochim. Biophys. Acta* **500**, 132–139
- Hardingham, T. E. & Muir, H. (1973) *Biochem. J.* **135**, 905–908
- Hardingham, T. E., Ewins, R. J. F. & Muir, H. (1976) *Biochem. J.* **157**, 127–143
- Hascall, V. C. & Heinegård, D. (1974a) *J. Biol. Chem.* **249**, 4232–4241
- Hascall, V. C. & Heinegård, D. (1974b) *J. Biol. Chem.* **249**, 4242–4249
- Hascall, V. C. & Sajdera, S. W. (1970) *J. Biol. Chem.* **245**, 4920–4930
- Hascall, V. C., Morales, T. I., Hascall, G. K., Handley, C. J. & McQuillan, D. J. (1983) *J. Rheumatol.* **10**, Suppl. **11**, 45–52
- Heinegård, D. (1977) *J. Biol. Chem.* **252**, 1980–1989
- Heinegård, D. & Axelsson, I. (1977) *J. Biol. Chem.* **252**, 1971–1979
- Heinegård, D. & Hascall, V. C. (1974) *Arch. Biochem. Biophys.* **165**, 427–441
- Heinegård, D. & Hascall, V. C. (1979) *J. Biol. Chem.* **254**, 927–934
- Heinegård, D., Paulsson, M., Inerot, S. & Carlstrom, C. (1981) *Biochem. J.* **197**, 355–366
- Hopwood, J. J. & Robinson, H. C. (1973) *Biochem. J.* **133**, 457–470
- Kimata, K., Oike, Y., Ito, K., Karasawa, K. & Suzuki, S. (1978) *Biochem. Biophys. Res. Commun.* **85**, 1431–1439
- Mathews, M. B. (1971) *Biochem. J.* **125**, 37–46
- Milroy, S. J. & Poole, A. R. (1974) *Ann. Rheum. Dis.* **33**, 500–508
- Mitchell, D. & Hardingham, T. E. (1982) *Biochem. J.* **202**, 387–395
- Stevens, R. L., Nissley, S. P., Kimura, J. H., Rechler, M. M., Caplan, A. I. & Hascall, V. C. (1981) *J. Biol. Chem.* **256**, 2045–2052
- Swann, D., Rowell, S. & Sotman, S. (1979) *J. Biol. Chem.* **254**, 945–954
- Wasteson, A. (1971) *J. Chromatogr.* **59**, 87–97