Biochem. J. (1983) **214**, 855–864
Printed in Great Britain

Proteoglycan aggregate formation by articular chondrocytes

Decrease in link-protein synthesis during culture

Anna H. K. PLAAS, John D. SANDY and Helen MUIR
Division of Biochemistry, Kennedy Institute of Rheumatology, London W6 7DW, U.K.

(*Received 18 April 1983/Accepted 24 June 1983*)

The synthesis of link-stabilized proteoglycan aggregates by rabbit articular chondrocytes was investigated by [^{35}S]sulphate labelling of primary monolayer cultures maintained for up to 21 days. (1) At all culture times the cells secreted a high-molecular-weight cartilage-type proteoglycan monomer of which 75%–80% formed aggregates with hyaluronic acid. (2) At 2 days of culture all of the aggregates were in a link-stabilized form, but by 21 days only 5% were link-stabilized, as shown by displacement of monomers from the aggregate by hyaluronic acid oligosaccharides. (3) The addition of purified link protein to 21-day culture medium increased the proportion of link-stable aggregate from 5% to 70%. (4) Analysis of [^{3}H]serine-labelled proteoglycan aggregates in the medium showed a marked decrease with culture time in the ratio of ^{3}H-labelled link protein to ^{3}H-labelled core protein present. The results suggest that the secretion of proteoglycan monomers and link protein by articular chondrocytes changes independently during prolonged monolayer culture.

The biological properties of hyaline cartilages are largely due to the macromolecular constituents of the extracellular matrix, namely proteoglycans and collagen. The proteoglycan monomers, which are largely composed of chondroitin sulphate and keratan sulphate attached to a protein core, are present as high-molecular-weight aggregates (Hardingham & Muir, 1972) in which a large number of monomers are non-covalently bound to hyaluronic acid via a specific binding region at one end of the core protein (Hascall & Heinegård, 1974). This interaction is further stabilized by link proteins (Hardingham, 1979), which are found in two distinct forms and which are known to interact with both proteoglycan monomer and hyaluronic acid (Caterson & Baker, 1978; Franzén *et al.*, 1981*a*).

The process by which the proteoglycan aggregate is synthesized and assembled in the extracellular space is largely unknown. Studies with chondrocytes isolated from the Swarm rat chondrosarcoma (Kimura *et al.*, 1979; Mitchell & Hardingham, 1982) have shown that all three components of the proteoglycan aggregate are synthesized by chondrocytes. Further, the formation of aggregates is apparently an extracellular event, occurring shortly after secretion in which a proteoglycan-monomer–link-protein complex forms, and this intermediate then binds to hyaluronic acid (Kimura *et al.*, 1980). Intracellular aggregation may be prevented through the existence of independent secretory pathways for the separate components (Kimura *et al.*, 1980; Mitchell & Hardingham, 1982; Björnsson & Heinegård, 1981*b*).

Although the basic mechanisms of proteoglycan aggregate synthesis have been established in chondrosarcoma-cell cultures, the aggregate components produced by these cells are not typical of mammalian chondrocytes in that they produce a proteoglycan monomer devoid of keratan sulphate (Lohmander *et al.*, 1980) and only one form of link protein (Caterson & Baker, 1979). On the other hand, little is known about aggregate synthesis by mammalian articular chondrocytes. These cells synthesize a matrix in which the proteoglycans are substituted with both chondroitin sulphate and keratan sulphate (Bayliss & Ali, 1978; Franzén *et al.*, 1981*b*) and which contains at least two forms of link protein (Treadwell *et al.*, 1980; Roughley *et al.*, 1982). Furthermore, although chondrocyte cultures established from rabbit (Srivastava *et al.*, 1974), bovine (Kuettner *et al.*, 1982*a*) and human (Oegema & Thompson, 1981) articular cartilage

Abbreviations used: GdmCl, guanidinium chloride; PUK unit, 1 PUK unit is that which produces, after hydrolysis of casein at 40°C in phosphate buffer, pH 7.4, for 10min, followed by precipitation of excess protein with trichloroacetic acid, an absorbance of 1.0 at 660nm with Folin and Ciocalteau's reagent.

have been used to study proteoglycan aggregate synthesis, the link stability of the aggregates has not been reported for these culture systems.

Articular chondrocytes are known to alter the type of matrix products formed during prolonged maintenance in culture. For example, the collagen types produced by rabbit articular chondrocytes is influenced by culture conditions (Benya *et al.*, 1977; Benya & Shaffer, 1982), and maintenance of bovine articular chondrocytes results in a decrease in the synthesis of aggregating proteoglycans and the preferential synthesis of low-molecular-weight proteoglycans (Kuettner *et al.*, 1982*b*).

In the present paper we describe the effect of the period of maintenance of rabbit articular chondrocytes in primary monolayer culture on the synthesis of link-stabilized proteoglycan aggregates.

Experimental

Materials

Collagenase (Type IA) (EC 3.4.24.3), and bovine serum albumin (fraction V) were from Sigma (Poole, Dorset, U.K.). Ovine testicular hyaluronidase was from Boehringer (Mannheim, Germany) and chondroitinase ABC was from Seikagaku Kogyo Co., Tokyo, Japan. Hyaluronic acid (from human umbilical cord) and Pronase (45000 PUK units/g) were from BDH Chemicals (Poole, Dorset, U.K.). Ham's F12 Medium, foetal-calf serum, gentamycin, glutamine, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, phosphate-buffered saline [137mM-NaCl/2.7mM-KCl/8.1mM-Na_2HPO_4/1.5mM-KH_2PO_4, pH 7.4 (Ca^{2+}- and Mg^{2+}-free)] and plastic culture dishes were from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Sepharose and Sephadex gels were from Pharmacia (Uppsala, Sweden). [^{35}S]Sulphate (carrier-free), L-[^{3}H]serine (11Ci/mmol) and the ^{14}C-labelled standard protein mixture were from Amersham International (Amersham, Bucks., U.K.). Lumagel was from Lumac Systems (Basel, Switzerland).

Ham's F12 medium was supplemented with 0.04M-$NaHCO_3$/20mM-Hepes, 10% (v/v) foetal-calf serum, 1mM-glutamine and 50μg of gentamycin/ml, and adjusted to pH 7.4.

Preparation of special materials

Hyaluronic acid oligosaccharides were prepared essentially as described by Kimura *et al.* (1979). Briefly, 2ml of a 7.5mg/ml solution of hyaluronic acid in 0.15M-NaCl/0.1M-sodium acetate, pH 5.5, was digested with 350μg [500 turbidity-reducing units (Dorfman, 1955)/mg] of hyaluronidase for 4h at 37°C, the solution was adjusted to pH 9.0 with NaOH and fractionated on a column (2.5cm × 160cm) of Bio-Gel P-30 eluted at about 10ml/h with 0.25M-pyridinium acetate, pH 6.7. Oligosaccharides of between about twenty and five disaccharide repeat units were eluted in fractions between $K_{av.} = 0.12$ and $K_{av.} = 0.64$. These fractions were combined, freeze-dried and the oligosaccharides were dissolved in water at 1mg of hexuronate/ml.

Bovine articular cartilage proteoglycan (A1) was prepared as described by Galloway *et al.* (1983). Pig laryngeal cartilage link protein was a gift from Dr. T. Hardingham of this Institute, and was prepared as described by him (Hardingham, 1979).

Rat-chondrosarcoma-cell conditioned culture medium was collected from cells maintained for 24h in the presence of 10% (v/v) foetal-calf serum under culture conditions as described by Mitchell & Hardingham (1981).

Preparation of primary cultures of rabbit articular chondrocytes

Articular cartilage was dissected from the knee and shoulder joints of 6-week-old New Zealand White rabbits, sliced and washed in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline, pH 7.0, and treated with hyaluronidase (5mg/g of tissue) in phosphate-buffered saline for 15min at 37°C. The tissue was then treated for 1h at 37°C with bacterial collagenase (3000–5000units/g of tissue) and Pronase (5mg/g of tissue) in Ham's F12 medium containing 4% (w/v) bovine serum albumin, pH 7.4. This digest was filtered through Nitex nylon mesh (75μm pore size), the filtrate discarded and the undigested tissue was treated with collagenase (4500–7500 units/g of tissue) for 2h at 37°C in the same medium. The second digest, which contained essentially no undigested cartilage, was filtered as described above and the cells in the filtrate were washed four times by suspension and centrifugation in phosphate-buffered saline. The final pellet was suspended to give 5×10^6cells/ml in phosphate-buffered saline, and portions containing 5×10^5 or 1×10^6 cells were transferred to 35mm- or 60mm-diam. dishes respectively at a final density of 5×10^5cells/ml of Ham's F12 medium supplemented as described above.

The cultures were maintained in a humidified CO_2/air (1:19) atmosphere at 37°C. Medium was replaced every 48h and primary cultures were used between 2 and 17 days for labelling experiments.

Incorporation of radiolabelled precursors into macromolecules in chondrocyte cultures

To determine DNA synthesis, cultures were labelled for 24h with 20μCi of methyl[^{3}H]thymidine/ml and [^{3}H]DNA was isolated from the cell layer as previously described (Sibatani, 1970).

[^{35}S]Sulphate (50μCi; 83.3μCi/μmol) and [^{3}H]-serine (100μCi; 1mCi/μmol) in fresh medium were added separately to plates and incorporation was allowed to proceed for 24h at 37°C. After incubation the medium was removed from cultures and a

small number of floating cells were removed by centrifugation. A mixture of proteinase inhibitors (10mM-sodium EDTA, 5mM-benzamidine hydrochloride, 0.1mM-phenylmethanesulphonyl fluoride and 100mM-6-aminohexanoic acid) was added to the medium before storage at −20°C until analysis. The cell layers were suspended in 2ml of 4M-GdmCl/0.5M-sodium acetate, pH 6.8 (containing proteinase inhibitors), mixed gently for 16h at 4°C, centrifuged to remove the insoluble residue, and the supernatant stored at −20°C until analysis. For both [^{35}S]sulphate- and [^{3}H]serine-labelled cultures the insoluble residue contained less than 8% of the total macromolecular radiolabelled material.

Isolation of radiolabelled proteoglycans

Medium and cell layer from ^{35}S-labelled cultures were adjusted to 4M-GdmCl/20mM-serine/0.25M-sodium sulphate, and 0.5–2.0mg of bovine articular cartilage proteoglycan (A1) was added. The mixture was left at 4°C for 4h and fractionated on a column (0.7cm × 18cm) of Sephadex G-50 eluted with 4M-GdmCl/0.5M-sodium acetate, pH 6.8 (containing proteinase inhibitors) to remove unincorporated [^{35}S]sulphate. Material eluting in the void volume fractions was combined and dialysed for 48h against 200vol. of 0.5M-GdmCl/0.5M-sodium acetate, pH 6.8 (containing proteinase inhibitors).

Medium and cell layer from ^{3}H-labelled cultures were adjusted to 4M-GdmCl/20mM-serine, and 5mg of bovine articular cartilage proteoglycan (A1) was added. Samples were fractionated on columns (0.7cm × 10cm) of Bio-Gel P-6 eluted with 2M-GdmCl/0.5M-sodium acetate/10mM-L-serine (containing proteinase inhibitors) to remove unincorporated [^{3}H]serine. Material eluting in the void-volume fractions was combined and dialysed for 18h against 200vol. of 0.5M-GdmCl/0.5M-sodium acetate, pH 6.8 (containing proteinase inhibitors).

CsCl gradient centrifugation

Samples of ^{3}H-labelled macromolecules in 0.5M-GdmCl/0.5M-sodium acetate, pH 6.8, were adjusted to 1.5g/ml with CsCl and centrifuged at 34000rev./min ($r_{av.}$ = 8.35cm) and 12°C for 40h in a Sorvall AH-650 swinging-bucket rotor. Tubes were fractionated into four equal fractions (A1, A2, A3, A4) with densities ranging from about 1.65g/ml in the A1 (bottom) to about 1.38g/ml in the A4 (top) fraction. The high-buoyant-density fraction (A1) was subsequently adjusted to 4M-GdmCl and 1.5g of CsCl/ml and centrifuged as above to give four equal dissociative fractions (A1D1, A1D2, A1D3 and A1D4). A1D4 samples were prepared for electrophoresis by dialysis against 1mM-Tris/HCl/0.1mM-EDTA, pH 8, followed by freeze-drying.

Slab-gel electrophoresis

Slab-gel electrophoresis was done essentially as described by Laemmli (1970). Dried samples were dissolved in the minimum volume of distilled water containing 1% (w/v) sodium dodecyl sulphate, 6% (w/v) sucrose and 0.005% (w/v) Bromophenol Blue. To each sample was added, as carrier, 7.5μl of a 10mg/ml solution of articular cartilage proteoglycan (A1) previously digested for 16h at 37°C with chondroitinase ABC (0.05 unit) in 0.04M-Tris/acetate, pH 7.4. All samples were heated at 100°C for 10min before application to gels. The slab gels [7.5% (w/v) acrylamide/0.2% bisacrylamide] were set in 0.04M-Tris/0.02M-sodium acetate/0.002M-EDTA, pH 7.4, and the running buffer was 0.04M-Tris/0.02M-sodium acetate/0.002M-EDTA, pH 7.4, containing 0.1% (w/v) sodium dodecyl sulphate. Gels were stained with Coomassie Brilliant Blue G250 (Sigma), prepared as described by Blakely & Boezi (1977), and destained in 7% (v/v) acetic acid. Fluorography of gels was done by the standard PPO (2,5-diphenyloxazole)/acetic acid procedure described by Skinner & Griswold (1983), and dried gels were exposed to preflashed Kodak X-ray film at −70°C for 10–30 days.

Results

Chondrocyte cultures

The method of isolating chondrocytes used here is based on similar studies with rabbit (Sokoloff *et al.*, 1970) and bovine articular cartilage (Kuettner *et al.*, 1982*a*). Routinely (4–6) × 10^{7} chondrocytes/g wet wt. of rabbit cartilage were obtained. The cells obtained were more than 95% viable as judged by Trypan Blue exclusion, and 80–90% adhered to the culture dishes within 24h of plating. The yield of viable, adherent cells was much improved by inclusion of Pronase and bovine serum albumin in the digestion mixture and the use of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline rather than medium for cell washing and suspension. After 2 days in culture the adherent cells [(0.2–0.5) × 10^{6}/35mm-diameter dish] had become polygonal and started to divide, as shown by [^{3}H]thymidine incorporation into DNA (about 7 × 10^{6}d.p.m./plate). After 7 days the cells [(0.6–1.0) × 10^{6}/35mm-diam. dish] had formed a confluent layer with morphological characteristics similar to those described previously for articular chondrocytes in monolayer culture (Srivastava *et al.*, 1974; Kuettner *et al.*, 1982*a*). At this time no further cell division was occurring, as shown by the constancy of cell numbers and a low rate of [^{3}H]thymidine incorporation into DNA (less than 7 × 10^{4}d.p.m./plate). At 17 days cell morphology was similar to 7 days and there was pronounced interterritorial matrix within cell groups, although no cartilage nodules were observed.

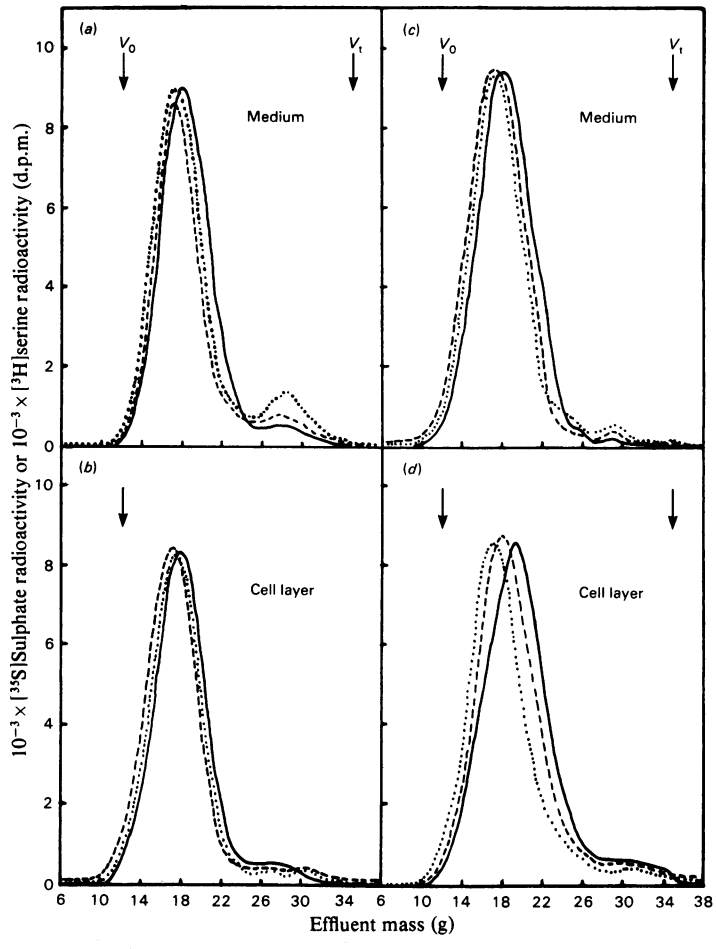

Fig. 1. *Fractionation of radiolabelled proteoglycans on Sepharose CL-2B under dissociative conditions*

^{35}S-labelled proteoglycans isolated from medium (*a*) and cell layer (*b*), and ^{3}H-labelled proteoglycans (A1D1 samples) from medium (*c*) and cell layer (*d*) were fractionated on a column (98 cm × 0.6 cm) of Sepharose CL-2B eluted at 1.8 ml/h with 2 M-GdmCl/0.5 M-sodium acetate, pH 6.8. Fraction sizes (about 0.7 ml) were determined by mass, and each fractionation was internally calibrated with $^{3}H_2O$ as a V_t marker. Proteoglycans were prepared from 2-day (····), 7-day (– – – –) and 17-day (——) cultures.

Proteoglycan structure and aggregation properties

^{35}S-labelled proteoglycans and ^{3}H-labelled proteoglycan monomers (A1D1) formed during 24 h of labelling at 2, 7 and 17 days were isolated from both the medium and GdmCl extract of the cell layer (see the Experimental section). For both radiolabels, between 40 and 60% of the newly synthesized proteoglycans were present in the medium at each culture period.

Fractionation of proteoglycans on Sepharose CL-2B under dissociative conditions (Fig. 1) showed that there was no marked difference in the size distribution of proteoglycan monomers synthesized at different culture times, although the monomers prepared from 17-day culture medium and cell layer appeared slightly smaller for both ^{35}S-labelled (Figs. 1*a* and 1*b*) and ^{3}H-labelled (Figs. 1*c* and 1*d*) products. This decrease in monomer size was most apparent for the ^{3}H-labelled monomers prepared from the cell layer (Fig. 1*d*). In none of the samples was there evidence for the synthesis of low-molecular-weight proteoglycans or for proteoglycan degradation fragments. In keeping with the constancy of monomer size, the [^{35}S]glycosaminoglycans prepared by alkaline cleavage from proteoglycans of medium or cell layer were also of a very similar size at 2, 7 and 17 days of culture, as assessed by chromatography on Sepharose CL-6B (Fig. 2).

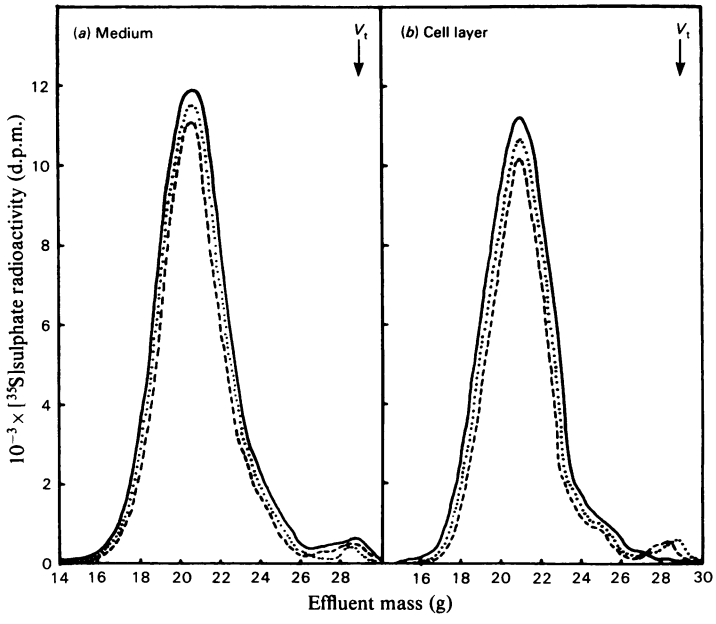

Fig. 2. *Fractionation of [^{35}S]glycosaminoglycans on Sepharose CL-6B*

^{35}S-labelled glycosaminoglycans were prepared from medium (*a*) and cell layer (*b*) by treatment of the isolated proteoglycans with 0.5 M-NaOH for 24h at 22°C. The alkaline hydrolysates were neutralized and fractionated on a column (94cm × 0.6cm) of Sepharose CL-6B eluted at 1.8ml/h with 2M-GdmCl/0.5M-sodium acetate, pH6.8. Fraction sizes (about 0.7ml) were determined by mass and each fractionation was internally calibrated with ^3H_2O as a V_t marker. Glycosaminoglycans were prepared from 2-day (••••), 7-day (– – – –) and 17-day (——) cultures.

Proteoglycans secreted into the medium in ^{35}S-labelling experiments were also analysed for their ability to form aggregates with endogenous hyaluronic acid (Fig. 3). In all cases 75–80% of the [^{35}S]proteoglycans were recovered from the void volume (effluent mass 10–14g), of the Sepharose CL-2B column in the form of proteoglycan aggregates. Therefore the size of the proteoglycan monomers (Fig. 1) and their substituent glycosaminoglycans (Fig. 2), as well as the hyaluronate-binding properties of the monomers formed (Fig. 3), did not change markedly during the culture period from 2 to 17 days.

Link stability of proteoglycan aggregates

Proteoglycan aggregates formed in the medium of chondrocyte cultures (Fig. 3) were tested for link stability by the addition of hyaluronic acid oligosaccharides before chromatography on Sepharose CL-2B (Fig. 4). Proteoglycan monomers that are present as link-stabilized aggregates are not displaced from the hyaluronate by oligosaccharides and are eluted in the void volume (elution mass 10–14g), whereas non-link-stabilized aggregates are dissociated and the monomers are eluted in the position

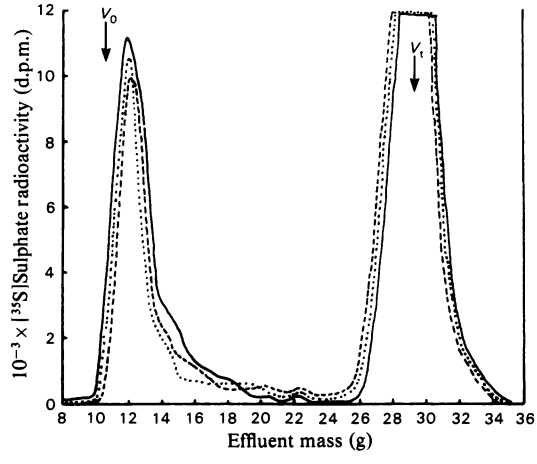

Fig. 3. *Fractionation of [^{35}S]proteoglycans on Sepharose CL-2B under associative conditions*

Samples (about 50μl) of medium from 2-day (••••), 7-day (– – – –) and 17-day (——) cultures were fractionated on a column (95cm × 0.6cm) of Sepharose CL-2B at 1.8ml/h with 0.5M-sodium acetate, pH6.8. Fraction sizes (about 0.7ml) were determined by mass.

of the free monomer between 14 and 20g of eluant (Hascall & Heinegård, 1974; Kimura *et al.*, 1979). The percentage of link-stabilized aggregate markedly decreased during the culture period, falling from 70–80% in 2-day culture medium to about 40% after 7 days and less than 10% after 17 days; 17-day cultures labelled in the absence of serum also produced very little link-stabilized aggregate.

In the present study the link-stability of aggregates was only determined in the culture medium. It has not been possible to assess accurately the percentage of link-stabilized aggregates in GdmCl extracts of the cell layers, since at all culture times these preparations contained very little aggregating proteoglycan. This is presumably due to the poor efficiency of reaggregation of proteoglycan at low concentrations after dissociation in GdmCl (Franzén *et al.*, 1981*a*).

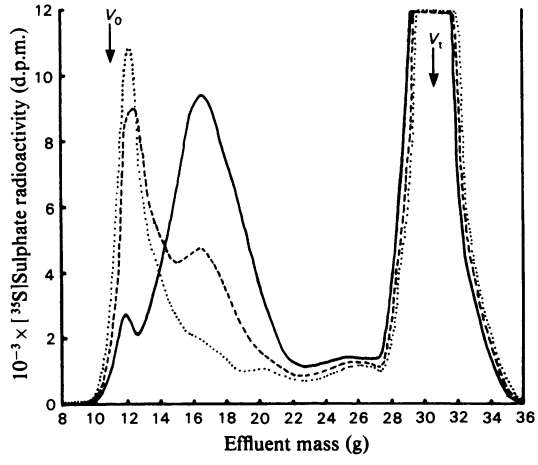

Fig. 4. *Determination of link stabilization of aggregates by chromatography on Sepharose CL-2B*

To samples (50μl) of medium from 2-day (····), 7-day (– – – –) and 17-day (——) cultures was added 10μl of the hyaluronic acid oligosaccharide preparation (see the Experimental section), and after 24h at 4°C the samples were fractionated on Sepharose CL-2B as described in the legend to Fig. 3.

Link-protein synthesis

The most likely explanation for the loss of link stability (Fig. 4) is a decrease in the amount of functional link protein relative to proteoglycan monomer present in the culture medium. To estimate the amount of functional link protein and proteoglycan core synthesized at each culture period, [^{3}H]serine-labelled medium samples were analysed (see the Experimental section) by sequential CsCl gradient centrifugation under associative and dissociative conditions (Tables 1 and 2).

Between day 2 and day 17 of culture (Table 1), [^{3}H]serine incorporation per plate into total protein does not change markedly; however, since the cell number per plate approximately doubles over this period, it appears that protein synthesis per cell is somewhat decreased. The percentage of the total protein synthesized that is associated with the proteoglycan aggregate fractions (A1) is increased between day 2 (about 21%) and days 7 to 17 (about 35%), indicating a preferential synthesis of proteoglycan protein over other proteins with increasing culture period.

Further, the composition of the A1 fraction changes markedly with time of culture (Table 2). Thus the percentage of [^{3}H]serine in A1 that is present as proteoglycan core protein (high-buoyant-density A1D1) increases from 67.7% at day 2 to about 79% at days 7 to 17. Conversely, the percentage of [^{3}H]serine in A1 that is present in the low-buoyant-density protein fraction (A1D4) decreases markedly from about 24% to 11.2% of the total over the culture period. Therefore, although there is a preferential synthesis of proteoglycan over other proteins as the culture is maintained (Table 1),

Table 1. *Associative CsCl gradient centrifugation of medium proteoglycans*

The total ^{3}H-labelled macromolecules isolated from the medium of duplicate cultures after 24h labelling at 2, 7 and 17 days of culture were fractionated by associative CsCl gradient centrifugation (see the Experimental section). In each case more than 82% of the applied radioactivity was recovered from the gradient. The values in parentheses are the percentages of the recovered radioactivity present in each gradient fraction.

Culture period (days)	Gradient fraction ...	[^{3}H]Serine incorporated/24h per plate (nmol)				
		A1	A2	A3	A4	Total
2		0.82 (20.6)	0.22 (5.6)	0.41 (10.5)	2.52 (63.3)	3.97
2		1.02 (21.4)	0.26 (5.5)	0.48 (10.2)	2.99 (62.9)	4.75
7		1.69 (33.9)	0.19 (3.8)	0.29 (5.8)	2.81 (56.3)	4.98
7		1.81 (39.4)	0.15 (3.3)	0.27 (5.8)	2.36 (51.3)	4.59
17		1.83 (36.5)	0.11 (2.2)	0.15 (3.1)	2.94 (58.3)	5.03
17		1.75 (34.0)	0.10 (2.0)	0.19 (3.7)	3.08 (60.2)	5.12

Table 2. *Dissociative CsCl gradient centrifugation of A1 preparations from the medium*
A1 samples (see Table 1) were fractionated by dissociative gradient centrifugation (see the Experimental section). In each case, more than 77% of the applied radioactivity was recovered from the gradient. The values in parentheses are the percentages of the recovered radioactivity present in each gradient fraction.

Culture period	Gradient fraction ...	[^{3}H]Serine incorporated/24 h per plate (nmol)			
		A1D1	A1D2	A1D3	A1D4
2		0.52 (67.9)	0.031 (4.1)	0.033 (4.3)	0.18 (23.6)
2		0.53 (67.5)	0.035 (4.5)	0.026 (3.3)	0.19 (24.7)
7		1.25 (81.4)	0.060 (4.1)	0.033 (2.2)	0.17 (12.5)
7		1.31 (78.6)	0.099 (5.9)	0.050 (3.0)	0.23 (13.8)
17		1.11 (78.6)	0.114 (8.1)	0.031 (2.2)	0.16 (11.2)
17		1.24 (78.2)	0.128 (8.1)	0.038 (2.4)	0.17 (11.2)

the proteoglycan aggregate formed is relatively deficient in associated low-buoyant-density proteins.

Analysis of low-buoyant-density proteins

The proteins present in A1D4 samples from the dissociative CsCl fractionation (Table 2) were analysed by slab-gel electrophoresis and fluorography. In samples from 2-day cultures there was one major discrete ^{3}H-labelled protein which entered the gel. This protein (corresponding to about 44000 mol.wt.) migrated with the Coomassie Blue-stained high-molecular-weight form of link protein prepared from A1 proteoglycan of bovine or rabbit articular cartilages (results not shown). This protein was also identical, in electrophoretic behaviour, with ^{125}I-labelled link protein from pig laryngeal cartilage. There was evidence on fluorographs for the presence of small amounts of radiolabelled low-molecular-weight link protein of about 41000 mol.wt.

The amount of ^{3}H-labelled link protein present in A1D4 samples decreased markedly between day 2 and day 7 of culture and was hardly detectable in preparations from day-17 cultures. This decrease in ^{3}H-labelled link protein with culture period is shown clearly in the densitometric scan of the fluorographs (Fig. 5). The labelled material that did not enter the gels was present in similar amounts in all samples and is presumably rich in low-buoyant-density aggregating proteoglycans.

Stabilization of aggregates with exogenous link protein

The deficiency of functional link protein in the medium of 17-day cultures (Fig. 5) was confirmed by showing that addition of exogenous link protein to such medium samples resulted in the formation of link-stable aggregates (Fig. 6). This effect was shown with either purified link protein from pig laryngeal cartilage or Swarm-rat-chondrosarcoma-cell conditioned medium, previously shown to contain excess

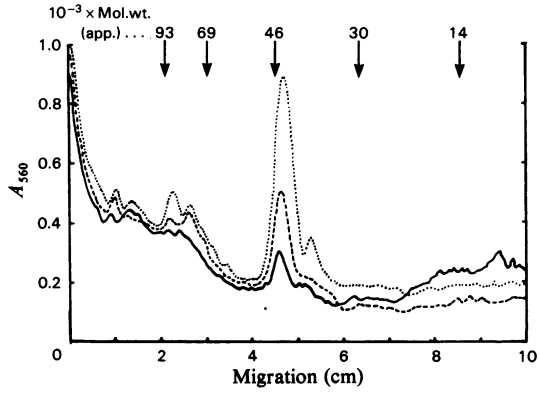

Fig. 5. *Densitometric scan of fluorographs of polyacrylamide-gel-electrophoretic separation of A1D4 proteins (20000 d.p.m. in each case) isolated from 2-day medium* (••••), *7-day medium* (----) *and 17-day medium* (——)
The position of migration of ^{14}C-labelled molecular-weight-marker proteins is also shown.

functional link protein (Kimura *et al.*, 1980). There was, however, no increase in the amount of stable aggregate in 17-day medium when it was incubated alone at 4°C or 37°C or when medium from 2-day rabbit chondrocyte cultures was added before incubation. There was therefore no evidence for excess link protein in 2-day culture medium, nor for an inactive precursor of link protein in 17-day culture medium.

Discussion

The first detailed study of proteoglycan-aggregate formation by chondrocytes used the Swarm rat chondrosarcoma cells (Kimura *et al.*, 1979, 1980). In these cells, maintained in culture for 2–4 days, all the [^{35}S]proteoglycan aggregates secreted into the medium were present in a link-stabilized form.

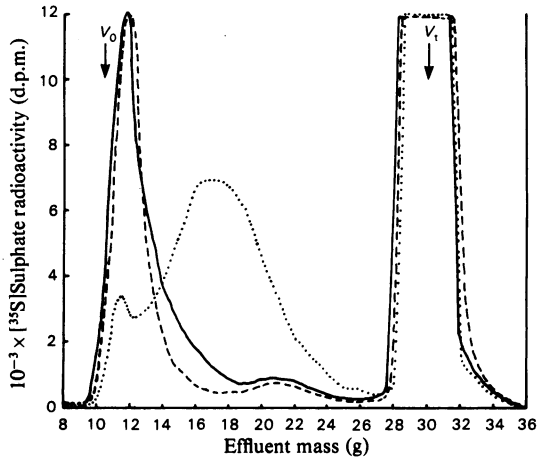

Fig. 6. *Stabilization of aggregates with exogenous link protein*

Samples (50μl) of medium from 17-day cultures were incubated at 4°C for 24h with 10μl of the hyaluronic acid oligosaccharides preparation (see the Experimental section) after the addition of 20μl of Ham's F12 culture medium containing 10% (v/v) foetal-calf serum (····), 20μl of chondrosarcoma-cell conditioned medium (– – – –), or 5μl of a 0.1μg/ml solution of pig laryngeal cartilage link protein in 2M-GdmCl/0.5M-sodium acetate, pH5.5 (——). The mixtures were then fractionated on Sepharose CL-2B as described in the legend to Fig. 3.

Furthermore, determination of [^{3}H]serine incorporation into link protein and proteoglycan core protein suggested that an approximate 1.8-fold molar excess of functional link protein over proteoglycan monomers was secreted.

Aggregate synthesis has also been studied in chondrocytes isolated from bovine foetal tracheal cartilage (Björnsson & Heinegård, 1981*b*). These cells, when kept in suspension for 2 days, synthesized proteoglycan monomers, 95% of which formed link-stable aggregates with hyaluronic acid. However, experiments with the same cells maintained for up to 2 weeks in monolayer culture in Ham's F12 medium containing foetal-calf serum indicated the secretion of proteoglycan aggregates that were deficient in link protein (Björnsson & Heinegård, 1981*a*). Kuettner *et al.* (1982*b*) studied the proteoglycans produced by monolayers of bovine articular chondrocytes and showed that, of the monomers synthesized on day 7 of culture, 73–76% were able to form aggregates with hyaluronic acid, but the degree of link stability of these aggregates was not reported. Further, monolayer cultures of both foetal-guinea-pig epiphyseal chondrocytes (Lohmander *et al.*, 1976) and rabbit auricular chondrocytes (Madsen & Lohmander, 1979) synthesized and secreted aggregating proteoglycans, but the link stability of these aggregates was not examined.

We here present the first comprehensive analysis of proteoglycan-aggregate synthesis in cultures of mammalian articular chondrocytes. Throughout the culture period of 17 days the rabbit chondrocytes produced a remarkably constant sulphated proteoglycan, as assessed by the monomer size (Fig. 1), sulphated-glycosaminoglycan chain size (Fig. 2) and the aggregation properties of the monomer (Fig. 3); however, the proportion of link-stabilized aggregate fell from 80% to less than 10% (Fig. 4).

The absence of link-stable aggregates observed in 17-day medium (Fig. 4) was apparently due to an insufficient supply of functional link protein, since the deficiency could be corrected for by the addition of exogenous link protein to the medium (Fig. 6). This is further supported by the electrophoretic analysis of radiolabelled A1D4 proteins present in late-stage cultures (Fig. 5), which showed a marked diminution of the high-molecular-weight link protein seen at earlier culture times.

Calculations based on the incorporation rate of [^{3}H]serine into A1D1 and A1D4 fractions, and assuming that (1) core protein of mol.wt. 220000 contains 11.5% serine, (2) link protein of mol.wt. 44000 contains 7% serine, and (3) 33% of the [^{3}H]serine in A1D4 is in link protein [calculated from the relative intensity of radiolabelled products seen on fluorography of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of A1D4 (Fig. 5)], indicate that about 2pmol each of core protein and link protein are synthesized/24h per culture dish at day 2 of culture. By contrast, on day 17, 5pmol of core protein and only 0.2pmol of link protein appear in the culture medium during the 24h labelling period.

This deficiency of link-protein supply in the medium of 17-day cultures might be explained in a number of ways. Firstly, degradation or inactivation of the link protein in late-stage culture medium seems an unlikely possibility, since this medium contains fully aggregating proteoglycan monomers of normal size with no evidence of degradation fragments (Figs. 1*a* and 1*c*); moreover, exogenous link protein when added to day-17 medium retains the capacity to stabilize aggregates for up to 24h at 37°C (results not shown).

Secondly, the observed deficiency in the medium at 17 days might be due to poor secretion of link protein from the cell. Inhibited secretion of extracellular products due to the disturbance of membrane systems by zwitterionic buffers, such as the Hepes used here, has been suggested by ultrastructural studies with late-stage chick-embryo chondrocyte cultures (Poole *et al.*, 1982). In the

present cultures, however, the rate of secretion of radiolabelled macromolecules does not change markedly between day 2 and day 17 of culture. Thus between 40 and 60% of the newly synthesized proteoglycans (^{35}S-labelled or ^{3}H-labelled) and total ^{3}H-labelled macromolecules are secreted into the medium at all culture times (results not shown). It therefore appears unlikely that major changes in the secretory function of the chondrocytes have occurred during the culture period.

Thirdly, link-protein synthesis itself may be repressed in the late-stage cultures along with the repression of type II collagen synthesis, which had been observed previously in adherent flattened cells in this system (Cheung *et al.*, 1976; Norby *et al.*, 1977). Such modification of collagen synthesis appears to be brought about by changes in cell shape, since flattened attached cells that synthesize Type I collagen recommence type II collagen synthesis upon transfer to suspension in agarose gels (Benya & Shaffer, 1982).

The finding of non-co-ordinated release of link protein and core protein into the medium of these cultures strongly indicates that, in articular chondrocyte cultures, the two are secreted independently from the cell, as suggested by similar studies with bovine tracheal chondrocytes (Björnsson & Heinegård, 1981*a*). Since proteoglycan monomers and hyaluronic acid appear to be secreted separately (Mitchell & Hardingham, 1982), it would follow that all three components of the proteoglycan aggregate are synthesized and secreted independently by chondrocytes, and that aggregate formation is an entirely extracellular process.

Finally, if chondrocytes of the type present in late-stage cultures, which secrete proteoglycan in the absence of link protein, arose in the hypertrophic zone of developing cartilages, the subsequent loss of stable high-molecular-weight aggregates might initiate the calcification process (Kuettner *et al.*, 1975; Buckwalter, 1983). Such a notion is supported by the finding that long-term maintenance of chick-embryo chondrocytes in monolayer culture is accompanied by the formation of alkaline phosphatase-rich matrix vesicles (Glaser & Conrad, 1981) and the deposition of calcium apatite-like crystals in the matrix adjacent to hypertrophic cells (Oakes *et al.*, 1977).

We thank Dr. T. Hardingham for encouragement during the progress of this work, Mr. C. Johnson for his expert technical assistance and the Medical Research Council (U.K.) for financial support.

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