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Articular cartilage cultured with interleukin 1

Increased release of link protein, hyaluronate-binding region and other proteoglycan fragments

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Pig articular cartilage was maintained in culture for 3 days with and without porcine interleukin 1. The proteoglycans remaining in the cartilage and those released into the medium were analysed by using radioimmunoassays for the hyaluronate-binding region, link protein and keratan sulphate. In interleukin 1-treated cultures after 3 days there was 38% release of total glycosaminoglycans into the medium, 18% release of binding region, 14% release of link protein and 20% release of keratan sulphate epitope, whereas in control cultures the proportions released were much less (16, 9, 10 and 7% respectively). Characterization of the proteoglycans in the media after 1.5 days and 3 days of culture showed that interleukin 1 promoted the release of proteoglycan of large average size and also the release of link protein and of low-M_r binding region which was unattached to proteoglycan. Both the link protein and binding region released were able to bind to exogenously added hyaluronate, whereas the proteoglycan in the medium was not. The proteoglycans extracted from cultured cartilage were similar to those from fresh cartilage: they contained a high proportion of aggregating proteoglycans and some low-M_r binding region. The proportion of this binding region extracted from the interleukin 1-treated cartilage was increased. The presence of interleukin 1 in the cultures therefore appeared to increase the rate of proteolytic degradation of proteoglycan in the matrix and to lead to a more rapid loss of intact binding region, of link protein and of large proteoglycan fragments into the medium.

INTRODUCTION

Proteoglycans and collagen are the major structural components of the extracellular matrix of articular cartilage; the collagen provides tensile strength and the proteoglycans give important compressive properties to the tissue (Muir, 1979; Kempson, 1980). Proteoglycans are complex glycoproteins of high M_r (1×10^6–4×10^6) (Hardingham, 1981) and they consist of an extended protein core (M_r 2×10^5–3×10^5) (Heinegård & Paulsson, 1984; Heinegård *et al.*, 1985; Schwartz *et al.*, 1985), to which are attached many glycosaminoglycan chains (chondroitin sulphate and keratan sulphate) as well as oligosaccharides. The protein core consists of three main regions: (i) an *N*-terminal region that forms two globular domains (Wiedemann *et al.*, 1984), one of which (M_r 66000) (Bonnet *et al.*, 1985) contains specific sites involved in aggregation, (ii) a trypsin-resistant keratan sulphate-rich region and (iii) an extended region bearing the chondroitin sulphate chains (Hardingham, 1981; Hascall, 1977; Heinegård, 1977). Proteoglycans in cartilage form large aggregates in which many proteoglycans bind to a chain of hyaluronate (Hardingham & Muir, 1972), and each proteoglycan–hyaluronate bond is stabilized by a separate link protein (M_r 40000) (Hardingham, 1979; Bonnet *et al.*, 1985).

Articular cartilage maintained in culture as explants releases into the medium a small amount of proteoglycan as a result of turnover and, at the same time, the chondrocytes synthesize matrix components similar to those found in normal tissue (Benya & Nimni, 1979; Tyler *et al.*, 1982; Tyler, 1985*a*,*b*; Hascall *et al.*, 1983). Cartilage explants therefore provide an appropriate model to study proteoglycan metabolism and the effect of agents that may influence regulation of the extracellular matrix. In this way retinol (Jubb & Fell, 1980), bacterial lipopolysaccharides (Morales *et al.*, 1984) and products of cultured synovium (Fell & Jubb, 1977; Dingle, 1979) were shown to induce resorption of the cartilage. One of these agents, interleukin 1(α), has been shown to affect the behaviour of many different cell types (Mizel *et al.*, 1981; Baracos *et al.*, 1983; Gowen *et al.*, 1984; Saklatvala *et al.*, 1984, 1985; Heath *et al.*, 1985). It has been purified from pig mononuclear leucocytes (M_r 21000, pI 5) (Saklatvala *et al.*, 1983). When articular cartilage was cultured in the presence of purified pig interleukin 1, proteoglycan synthesis by the chondrocytes was inhibited (Tyler, 1985*b*) and proteoglycan release into the medium was stimulated (Tyler, 1985*a*). However, the released proteoglycans, unlike those in the cartilage matrix, were unable to aggregate. The aim of the present study was to determine the change in structure of proteoglycans in cartilage cultured in the presence of interleukin 1 and thereby to assess possible mechanisms involved in cartilage turnover and degeneration.

MATERIALS AND METHODS

Materials

Iscove's Modified Eagle's Medium, supplements and serum were from Gibco, Paisley, Renfrewshire, Scotland, U.K. Chondroitin sulphate and *Staphylococcus aureus* (Cowan strain 1) were from Miles Laboratories, Colnbrook, Slough, Berks., U.K. 1,9-Dimethyl Methylene Blue was from Serva Feinbiochemica, Heidelberg, Germany. Sepharose 2B was from Pharmacia, Uppsala, Sweden. $Na^{125}I$ was obtained from Amersham International (Amersham, Bucks., U.K.). Protein A was from Sigma, Poole, Dorset, U.K. High-M_r hyaluronate was generously given by Dr. E. A. Balazs (Biomatrix,

Ridgefield, NJ, U.S.A.). All other reagents were of analytical grade.

Tissue culture

Cartilage was cultured as described previously (Tyler, 1985*a*). Briefly, cartilage slices (approx. 12 mm × 12 mm × 0.3 mm) from the metacarpophalangeal joints of pigs aged approx. 6 months were cultured for 48 h to stabilize them and then cut into halves. The equivalent halves were placed in separate Petri dishes (45 mm) which contained 2 ml of Iscove's medium supplemented with bovine serum albumin (60 mg/ml), human transferrin (5 mg/ml), soya-bean lipid (15 mg/ml), ascorbic acid (75 mg/ml), streptomycin (100 mg/ml) and penicillin (150 units/ml). One set of halves were cultured with the addition of pig interleukin 1 in the medium. Cultures were incubated at 37 °C in an atmosphere of CO_2/air (1:19) and the medium, changed every 6–12 h, was collected separately, mixed with proteinase inhibitors [pepstatin (1 μg/ml), phenylmethanesulphonyl fluoride (5 mM), 1,10-phenanthroline (1 mM) and iodoacetic acid (1 mM)] and stored frozen before analysis. The interleukin 1 used for these experiments was isolated from the conditioned medium of cultured pig mononuclear cells stimulated with lectins by the method of Saklatvala *et al.* (1985). It was of M_r 21000 and pI 4.9 and is homologous with human interleukin (α).

To study the time-dependent release of proteoglycan components into the medium, four parallel cultures were established with eight halves of cartilage (approx. 50 mg wet wt.) in each. Two were cultured with interleukin 1 (5 ng·ml^{-1}) and two without for 3 days. The proteoglycans remaining in the cartilage were then extracted in 4 M-guanidinium chloride (approx. 50 mg wet wt. of cartilage·ml^{-1}) in the presence of proteinase inhibitors and the concentrations in the media and in the extracts of glycosaminoglycans, binding region, link protein and keratan sulphate epitope were determined.

To characterize the proteoglycan components in the media and in the extracts, two cultures each containing 95 halves of cartilage (approx. 500 mg wet wt.) were incubated with or without interleukin 1 (10 ng·ml^{-1}) as used previously (Tyler, 1985*a*), and the early three media (0–1.5 days) and the later three media (1.5–3 days) were pooled separately for analysis. At the end of the culture period the proteoglycans were extracted from the cartilage and the media and the extracts were analysed by associative and dissociative chromatography.

Extraction of proteoglycans

The cartilage pieces were frozen and sliced to 30 μm thickness with a cryostat. The sections from control or treated cartilage were sequentially extracted twice with 0.05 M-guanidinium chloride in sodium acetate buffer, pH 5.8, then twice with 4 M-guanidinium chloride in 0.05 M-sodium acetate buffer, pH 5.8, at 4 °C for 24 h, and the extracts were pooled. Extraction of the glycosaminoglycans from the cartilage was > 90%.

Gel chromatography

In order to test the ability of proteoglycans to aggregate, samples of medium were incubated with high-M_r hyaluronate [2% (w/w) per sample of glycosaminoglycan] for 4 h at room temperature and applied to a column (120 cm × 0.6 cm) of Sepharose 2B and eluted with 0.5 M-sodium acetate, pH 5.8, at 4 °C. Aggregation was assessed by elution in the void volume of the column (Hardingham & Muir, 1972). The size of the proteoglycans in the medium and in the extracts was determined by gel chromatography under dissociative conditions on a column (120 cm × 0.6 cm) of Sepharose 2B in the presence of 2 M-guanidinium chloride/0.5 M-sodium acetate, pH 5.8, at 4 °C. Samples (300 μl) were applied to the columns and the recovery from the columns was > 95%. The effluent fractions were analysed for total glycosaminoglycans and by radioimmunoassay for binding region, link protein and keratan sulphate.

Determination of glycosaminoglycan concentration

Glycosaminoglycan concentration was determined by using an automated modification of the method of Farndale *et al.* (1982), which employs the dye 1,9-dimethyl Methylene Blue. Shark chondroitin sulphate was used as a standard. The linear range of the assay was 5–50 μg/ml.

Antibodies and radioimmunoassay procedures

The antisera to the hyaluronate-binding region of proteoglycan and to link protein, and the radioimmunoassays using these antibodies, were as described previously (Ratcliffe & Hardingham, 1983). The antibodies have been shown to be specific and do not cross-react with any other cartilage component (Ratcliffe & Hardingham, 1983; Ratcliffe *et al.*, 1984). Competition in these assays was with purified antigens isolated from pig laryngeal cartilage (Bonnet *et al.*, 1985) and ^{125}I-labelled, which showed complete cross-reaction with the corresponding components from articular cartilage (Ratcliffe & Hardingham, 1983; Ratcliffe *et al.*, 1984). The antigenic determinants of link protein and, to a lesser extent, binding region, are masked when they are present as link-stabilized aggregates (Ratcliffe & Hardingham, 1983). To allow quantitative determination of binding region and link protein, samples were diluted in assay buffer and incubated at 80 °C for 15 min in the presence of 0.025% SDS before analysis in the radioimmunoassays in order to dissociate any proteoglycan aggregates that may be present. The monoclonal antibody MZ15 has been shown to be specific for keratan sulphate and was used in radioimmunoassay as described previously (Zanetti *et al.*, 1985). It has been shown (Mehmet *et al.*, 1986) that MZ15 binds to a pentasulphated hexasaccharide and larger related oligosaccharides of keratan sulphate, and this suggests that it selectively binds to highly sulphated sequences. The keratan sulphate epitope was determined by competition with a purified proteoglycan monomer from pig laryngeal cartilage and results were therefore expressed in mol of proteoglycan (Zanetti *et al.*, 1985).

Polyacrylamide-gel electrophoresis and immunoblotting

SDS/polyacrylamide-gel electrophoresis was performed as described by Fairbanks *et al.* (1971), with a 5% (w/v) gel run under non-reducing conditions. Immunoblotting with the anti-(binding region) antiserum (1-in-40 dilution) and using ^{125}I-labelled protein A to detect the location of the antibodies was performed as described by Burnette (1981). The nitrocellulose sheet was preincubated in 0.88% NaCl/10 mM-Tris/HCl, pH 7.4, containing 0.5% deoxycholate, 0.1% Nonidet P40, 0.03% NaN_3 and 3% (w/v) bovine serum albumin (in order to decrease

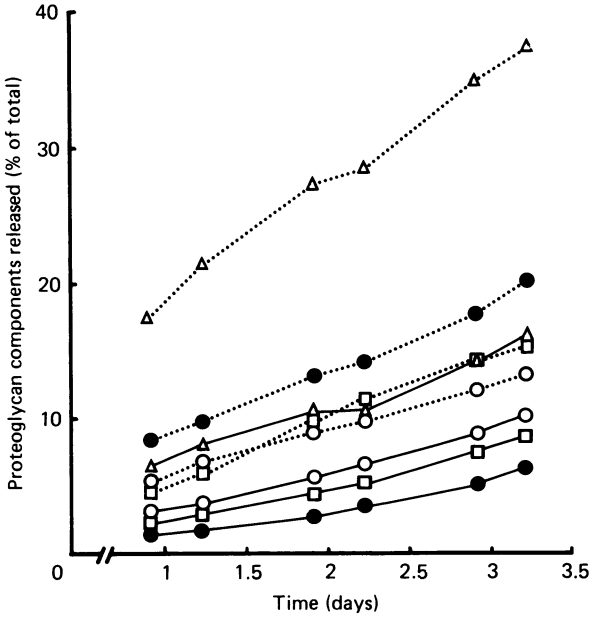

Fig. 1. Release of proteoglycan components from cartilage explants

Cartilage was cultured for 3 days in the absence (——) or presence (·····) of interleukin 1 (5 ng·ml^{-1}) and the media were analysed for glycosaminoglycan (△), binding region (□), link protein (○) and keratan sulphate epitope (●). Results are from a single experiment using duplicate cultures. Each value represents the mean of several determinations (at least four) for which the S.D. was $< \pm 1.5\%$. Similar results were obtained from other independent experiments (results not shown).

background staining), and the immunoblotting and washing procedures were carried out with a buffer similar but containing 0.1% bovine serum albumin.

RESULTS

Time-dependent release of proteoglycan components from cartilage explants

Cartilage was cultured with or without pig interleukin 1 (5 ng·ml^{-1}) as described in the Materials and methods section and the medium was analysed (Fig. 1). In the absence of interleukin 1 there was a steady release of all proteoglycan components from the explants, and by day 3 there had been 16% release of glycosaminoglycans, 9% release of binding region, 10% release of link protein and 7% release of keratan sulphate epitope. When cartilage was cultured continuously in the presence of interleukin 1, the amount of the glycosaminoglycans appearing in the medium was increased. After 20 h, 17% of the total glycosaminoglycans had been released, which was 2.8 times that in control cultures, and by day 3, 38% was released in the medium, which was 2.5 times that of control cultures. There was also a stimulation of the release of binding region and link protein, but overall this was not as pronounced as the release of the glycosaminoglycans. After 20 h, the release of both binding region and link protein was 1.7 times that of control cultures and was similarly increased when assayed at day 3. The release of keratan sulphate epitope in the presence of interleukin 1 was also stimulated, to a greater extent than that of binding region and link protein, but by day 3 was still only 54% of the release of the total glycosaminoglycans.

From the total amount of glycosaminoglycans in the medium and tissue extract, the number of mol of proteoglycan was calculated by assuming an M_r of 1.2×10^6 and a composition with 78% (w/w) glycosaminoglycan, 17% (w/w) protein (protein core M_r 2×10^5) and 5% (w/w) oligosaccharide. In control cultures (Table 1) the total glycosaminoglycan corresponded to 1288 pmol of proteoglycan per culture. This was somewhat larger than the total amount of binding region (891 pmol) and link protein (820 pmol), but does include some non-aggregating proteoglycans, which can comprise up to 30% (w/w) of the total glycosaminoglycan (see Fig. 5*a*). In interleukin 1-treated cultures the total amounts of binding region and link protein detected was significantly decreased. When calculated relative to the total glycosaminoglycan, binding region was 28% less and link protein was 30% less than in control cultures. In contrast, by similar calculation, keratan sulphate was only 9% less. More extensive proteolytic degradation resulting in some net loss of binding-region and link-protein antigens may therefore have occurred in the interleukin 1-treated cultures.

Proteoglycan components released from cartilage cultured with interleukin 1

The early medium (0–1.5 days) and late medium (1.5–3 days) from the interleukin 1-treated cartilage were chromatographed under associative conditions (Figs. 2*a* and 2*b*) in the presence of hyaluronate (to promote

Table 1. Release of proteoglycan components from cartilage explants into medium when cultured for 3 days with or without interleukin 1 (5 ng/ml)

	Amount released (glycosaminoglycans, μg; others, pmol per culture)							
	Control cultures				Cultures with interleukin 1			
	Glycosamino-glycans	Binding region	Link protein	Keratan sulphate	Glycosamino-glycans	Binding region	Link protein	Keratan sulphate
Released into medium	195 (16.2%)	78 (8.7%)	82 (10.1%)	52 (6.7%)	504 (37.8%)	132 (18.4%)	85 (13.5%)	161 (20.3%)
Cartilage extract	1011 (83.8%)	816 (91.3%)	731 (89.9%)	730 (93.3%)	830 (62.2%)	582 (81.6%)	544 (86.5%)	632 (79.7%)
Total	1206	894	813	782	1334	714	629	793

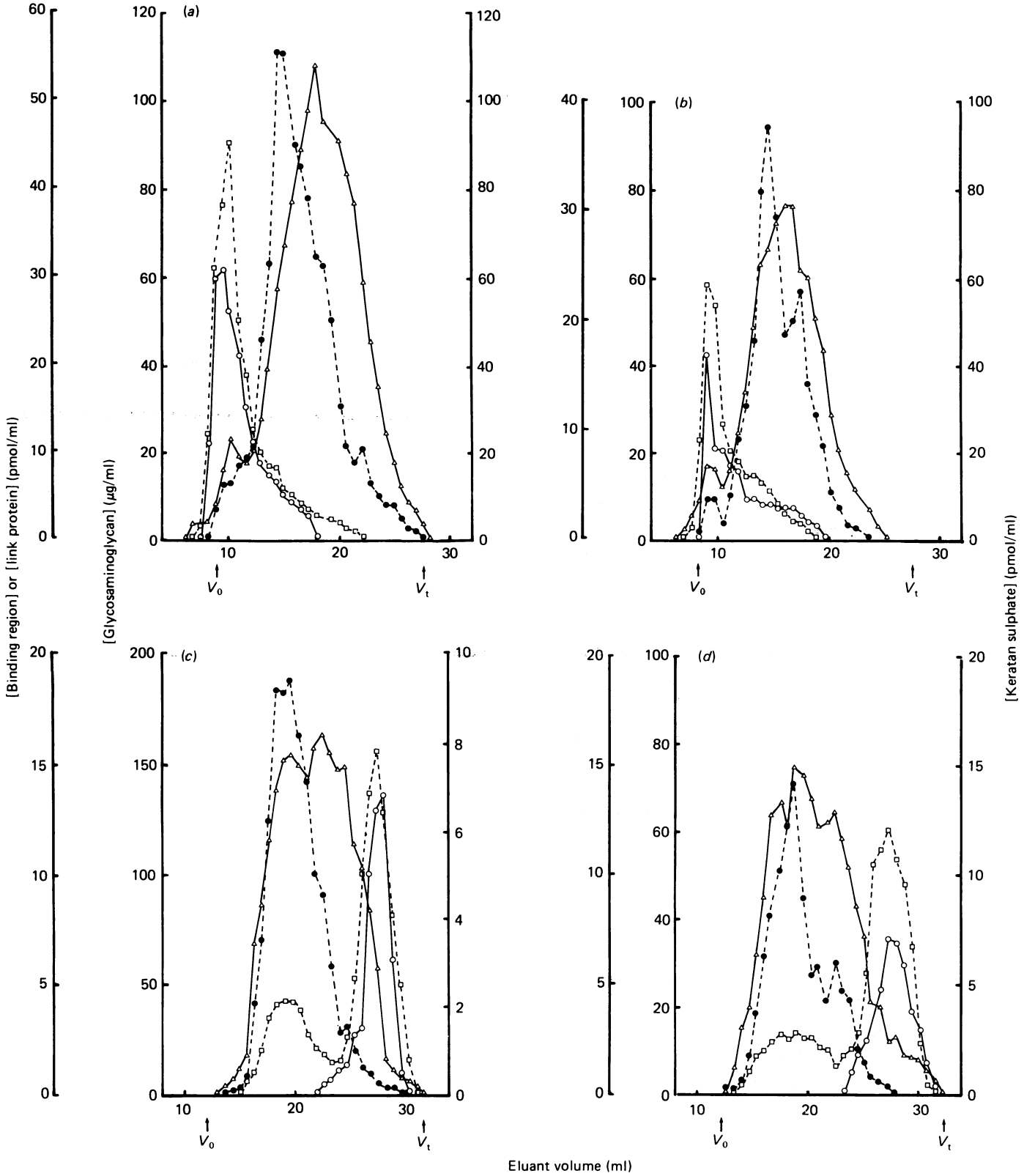

Fig. 2. Gel chromatography of media from cartilage cultured with interleukin 1

The early medium (*a* and *c*) and later medium (*b* and *d*) from interleukin 1-treated cartilage were chromatographed on a column of Sepharose 2B under associative conditions (*a* and *b*) after incubation with high-M_r hyaluronate, and under dissociative conditions (*c* and *d*) as described in the Materials and methods section. The eluate fractions were analysed for glycosaminoglycans (△), binding region (□), link protein (○) and keratan sulphate (●). The elution profiles shown are from a single experiment. Comparable results were obtained in two other experiments (results not shown).

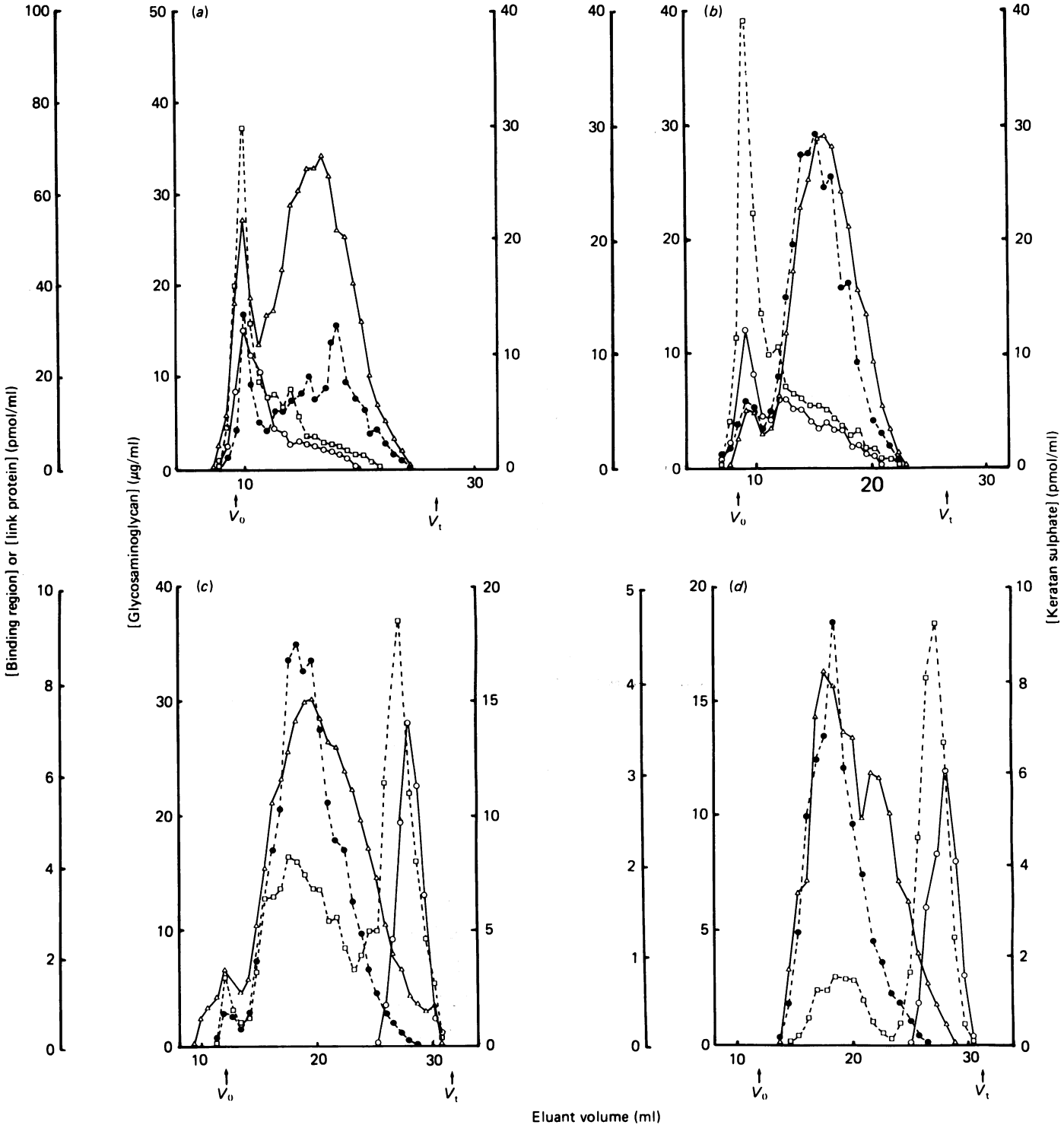

Fig. 3. Gel chromatography of media from control cultured cartilage

The early medium (*a* and *c*) and later medium (*b* and *d*) from control cartilage were chromatographed on a column of Sepharose 2B under associative conditions (*a* and *b*) after incubation with high-M_r hyaluronate, and under dissociative conditions (*c* and *d*) as described in the Materials and methods section. The eluate fractions were analysed for glycosaminoglycans (△), binding region (□), link protein (○) and keratan sulphate (●). The elution profiles are from a single experiment. Comparable results were obtained in two other experiments (results not shown).

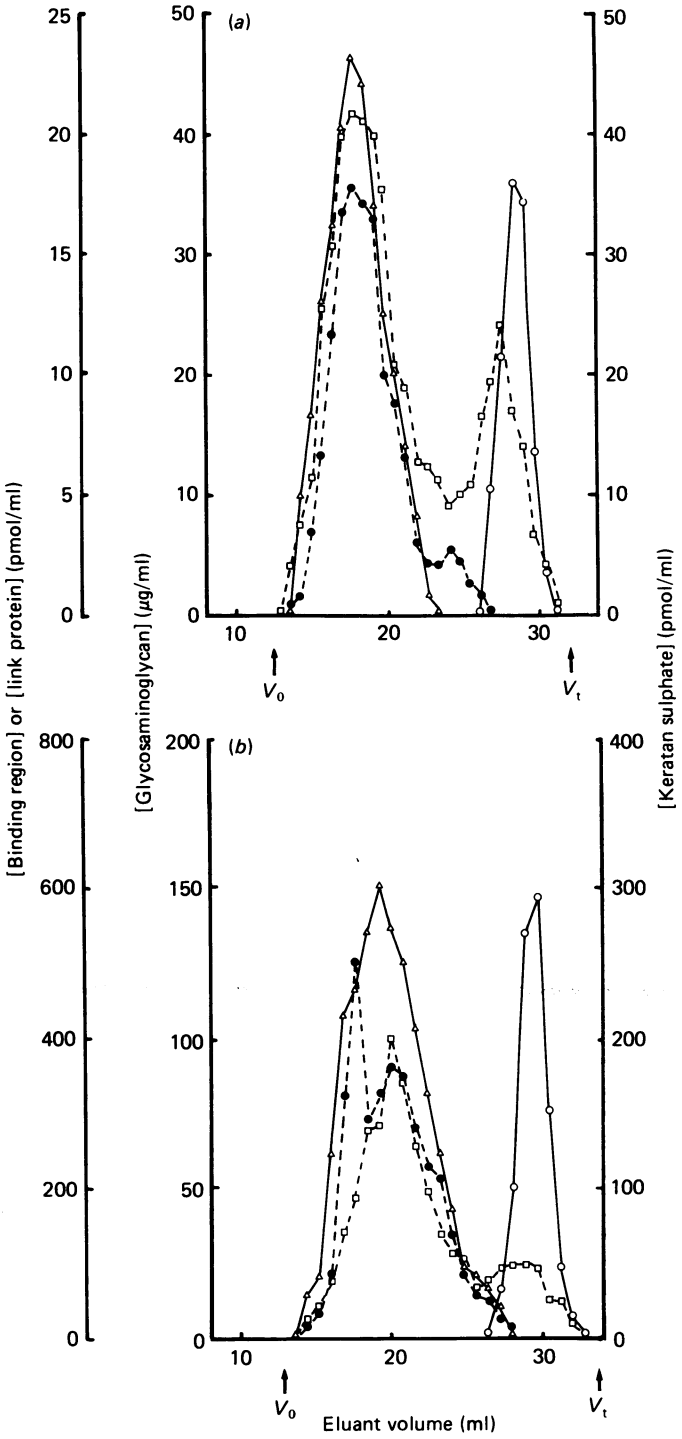

Fig. 4. Gel chromatography of 4 M-guanidinium chloride extracts of the cultured cartilage

The 4 M-guanidinium chloride extracts of the interleukin 1-treated cartilage (*a*) and control cartilage (*b*) were chromatographed on a column of Sepharose 2B under dissociative conditions as described in the Materials and methods section. The eluate fractions were analysed for glycosaminoglycans (△), binding region (□), link protein (○) and keratan sulphate (●).

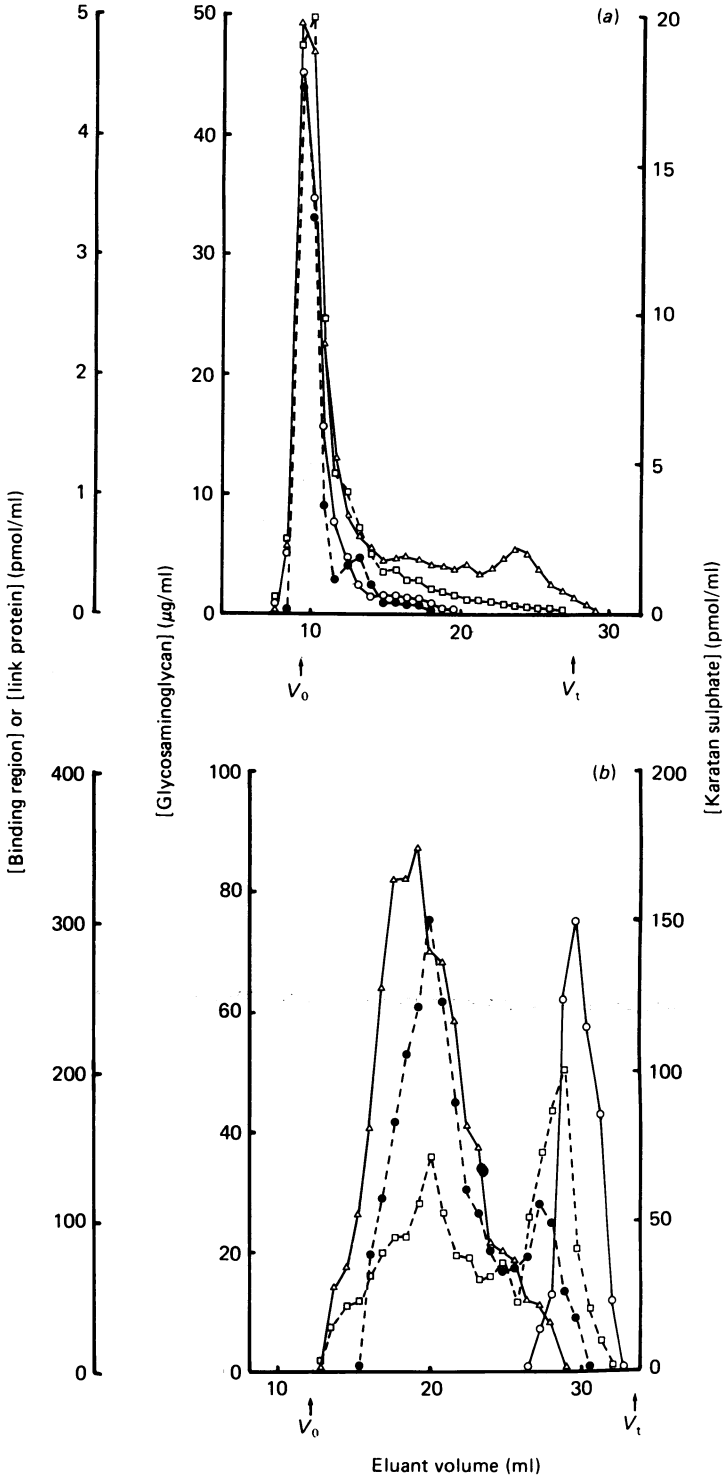

Fig. 5. Gel chromatography of a 4 M-guanidinium chloride extract of non-cultured cartilage

A 4 M-guanidinium chloride extract of cartilage was chromatographed on a column of Sepharose 2B under (*a*) associative and (*b*) dissociative conditions, as described in the Materials and methods section. The eluate fractions were analysed for glycosaminoglycan (△), binding region (□), link protein (○) and keratan sulphate (●).

aggregation), and the glycosaminoglycan elution profile showed that almost all of the released proteoglycans (93% in both the early and late media) were unable to aggregate, being eluted as a broad peak in the included part of the column. The keratan sulphate epitopes showed a similar elution profile, but appeared more evident in the higher-M_r proteoglycans. In contrast, analysis of the column fractions for binding region and link protein showed them to be eluted close to the void volume of the column, indicating that they were bound to hyaluronate, although they were separate from most of the proteoglycans.

Analysis of these media by dissociative chromatography (Figs. 2*c* and 2*d*) showed that the glycosaminoglycans were eluted with a polydisperse size as a broad peak, with the leading part of the peak being eluted as high-M_r proteoglycan but with a proportion of the total glycosaminoglycans being eluted as low-M_r proteoglycan fragments. In the early medium the keratan sulphate epitopes appeared in the position of the large proteoglycan monomers and were absent from the smaller proteoglycan fragments, but in later medium a proportion of the epitope was eluted later with the lower M_r species. The elution profile of binding region showed it to be present as two species, one being eluted in the position of large proteoglycan monomer, but most (70% in the early medium and 73% in the later medium) being eluted just before link protein as a low-M_r species.

Proteoglycan components released from control cartilage

Analysis of the early medium and the later medium from the control cartilage by associative chromatography (Figs. 3*a* and 3*b*) showed that a high proportion of the glycosaminoglycans in the medium (82% of the glycosaminoglycans in the early medium and 95% in the late medium) were unable to aggregate. The keratan sulphate epitopes were mainly with the non-aggregating components, but, in contrast, binding region and link protein were eluted close to the void volume of the column, indicating that they were able to bind to hyaluronate.

When these media were analysed by dissociative chromatography (Figs. 3*c* and 3*d*), the glycosaminoglycans in the early medium were eluted as a broad peak composed of molecules with a polydisperse size, but with a relatively high average M_r, and the glycosaminoglycans of the later medium were eluted similarly. Analysis for keratan sulphate showed the epitope to be associated with the larger proteoglycan fragments in both the early and late media. Binding region was eluted as two peaks: in the early medium 50% was eluted with the high-M_r proteoglycans, and the remainder was eluted just before link protein as a low-M_r species, and in the later medium 78% of the binding region was eluted as a low-M_r species.

Proteoglycans in the 4M-guanidinium chloride extracts of the cultured cartilage

Proteoglycans remaining in cartilage after culture were extracted in 4 M-guanidinium chloride and analysed by associative gel chromatography. A high proportion of the proteoglycan components from both the control and interleukin 1-treated cartilage was eluted near the void volume of the column as aggregating proteoglycan (results not shown). Dissociative chromatography of both extracts (Figs. 4*a* and 4*b*) showed that the glycosaminoglycans were eluted as high-M_r proteoglycan, which also contained the keratan sulphate epitope. Binding region was eluted as two species; however, in the extract from control cartilage, only 16% of extract was of low M_r, whereas in the extract of the interleukin 1-treated cartilage the proportion was 29%.

Proteoglycans extracted from cartilage

Proteoglycans were extracted from fresh (non-cultured) cartilage in 4 M-guanidinium chloride and chromatographed under the associative and dissociative conditions on Sepharose 2B. A high proportion of the proteoglycans (70% of the total glycosaminoglycans) were able to aggregate (Fig. 5*a*). The binding region, link protein and keratan sulphate epitope were also eluted close to the void volume of the column as components of aggregating proteoglycan. Dissociative chromatography (Fig. 5*b*) showed the proteoglycans to be eluted as monomers in a single peak of high average M_r and the keratan sulphate epitope to be eluted in the same position. The binding region was present as two species: 75% was eluted with proteoglycan, but the remainder was eluted separately at low M_r. Link protein formed a single peak of slightly lower M_r than that of the free binding region.

Characteristics of the released binding region and link protein

The associative column fractions of the interleukin 1-treated media were analysed by radioimmunoassay for link protein, but the pre-treatment of the samples with SDS and heat (in order to dissociate aggregates) was omitted. Detection of link protein was diminished by $>95\%$. The antigenic determinants were therefore masked, suggesting that link protein was bound with binding region and hyaluronate as complexes (Ratcliffe & Hardingham, 1983; Bonnet *et al.*, 1985). However, these complexes were dissociated by incubation with oligosaccharides (containing 10–16 saccharide units) of hyaluronate (results not shown). So although the binding region and link protein were able to bind together with hyaluronate, they were less tightly bound than in native proteoglycan aggregates (Hardingham, 1979). Preliminary analysis of the later culture medium by SDS/polyacrylamide-gel electrophoresis and immunoblotting using the anti-(binding region) antiserum detected a single low-M_r proteoglycan fragment containing binding region which was of M_r 115000.

DISCUSSION

Previous experiments (Tyler, 1985*a*,*b*) showed that, when cartilage was maintained in culture in chemically defined medium, there was continued synthesis of proteoglycans similar to those produced *in vivo*, and there was a slow and steady release of proteoglycan components into the medium. This appeared to reflect the normal processes of proteoglycan turnover in the matrix. The proteoglycans released into the medium were of similar size to the high-M_r aggregating proteoglycan in the matrix, but were no longer able to bind to hyaluronate. Treatment of the cultures with interleukin 1 over several days resulted in a greatly increased release of this type of proteoglycan from the matrix, together with some inhibition of proteoglycan synthesis. Both

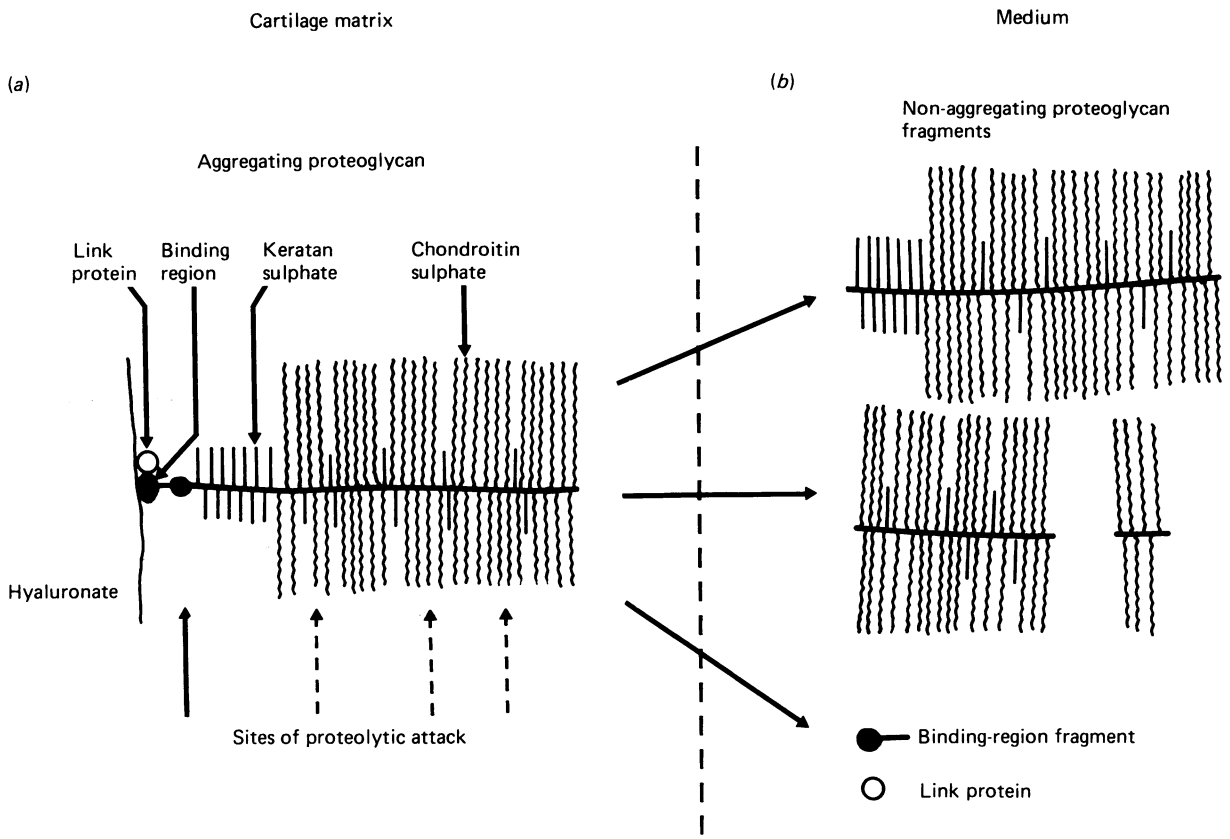

Fig. 6. Aggregating proteoglycan and derived degradation products of articular-cartilage explants

(*a*) Schematic model of aggregating cartilage proteoglycan structure (see Hardingham *et al.*, 1986). (*b*) Fragments derived from aggregating cartilage proteoglycan released into the medium of the cartilage explant cultures. The major site of proteolytic attack is in the protein core close to the binding region involved in aggregation. Major products are the large non-aggregating proteoglycan fragment, which contains the keratan sulphate-rich region, and a binding-region fragment. Further limited cleavage within the major glycosaminoglycan-bearing regions of the protein core produces some smaller proteoglycan fragments. Keratan sulphate remains with the larger proteoglycan fragments. Link protein is also released from the cartilage matrix in similar amounts to the binding-region fragment.

these effects were shown to result from interleukin 1 action on the chondrocytes.

In the present study the proteoglycan components released into the medium have been characterized by using specific antibodies to binding region, to link protein and to a keratan sulphate epitope. In cartilage cultured with pig interleukin 1 there was a steady release of proteoglycans, most of which were of high M_r, but lacked binding-region epitopes. In the same cultures there was a slower release of link protein and binding region, which was unattached to proteoglycan and of low M_r. This binding region, therefore, represented a fragment of the protein core cleaved in the protein region between the binding region and the major chondroitin sulphate- and keratan sulphate-bearing regions (see Fig. 6). Similar products were released at a slower rate in the control cultures. The high initial rate of release of glycosaminoglycans (see Fig. 1) as large non-aggregating proteoglycan, when compared with the release of binding region, indicates that the proteolytic attack results in the separation of those parts of the structure involved in aggregation from those contributing the major physical properties (Fig. 6) and is an important step in both the normal turnover and the stimulated degradation of proteoglycan.

Analysis of 4 M-guanidinium chloride extracts of fresh cartilage before culture showed that 25% of the binding region was already present in the matrix as a separate low-M_r component and, presumably, represented a normal intermediate in proteoglycan turnover. At the end of the culture period of control cartilage only 16% of binding region in the matrix was of low M_r, whereas most of the binding region released into the medium was of low M_r. In interleukin 1-treated cartilage there was an increased appearance of low-M_r binding-region fragment, which resulted in some accumulation within the cartilage matrix and also increased release into the medium. Therefore, when compared with binding region of aggregating proteoglycan, the low-M_r binding-region fragment of proteoglycan was preferentially lost into the medium.

In view of the parallel release of link protein and binding region from both interleukin 1-treated and control explants, it was surprising to find that both components were able to bind to high-M_r hyaluronate added to the medium. Previous results would suggest that binding region and link protein stably bound in aggregate structures are unlikely to dissociate and diffuse out of the matrix (Muir & Hardingham, 1975). The appearance of functional binding region and link protein

in the medium therefore suggests that they arose from components that were able to aggregate but were not stably bound in the matrix (i.e. not bound to hyaluronate or only weakly bound). This might occur if proteoglycan secreted from the chondrocyte into the cartilage matrix failed to make contact with available hyaluronate and remained as free monomer in the matrix. A delay in the aggregation of newly synthesized and secreted proteoglycan has been shown to occur in human articular cartilage (Bayliss *et al.*, 1983, 1984). This was suggested to be caused by the slow formation of intramolecular disulphide bonds in the binding region, which resulted in only low-affinity binding to hyaluronate. Free monomer or weakly bound monomer in the cartilage matrix might be particularly sensitive to proteolytic attack and could therefore be a source of the different fragments that diffuse from the matrix into the medium.

The fragments may also be derived from proteoglycans stably bound in aggregates if the proteolytic attack not only cleaves the protein core to release the major glycosaminoglycan-bearing fragment, but also attacks within the binding-region domain and link protein to weaken the interactions of the protein conformations involved in aggregation. The ability of oligosaccharides to dissociate binding region and link protein from hyaluronate showed that their interaction was not as stable as in native aggregates, although the masking of the link-protein antigenic sites showed that they were bound together in binding-region–link-protein–hyaluronate complexes. Reversible dissociation of these components may therefore lead to their increased mobility within the matrix. The possibility that hyaluronidase activity may be responsible for the release of binding region and link protein with these components still attached to short cleaved sections of the hyaluronate chain would seem unlikely, as such fragments, if bound to the short-chain hyaluronate, would not be free to bind in the medium to the exogenously added hyaluronate (Hardingham, 1979). Hyaluronidase action is therefore unlikely to be the main cause for the release of these components, although its presence at a low level is not precluded by the results.

In the interleukin 1-treated cultures there was increased release of fragments that were similar to those released from control cultures. The total amount of binding region and link protein detected in the interleukin 1-treated cultures was less than that detected in the control cultures. This decrease may result from more extensive proteolytic cleavage of binding region and link protein, leading to a loss of detectable epitopes. However, direct analysis of the size of the binding-region fragment from interleukin 1-treated cultures showed it to be significantly larger (apparent M_r 115000) than the binding-region domain produced by extensive trypsin digestion of proteoglycan aggregate (M_r 65000) (Bonnet *et al.*, 1985), and comparison of gel-elution profiles suggested no significant decrease in the average size of this fragment as a result of interleukin 1 treatment. The results thus suggest that interleukin 1 greatly stimulates the selective cleavage of the protein core seen in untreated cartilage, but there is no evidence of a radical switch in the type of activity involved.

The results obtained by radioimmunoassay for keratan sulphate contribute to understanding the fragmentation of the proteoglycans. Analysis of the specificity of the monoclonal antibody MZ15 suggested that the affinity of the antibodies for keratan sulphate is highest for heavily sulphated sequences (Mehmet *et al.*, 1986) that may not be uniformly present in all keratan sulphate chains. The detection of the epitope may not therefore reflect precisely that of keratan sulphate (Gal-GlcNAc) disaccharides, but provides a marker of a specific structure within keratan sulphate chains. In control and interleukin 1-treated cultures the proportion of keratan sulphate epitope released was significantly less than the proportion of total glycosaminoglycans released, and suggests that proteoglycans poor in keratan sulphate epitope may be turned over more rapidly. The keratan sulphate released was all present in proteoglycan fragments, which were large, but lacked functional binding region (Fig. 6). It was not detected as a separate component in the M_r range corresponding to the keratan sulphate region released from proteoglycan by trypsin digestion (approx. M_r 1.4×10^5) (Bonnet *et al.*, 1985). The release of functional binding region and link protein, and of large proteoglycan fragments still containing a high proportion of the keratan sulphate epitope, suggests that few protein cleavages are involved in their release from the matrix. This may reflect the specificity of the enzyme(s) involved, but may also be greatly influenced by conditions within the matrix, where the enzymes may be surrounded by an extremely high concentration of proteoglycan, which may limit digestion to only those most sensitive substrate sites.

The effect of interleukin 1 on cartilage in culture is mediated by its action on viable chondrocytes in the matrix (Dingle *et al.*, 1979). The increased proteinase activity in the matrix may be the result of the production by the cells of less proteinase inhibitors or more active enzyme(s); and the enzyme released may act directly in the matrix or indirectly by activating other latent proteinases already present. Whatever the precise mechanism, the present results give no indication that interleukin 1 initiates a new mechanism of proteoglycan degradation radically different from that occurring in the control cultures. It seems more likely that it increases the rate and extent of normal proteinase action involved in proteoglycan turnover. Combined with the ability of interleukin 1 to inhibit partially proteoglycan biosynthesis in chondrocytes (Tyler, 1985*b*), it is apparent that the exposure of cartilage to interleukin 1 may readily lead to the progressive depletion of proteoglycan in the matrix.

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