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Turnover of proteoglycans in articular-cartilage cultures

Characterization of proteoglycans released into the medium

Margaret A. CAMPBELL,* Christopher J. HANDLEY† and Stanley E. D'SOUZA‡
Department of Biochemistry, Monash University, Clayton, Vic. 3168, Australia

By using an e.l.i.s.a. method it was demonstrated that the majority of proteoglycans released into the medium of both control and retinoic acid-treated explant cultures of bovine articular cartilage did not contain a hyaluronate-binding region. This supports our previous findings [Campbell & Handley (1987) Arch. Biochem. Biophys. **258**, 143–155] that proteoglycans released into the medium of both cultures were of smaller hydrodynamic size, more polydisperse and unable to form aggregates with hyaluronate. Analysis of ^{35}S-labelled core proteins associated with proteoglycans released into the medium of both cultures by using SDS/polyacrylamide-gel electrophoresis and fluorography indicated the presence of a series of core-protein bands (M_r approx. 300000, 230000, 215000, 200000, 180000, 140000, 135000, 105000, 85000 and 60000) compared with three core proteins derived from the proteoglycans remaining in the matrix (M_r 300000, 230000 and 215000). Further analysis of the core proteins released into the medium indicated that the larger core proteins associated with medium proteoglycans contain both chondroitin sulphate and keratan sulphate glycosaminoglycans whereas the smaller core proteins contain only chondroitin sulphate chains. These experiments provide definitive evidence that the loss of proteoglycans from the matrix involves proteolytic cleavage at various sites along the proteoglycan core protein.

INTRODUCTION

Articular cartilage is an avascular tissue in which the chondrocytes are sparsely distributed in an extracellular matrix that is synthesized by these cells and consists primarily of collagen and proteoglycans. Proteoglycans are large polyanionic hydrated macromolecules that are restrained by the insoluble network of collagen fibres and provide cartilage with the unique ability to resist both compressive and shear loads (Kempson, 1980).

In normal healthy cartilage, chondrocytes regulate the synthesis and turnover of proteoglycans, to maintain constant concentrations of proteoglycans within the extracellular matrix. Previous studies have demonstrated that bovine articular cartilage can be maintained in culture for up to 3 weeks (Handley *et al.*, 1986). After 5 days in culture in the presence of serum, steady-state rates of synthesis and turnover of proteoglycans are attained (Hascall *et al.*, 1983; Campbell *et al.*, 1984; Handley *et al.*, 1986). Addition of analogues of retinol can perturb this balance by stimulating chondrocytes to resorb their own matrix. It has also been suggested that the retinoic acid-mediated stimulation of proteoglycan loss in bovine articular-cartilage cultures may be due to an enhancement of the turnover process that occurs in control cartilage (Campbell & Handley, 1987*b*). In contrast with proteoglycans remaining in the matrix of both control and retinoic acid-treated cultures, proteoglycans released into the medium were smaller in hydrodynamic size, more polydisperse and not capable of forming aggregates (Campbell *et al.*, 1984; Campbell & Handley, 1987*b*).

The aim of the present study was to perform a more extensive characterization of the proteoglycans released into the medium of control and retinoic acid-treated cultures and their constituent core proteins, in order to investigate the possible mechanisms involved in proteoglycan turnover. Clearly, the elucidation of the intricate mechanisms by which chondrocytes remove and replace the matrix in a well-defined culture system, such as the one described, is of potential significance in the understanding of diseases such as degenerative arthritis.

EXPERIMENTAL

Materials

$Na_2{}^{35}SO_4$ (5 mCi/μg of S) and Amplify were purchased from Amersham International (Amersham, Bucks., U.K.). Sepharose CL-2B and Sephadex G-50 (medium grade) were obtained from Pharmacia (Uppsala, Sweden). Tissue-culture flasks (200 ml) were purchased from Nunclon (Roskilde, Denmark). Dulbecco's modified Eagle's medium and fetal-calf serum were purchased from Gibco (Auckland, New Zealand). Eagle's non-essential amino acids were purchased from CSL (Melbourne, Vic., Australia). Retinoic acid, guanidinium chloride, chondroitin ABC lyase, dithiothreitol, trypsin, Tween 20 (polyoxyethylenesorbitan monolaurate) and *p*-nitrophenol phosphate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Endo-β-galactosidase (from *Pseudomonas* sp.) was purchased from Seikagaku Kogyo (Tokyo, Japan). Iodoacetamide was obtained from BDH Chemicals (Port Fairy, Vic., Australia). Flat-bottom Titertek poly(vinyl chloride) microplates (γ-irradiated) were purchased from Flow Laboratories (Melbourne, Vic., Australia). Alkaline phosphatase-conjugated sheep anti-(mouse IgG) antibody was purchased from New England Nuclear (Boston, MA, U.S.A.). A monoclonal antibody specific for the hyaluronic acid-binding region of the proteoglycan monomer, designated 12/21/I-C-6 (Stevens *et al.*, 1984), was a gift from Dr. Bruce Caterson, University of West Virginia, Morgantown, WV, U.S.A.

* Present address: Bone Research Branch, National Institute of Dental Health, National Institutes of Health, Bethesda, MD 20892, U.S.A.
† To whom correspondence should be addressed.
‡ Present address: Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037, U.S.A.

Methods

Cartilage cultures. Articular cartilage from a single metacarpal–phalangeal joint of a 1–2-year-old steer was maintained in medium containing 20% (v/v) fetal-calf serum for 5 days and then incubated for 6 h with 10 ml of medium containing 2 mCi of [^{35}S]sulphate and 20% (v/v) fetal-calf serum (Campbell *et al.*, 1984). The tissue was then washed three times in medium alone, and some tissue was immediately extracted at 4 °C for 48 h with 15 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, in the presence of proteinase inhibitors (Hascall & Kimura, 1982). The remainder of the tissue was distributed into 200 ml plastic culture flasks with either medium alone or medium containing 1 μM-retinoic acid (Campbell & Handley, 1987*a*). The medium from each culture was collected every 24 h and replaced with 20 ml of fresh medium. Medium fractions were stored at 4 °C in the presence of proteinase inhibitors. After a further 5 days in culture the tissue was extracted with 15 ml of 4 M-guanidinium chloride for 24 h at 4 °C.

Quantification of the hyaluronic acid-binding region present in medium and matrix proteoglycans. Pooled medium fractions and 4 M-guanidinium chloride extracts from articular-cartilage cultures were concentrated individually by ultrafiltration on a membrane with an exclusion limit of M_r approx. 30000. The retained portions (3 ml) were each mixed with an equal volume of 8 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, in the presence of proteinase inhibitors, and solid CsCl was added to give a density of 1.47 g/ml. After centrifugation in a Beckman L8 ultracentrifuge with a type 65 fixed-angle rotor at 100000 *g* for 45 h at 10 °C, the gradient was fractionated into four equal parts (Hascall & Sajdera, 1969). The bottom one-fourth of each of the dissociative gradients (D1) contained the majority of the proteoglycan monomers, and these fractions were dialysed extensively against distilled water to remove CsCl, freeze-dried and dissolved in 20 mM-ammonium bicarbonate buffer, pH 8.0. Portions (1 ml) of each preparation were applied to a Sepharose CL-2B column (1 cm × 70 cm) eluted with 0.5 M-sodium acetate buffer, pH 7.0, at a flow rate of 4.5 ml/h. Each fraction (approx. 2.0 ml) was analysed for hexuronate content (Heinegård, 1973) before reduction in 5 mM-dithiothreitol for 1 h at 70 °C followed by alkylation with 35 mM-iodoacetamide. Analysis for presence of hyaluronate-binding region was then determined by using the e.l.i.s.a. method described below.

E.l.i.s.a. procedure. Trypsinized, reduced and alkylated bovine articular-cartilage proteoglycan was used to coat poly(vinyl chloride) micro-titre plates. This was prepared by partially digesting a D1 preparation of bovine articular-cartilage proteoglycan (100–500 μg/ml) in 0.1 M-Tris/HCl/0.1 M-sodium acetate buffer, pH 7.3, with trypsin (final concn. 13 mg/ml). After 5 h at 37 °C the incubation was stopped with 0.1 mM-phenylmethanesulphonyl fluoride at 100 °C for 5 min, after which the sample was reduced and alkylated as described above. Wells of micro-titre plates were coated with 3.4 μg of coating antigen in 20 mM-sodium carbonate buffer, pH 9.6, overnight at 4 °C. These conditions of partial trypsin digestion of the proteoglycan monomer were found to be optimal for binding of the epitope to the micro-titre plate.

After coating and in all subsequent steps, the plates were washed six times with PBS/Tween [0.05% (v/v) Tween 20 in 0.15 M-NaCl/20 mM-sodium phosphate buffer, pH 7.0]. Unoccupied adsorption sites on the plate were then blocked by the addition of 0.5% (w/v) bovine serum albumin in PBS/Tween. For inhibition assays, various concentrations (0.01–10 μg of hexuronate) of standard antigen (reduced and alkylated bovine articular-cartilage proteoglycan monomer) or of reduced and alkylated fractions from Sepharose CL-2B were incubated for 2 h at 37 °C with an excess of antibody 12/21/1-C-6, specific for the hyaluronic acid-binding region of the cartilage proteoglycan monomer (Stevens *et al.*, 1984). Initial experiments showed that the antibody dilution 1:8000 was optimal for inhibition assays. The mixture (100 μl) was then added to the antigen-coated plate for 1 h at 37 °C. Appropriate controls without antibody and/or coating antigen were also included in the procedure. The second antibody (100 μl), alkaline phosphatase-conjugated sheep anti-(mouse IgG) antibody (1:500 dilution), was added to each well and left for 1 h at 37 °C. A 100 μl portion of substrate, 1 mg of *p*-nitrophenyl phosphate/ml in 1 M-ethanolamine/HCl buffer, pH 9.3, was added to each well and incubated for 1 h at 37 °C. The reaction was terminated by the addition of 50 μl of 5 M-NaOH, and the colour produced was measured at 405 nm with a Titretek Multiscan micro-titre-plate reader.

Analysis of core proteins from medium and matrix proteoglycans by SDS/polyacrylamide-gel electrophoresis and fluorography. Medium fractions and 4 M-guanidinium chloride extracts from articular-cartilage cultures containing ^{35}S-labelled proteoglycans were precipitated by the addition of 3 vol. of ethanol and stored overnight at 4 °C. The precipitates were resuspended in 3 ml of 0.1 M-Tris/HCl/0.1 M-sodium acetate buffer, pH 7.3. Portions of each extract containing approx. 4×10^6 d.p.m. of [^{35}S]macromolecular radioactivity were digested overnight at room temperature with chondroitin ABC lyase (0.5 unit/ml) and endo-β-galactosidase (0.1 unit/ml) in the presence of proteinase inhibitors (Oike *et al.*, 1980). Extracts were applied to Sephadex G-50 columns (0.8 cm × 20 cm) equilibrated and eluted with 20 mM-ammonium bicarbonate buffer, pH 8.0. The material in the excluded-volume fractions was pooled, freeze-dried and subjected to electrophoresis under reducing conditions in 5–20%-polyacrylamide-gradient slab gels (1.5 mm thick) as previously described (Laemmli, 1970; Laemmli & Favre, 1973). After the gels had been fixed in 30% (v/v) methanol/10% (v/v) acetic acid they were washed with three changes of distilled water and soaked in Amplify for 30 min before being dried under vacuum. The gels were then exposed to Kodak X-Omat AR films for periods of up to 14 days at −70 °C (Bonner & Laskey, 1974).

RESULTS

Quantification of the hyaluronic acid-binding region present in medium and matrix proteoglycans

E.l.i.s.a. assays were performed with a monoclonal antibody, 12/12/1-C-6, against the hyaluronic acid-binding region of the proteoglycan monomer (Stevens *et al.*, 1984), in order to compare the amounts of hyaluronic acid-binding region present on proteoglycans released

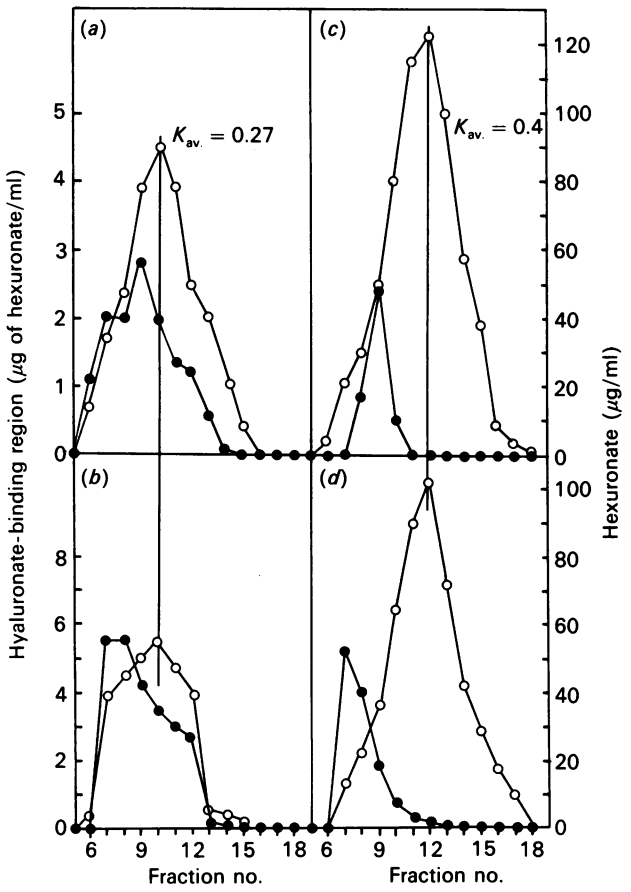

Fig. 1. Quantification of the amount of hyaluronic acid-binding region present on proteoglycans remaining in the matrix (*a* and *b*) or released into the medium (*c* and *d*) of cartilage cultures

Proteoglycans were prepared from matrix and pooled medium extracts of articular cartilage by density-gradient centrifugation. The proteoglycans in the bottom one-fourth of each gradient were applied to Sepharose CL-2B eluted with 0.5 M-sodium acetate, pH 7.0 (V_0 = 12 ml, V_t = 42 ml). Fractions were analysed for hexuronate content (○) and after reduction and alkylation for hyaluronate-binding region (●). Profiles are shown for proteoglycans extracted from the matrix of (*a*) control or (*b*) retinoic acid-treated cultures or released into the medium of (*c*) control or (*d*) retinoic acid-treated cultures.

into the medium with those on macromolecules remaining in the matrix. Medium fractions and 4 M-guanidinium chloride extracts were prepared as described in the Experimental section before elution on a column of Sepharose CL-2B with 0.5 M-sodium acetate buffer, pH 7.0. Fig. 1 shows the profile for the concentration of hyaluronic acid-binding region (● symbols) and hexuronic acid (○ symbols) for proteoglycans extracted from the matrix or released into the medium of control and retinoic acid-treated cultures. The hydrodynamic size of proteoglycans released into the medium of control (Fig. 1*c*) or retinoic acid-treated cultures (Fig. 1*d*) was smaller (elution from Sepharose CL-2B with a $K_{av.}$ of 0.4) compared with proteoglycans remaining in the matrix of both cultures (Figs. 1*a* and 1*b*; $K_{av.}$ 0.27). The majority of the proteoglycans extracted from the matrix of control (Fig. 1*a*) or retinoic acid-treated cultures (Fig. 1*b*) contained a hyaluronic acid-binding region. Conversely, the majority of the proteoglycans released into the medium of control cultures (Fig. 1*c*) or retinoic acid-treated cultures (Fig. 1*d*) did not contain or had significantly smaller proportions of hyaluronic acid-binding region. There is a small proportion of proteoglycans released into the medium that contain a hyaluronic acid-binding region. Proteoglycans from the retinoic acid-treated cultures containing hyaluronic acid-binding region were observed to be eluted close to the void volume of the column and represent proteoglycans aggregated with hyaluronate, the hyaluronate being a contaminant coming through with the proteoglycans in the CsCl-density-gradient step.

Analysis of the core proteins derived from proteoglycans extracted from the matrix or released into the medium

An experiment was designed to determine the apparent M_r values of the ^{35}S-labelled core proteins isolated from proteoglycans extracted from the matrix or released into the medium. ^{35}S-labelled matrix and medium proteo-

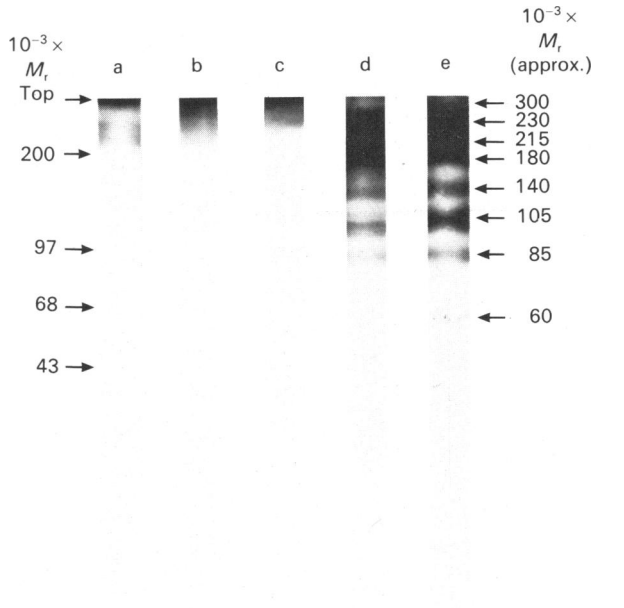

Fig. 2. Fluorogram of a 5–20%-gradient slab gel of ^{35}S-labelled core proteins derived from medium and matrix proteoglycans

^{35}S-labelled proteoglycans from pooled medium or guanidinium chloride extracts from articular cartilage were precipitated with ethanol and then digested with chondroitin ABC lyase and endo-β-galactosidase. The lanes show ^{35}S-labelled core proteins from proteoglycans (lane a) extracted from control cultures on day 5, (lane b) extracted from control cultures on day 10, (lane c) extracted from retinoic acid-treated cultures on day 10, (lane d) released into the medium of control cultures between days 6 and 10, and (lane e) released into the medium of retinoic acid-treated cultures between days 6 and 10. The approximate M_r values of the major ^{35}S-labelled core proteins are shown for each sample.

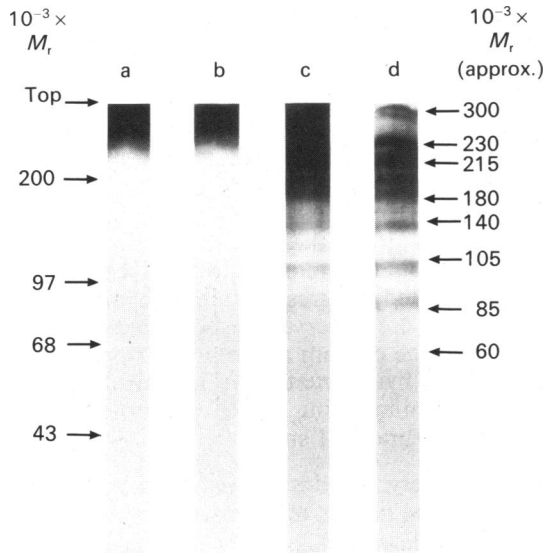

Fig. 3. Fluorogram of a 5–20%-gradient slab gel after digestion of ^{35}S-labelled proteoglycans released into the medium with different combinations of endoglycosidases

^{35}S-labelled proteoglycan from pooled medium samples of articular-cartilage explants were concentrated by ethanol precipitation and then digested with different endoglycosidases. Lane a shows intact medium proteoglycan from control cultures. The other lanes show ^{35}S-labelled core proteins obtained after digestion of medium proteoglycans with (lane b) endo-β-galactosidase, (lane c) chondroitin ABC lyase, and (lane d) both endoglycosidases. The approximate M_r values of the major ^{35}S-labelled core proteins are shown for each sample.

glycans were prepared from bovine articular-cartilage cultures as described in the Experimental section. Samples of each proteoglycan were treated with endo-β-galactosidase and chondroitin ABC lyase in the presence of proteinase inhibitors. Each sample was then subjected to SDS/polyacrylamide-gel electrophoresis on a 5–20%-gradient slab gel followed by fluorography to detect the presence of ^{35}S-labelled core proteins. In control experiments repetitive digestion with endo-β-galactosidase and chondroitin ABC lyase in the presence of proteinase inhibitors did not change the pattern of the core-protein bands obtained. Treatment of ^{35}S-labelled proteoglycans with the two endoglycosidases resulted in approx. 10% of the ^{35}S label remaining associated with the core protein of these macromolecules. Fig. 2 (lanes a–c) demonstrates that a major ^{35}S-labelled core protein with an apparent M_r of 300000 and two minor core proteins with M_r values of 230000 and 215000 were extracted from the matrix of control and retinoic acid-treated cultures. In addition to these bands, a series of ^{35}S-labelled core proteins of smaller size with apparent M_r values of 180000, 140000, 105000, 85000 and 60000 were also detected from proteoglycans released into the medium of both control (Fig. 2, lane d) and retinoic acid-treated cultures (Fig. 2, lane e).

An experiment was designed to investigate the apparent M_r values of the ^{35}S-labelled core protein fragments obtained after the digestion of medium proteoglycans with different combinations of endoglycosidases. Samples of the proteoglycans released into the medium of control cultures from the experiment described in Fig. 2 were treated as indicated: (*a*) no digestion with either endoglycosidase; (*b*) digestion with endo-β-galactosidase; (*c*) digestion with chondroitin ABC lyase; (*d*) digestion with both endoglycosidases. Each sample was then subjected to SDS/polyacrylamide-gel electrophoresis on a 5–20%-gradient slab gel, followed by fluorography. Fig. 3 demonstrates that ^{35}S-labelled medium proteoglycans in the intact form (lane a) or after digestion with endo-β-galactosidase (lane b) did not enter the gel. Analysis of the ^{35}S-labelled core proteins obtained after digestion of medium proteoglycans with chondroitin ABC lyase alone indicated a series of fragments that were larger and in broader bands (with M_r values ranging from 140000 to 300000) than core-protein fragments obtained after digestion with both endo-β-galactosidase and chondroitin ABC lyase (lane d, M_r approx. 300000, 230000, 215000, 200000, 180000 and 140000), together with a series of core proteins that were smaller in size similar to those obtained after digestion with both endoglycosidases (M_r approx. 105000, 85000 and 60000).

DISCUSSION

In comparison with the proteoglycans that are extracted from the matrix of articular-cartilage cultures, the majority of the large aggregating proteoglycans that are released into the medium do not contain a hyaluronic acid-binding region. These findings are in agreement with previous observations (Campbell *et al.*, 1984; Ratcliffe *et al.*, 1986; Campbell & Handley, 1987*b*) suggesting that most proteoglycans appearing in the medium of cartilage explant cultures do not contain or have diminished amounts of functional hyaluronic acid-binding region. There is, however, a small proportion of high-M_r proteoglycans released into the medium that contain a hyaluronic acid-binding region. These macromolecules constitute a small population of intact large aggregating proteoglycans that are released passively from cartilage explants (S. Bolis & C. J. Handley, unpublished work).

Analysis of the core proteins of proteoglycans extracted from the extracellular matrix of cartilage immediately after incubation of the tissue with [^{35}S]sulphate and from the same tissue maintained in culture for 5 days in medium alone or in medium containing retinoic acid demonstrated the presence of three core proteins with apparent M_r values larger than 200000. The presence of more than one population of aggregating proteoglycan has been reported to be present in adult bovine articular cartilage (Thonar *et al.*, 1986); however, it is not clear whether these core proteins are separate gene products or are derived from the same gene product. In fluorograms of matrix proteoglycans exposed for longer periods of time, a core protein fragment with M_r approx. 43000, which represents the core protein of the small non-aggregating proteoglycans of articular cartilage, was apparent (Campbell *et al.*, 1984; Campbell & Handley, 1987*b*; M. A. Campbell & C. J. Handley, unpublished work). Analysis of core proteins of proteoglycans appearing in the medium indicated the presence of a series of core proteins with various apparent M_r values. This demonstrated that the decreased hydrodynamic size and increased polydispersity of medium proteoglycans can be attributed to the proteolytic cleavage of the core protein

of the large aggregating proteoglycan. Moreover, it is apparent that the larger core proteins associated with the medium proteoglycans are devoid of a hyaluronic acid-binding region but contain both keratan sulphate and chondroitin sulphate chains. The smaller core proteins also present in the medium contain only chondroitin sulphate chains and must therefore be derived from the chondroitin sulphate regions of the large aggregating proteoglycans, since these core proteins are larger than the core protein of the small non-aggregating proteoglycan. This implies that the catabolism of the large aggregating proteoglycans in the matrix of cartilage involves the proteolytic cleavage of these macromolecules not only in the hyaluronic acid-binding region but also at various sites along the core protein.

Since the sizes of the core proteins from proteoglycans appearing in the medium of both control and retinoic acid-treated cultures were identical, it can be suggested that retinoic acid stimulates the normal mechanism involved in proteoglycan catabolism. A similar suggestion has been made about the action of interleukin-1 on pig articular cartilage (Ratcliffe *et al.*, 1986). This interpretation assumes that proteoglycan catabolism involves the action of one or more proteinases that cleave the core protein in precise regions. It has been suggested that there may be a site common to a number of proteinases in the hyaluronic acid-binding region, and a similar argument may be made for sites in other parts of the macromolecule, especially since there is evidence for the clustering of glycosaminoglycan chains in the chondroitin sulphate-rich and keratan sulphate-rich regions of the core protein of the large aggregating proteoglycan (Heinegård & Hascall, 1974; Oldberg *et al.*, 1987; Doege *et al.*, 1987).

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