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Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads

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SUMMARY

Articular chondrocytes embedded in alginate gel produce de novo a matrix rich in collagens and proteoglycans. A major advantage of this culture system is that the cells can be recovered by chelating the calcium, which otherwise maintains the alginate in its gel state. Chondrocytes thus released are surrounded by tightly bound cell-associated matrix, which seems to correspond to the pericellular and territorial matrices identified in cartilage by electron microscopy. The cells and their associated matrix can be easily separated by mild centrifugation from more soluble matrix components derived principally from the 'interterritorial' matrix. This new cell culture system thus makes it possible to study the assembly and turnover of molecules present in two distinct matrix pools. Importantly, a significant proportion of the aggrecan molecules in each of these two pools can be extracted using a non-denaturing solvent, thereby making possible studies of the metabolism and turnover of native proteoglycan aggregates. We show in this report that chondrocytes isolated from the full depth of adult bovine articular cartilage and maintained for 8 months in alginate gel are still metabolically active and continue to synthesize cartilage-specific type II collagen and aggrecan. The cells did not synthesize large amounts of type I collagen or of the small nonaggregating proteoglycans as usually occurs when chondrocytes lose their phenotypic stability. After this extended period of time in culture, the cells were present as two populations exhibiting differences in size, shape and amount of extracellular matrix surrounding them. The first population was found only near the surface of the bead: these cells were flattened and surrounded by a matrix sparse in proteoglycans and collagen fibrils. The second population was found throughout the remaining depth of the bead: the cells were more round and almost always surrounded by a basket-like meshwork consisting of densely packed fibrils running tangential to the surface.

Key words: alginate, articular cartilage, chondrocyte, collagen, proteoglycan

INTRODUCTION

Adult articular cartilage is sparsely populated by chondrocytes, which are embedded in an abundant extracellular hydrated matrix comprising collagens (predominantly type II with smaller amounts of types VI, IX and XI) (Eyre et al., 1992), proteoglycans (PGs) (predominantly aggrecan with smaller amounts of decorin, biglycan and fibromodulin) and only small amounts of noncollagenous matrix proteins (Heinegård and Rosa-Pimentel, 1992). The collagens are organized into an insoluble fibrous network of high tensile strength, which helps contain the soluble deformable PGs that give the tissue its ability to undergo reversible deformation (Sah et al., 1992).

A variety of culture systems has been used to elucidate the complex mechanisms regulating the synthesis of PGs, to examine how these become organized into the extracellular matrix and to learn more about the processes involved in their turnover. For example, much of what we know today concerning the structure and synthesis of cartilage PGs was obtained using chondrocytes cultured as high density monolayers (Kimura et al., 1980; Benya and Shaffer, 1982; Solursh et al., 1982; Thonar et al., 1983). In this system, PGs synthesized in the presence of radiolabeled precursors are rapidly lost from the cell-associated matrix and thus can be recovered from the medium in relatively large amounts. A major disadvantage of this approach is that the cells, which are attached to the plastic of the culture dish, usually remain phenotypically stable for only a few weeks (Thonar et al., 1986). Further, as the newly synthesized radiolabeled PGs spend only a short time in the cell-associated matrix, it is not possible to examine how they are turned over (Thonar et al., 1986). As articular cartilage explants can be maintained in steady state for several weeks

(Handley et al., 1986), this type of culture system has been used not only to examine how newly synthesized radiolabeled molecules are incorporated into the existing cartilage matrix but also how they are turned over (Campbell et al., 1984). The average half-life of PGs in explants of articular cartilage from different sources and ages varies from 10 to 25 days (Campbell et al., 1984; Handley et al., 1986; Barone-Varelas et al., 1991); this average half-life is believed to be shorter than that in vivo (Maroudas and Urban, 1980). A disadvantage of the explant culture system is that the newly synthesized aggregating PG (aggrecan) molecules can be recovered in high yield only by using solvents, which cause the dissociation of the large molecular mass aggregates (Campbell et al., 1984). Although studies have shown that native aggregates can be recovered in relatively good yields from very thin sections of articular cartilage extracted with nondenaturing solvents (Bayliss et al., 1983), this approach requires access to a microtome and is labor intensive. The explant system thus has seldom been used as the method of choice for those interested in elucidating some of the complex extracellular interactions that lead to the formation of PG aggregates.

The entrapment of chondrocytes within agarose (Benya et al., 1978; Aydelotte et al., 1986; Bruckner et al., 1989) or on agarose (Archer et al., 1990) has become a popular culture system. Most of the PGs are retained in the vicinity of the cells; the latter accumulate, with time, a matrix that appears to share many of the physicochemical properties of normal cartilage matrix (Buschmann et al., 1992). A significant advantage of this culture system is that the matrix produced by individual cells can be characterized, using techniques such as immunohistochemistry (Aydelotte et al., 1992). Importantly, several studies have now established that the entrapment of isolated chondrocytes in the agarose gel helps promote the synthesis of collagen type II and of keratan sulfate-containing aggrecan, markers of the chondrocytic phenotype (Benya and Shaffer, 1982; Aydelotte et al., 1991; Aydelotte et al., 1992). Recent studies have shown that chondrocytes also can be entrapped in alginate, a linear polysaccharide (mannuronic acid-guluronic acid)$_n$, which forms a gel in the presence of calcium or other divalent cations (Kupchik et al., 1983; Guo et al., 1989; Häuselmann et al., 1992). The alginate-based culture system appears to offer all of the advantages of the agarose system (Häuselmann et al., 1992). However, as the gel can be solubilized by the addition of a chelating agent, it is relatively easy to separate the cells surrounded by a tightly bound cell-associated matrix from components from the 'interterritorial' matrix, which is further-removed from the cells (Häuselmann et al., 1992). This makes it possible to study the distribution and turnover of macromolecules such as PGs in these two distinct matrix pools. Further, as most of the PGs present in these two pools can be extracted with non-denaturing solvents (Häuselmann et al., 1992) the alginate-based system appears to be an ideal tool for studying these molecules in their native aggregated state.

In this paper, we present evidence that bovine adult articular chondrocytes cultured for 8 months in alginate beads have retained their phenotype and are synthesizing collagens and PGs that are typical of those found in the cartilage matrix from which the cells were isolated. We also show that the cells are present as two populations that differ in shape depending upon their location within the alginate bead.

MATERIALS AND METHODS

Materials

Pronase was purchased from Calbiochem, La Jolla, CA; collagenase (*Clostridium histolyticum*, type CLS-2) from Cooper Biomedicals, Cappel Worthington, Malvern, PA; chondroitin ABC lyase and keratanase I from ICN Immuno Biologicals, Costa Mesa, CA; natural *N*-glycanase from Genzyme, Boston, MA; and keratanase II from Seikagagu, Japan. Pepsin, sodium cacodylate, Chaps and Triton X-100 were purchased from Sigma, St Louis, MO. Fetal bovine serum (FBS) was from Hyclone, Logan, UT; Ham's F12/DMEM medium from Gibco, Grand Island, NY; and low viscosity alginate (Keltone LV) from Kelco, Chicago, IL. Poly/Bed 812 was purchased from Polysciences, Warrington, PA; ruthenium hexammine trichloride (RHT) from Aesar, Johnson Matthey Inc., Herts, United Kingdom, and osmium tetroxide (4% aqueous solution) from Electron Microscopy Sciences, Fort Washington, PA. Highly pure rooster comb-derived hyaluronic acid (Healon), Sepharose CL-2B, Sephadex G-25 (as prepacked PD-10 columns), DEAE-Sephacel A-25, Octyl-Sepharose and Superose 12 were from Pharmacia, Piscataway, NJ. Purified bovine link protein was a gift from Dr Lawrence Rosenberg, Montefiore Medical Center, New York, NY. The anti-keratan sulfate (1/20/5-D-4) and anti-hyaluronic acid-binding region (1-C-6) monoclonal antibodies were a gift from Dr B. Caterson, University of N. Carolina, Chapel Hill, NC. Radiolabeling was performed with [^{35}S]sulfate (25-40 Ci/mg), and L-[2,3,4,5-^{3}H]proline (20-50 Ci/mmole) purchased from Amersham, Arlington Heights, IL. All other chemicals were reagent grade and purchased from several different companies.

Cartilage sampling and cell culture in alginate

Metacarpophalangeal joints of steers (18-24 months old) were obtained from a local slaughterhouse. The joints were opened and full-thickness articular cartilage slices were aseptically collected and immediately placed in Ham's F12 medium containing 5% FBS and 50 μg/ml gentamicin. In all experiments, cartilage from at least two hooves was pooled to reduce potential individual variation. Chondrocytes were isolated from slices of bovine articular cartilage as previously described (Kuettner et al., 1982). The isolated cells were encapsulated in alginate beads at a density of 4×10^6 cells/ml of gel. The preparation of chondrocytes in alginate beads was performed as described by (Guo et al., 1989), with the slight modifications recently described (Häuselmann et al., 1992). Briefly, the cells were suspended in sterile 0.15 M NaCl containing low-viscosity alginate gel (1.2%), then slowly expressed through a 22 gauge needle in a dropwise fashion into a 102 mM $CaCl_2$ solution. After instantaneous gelation the beads were allowed to polymerize further for a period of 10 minutes in the $CaCl_2$ solution. After 1 wash in 10 volumes of 0.15 M NaCl and 3 washes in 10 volumes of Ham's F12/DMEM medium, the beads were finally placed in complete culture medium: 9 beads (containing an average number of $44\pm2\times10^3$ cells/bead) were cultured in each well of a 24-well plate. The cells were maintained by feeding daily with 0.4 ml medium/well (Ham's F-12/DMEM medium: 50/50) with 10% FBS, 50 μg/ml gentamicin and 25 μg/ml ascorbic acid) during the first month, then with 1.0 ml medium/well twice a week from the second to the eighth month. The cultures were incubated at all stages in a humidified atmosphere of 5% CO_2 in air at 37°C.

Quantitative and qualitative assessment of [^{3}H]proline-labeled proteins present in different compartments of the alginate beads

After 8 months of culture, the chondrocytes were fed with fresh Ham's F-12/DMEM medium containing all the supplements described above, [^{3}H]proline (final concentration 50 μCi/ml) and BAPN-fumarate (100 μg/ml) to inhibit crosslinking of collagen. After a period of 16 hours, the medium was harvested and stored at 4°C.

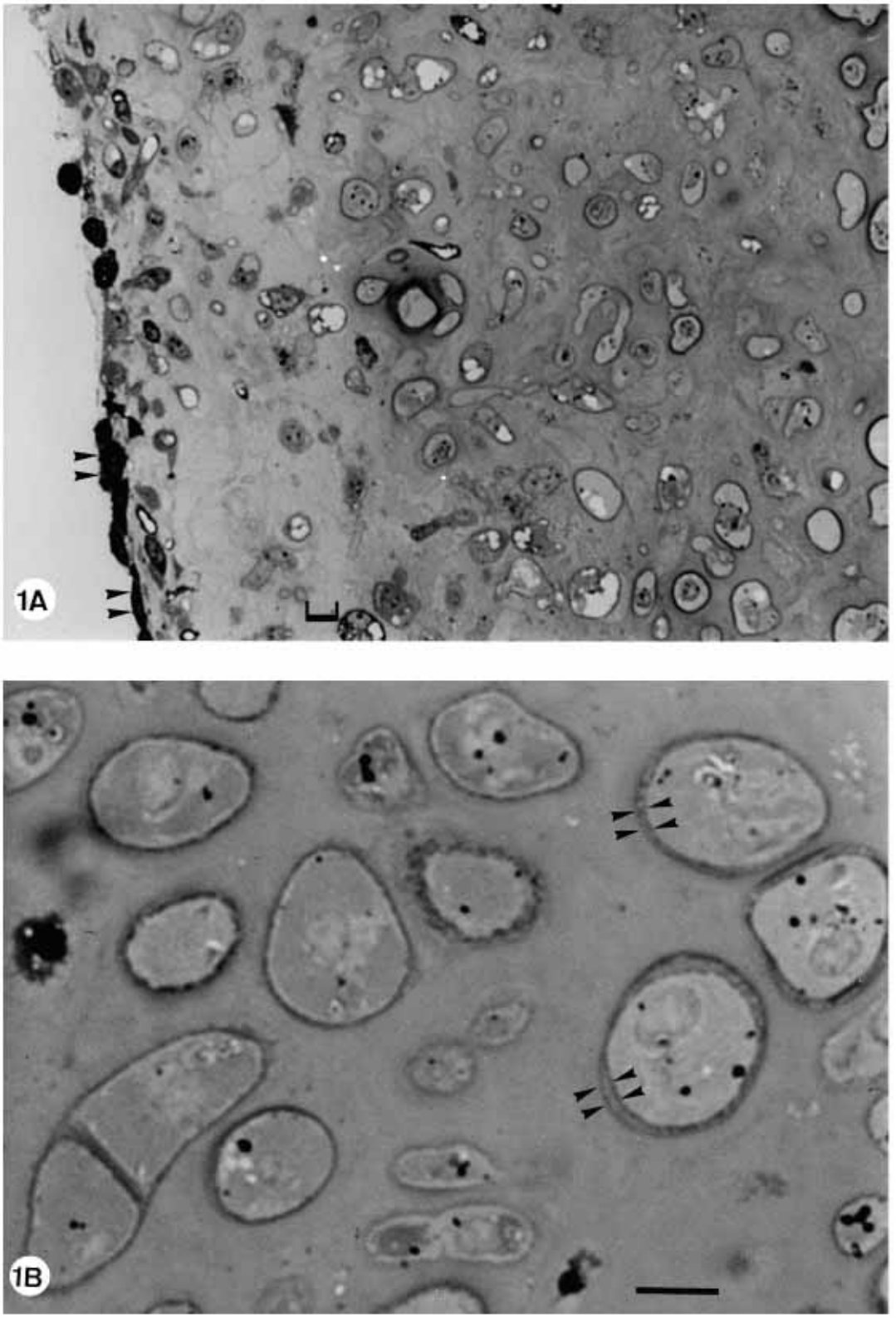


Fig. 1. Semithin sections of peripheral and more central regions of an alginate bead after 8 months of culture, demonstrating the presence of two types of chondrocytes. The beads were fixed in the presence of RHT and sections were stained with uranyl acetate and lead citrate, as described in Materials and Methods. (A) Dark, flattened cells at the free surface of the bead. The arrows point to individual flattened cells. ×550; bar, 10 μm. (B) Round cells in the central portion of the bead, surrounded by a clearly defined newly synthesized cell-associated matrix. The arrows delineate the inner and outer border of the cell-associated matrix. ×1650; bar, 10 μm.

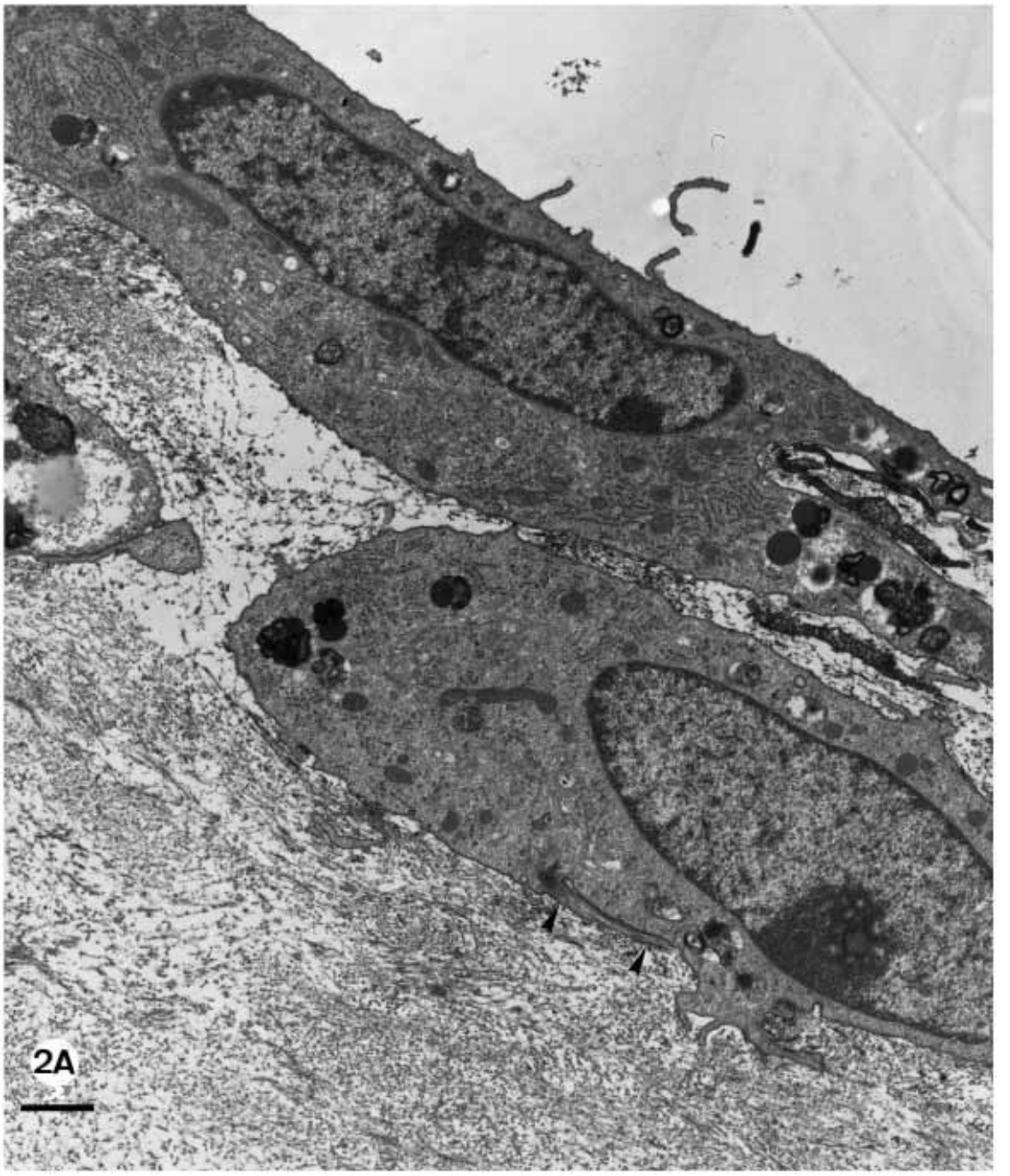
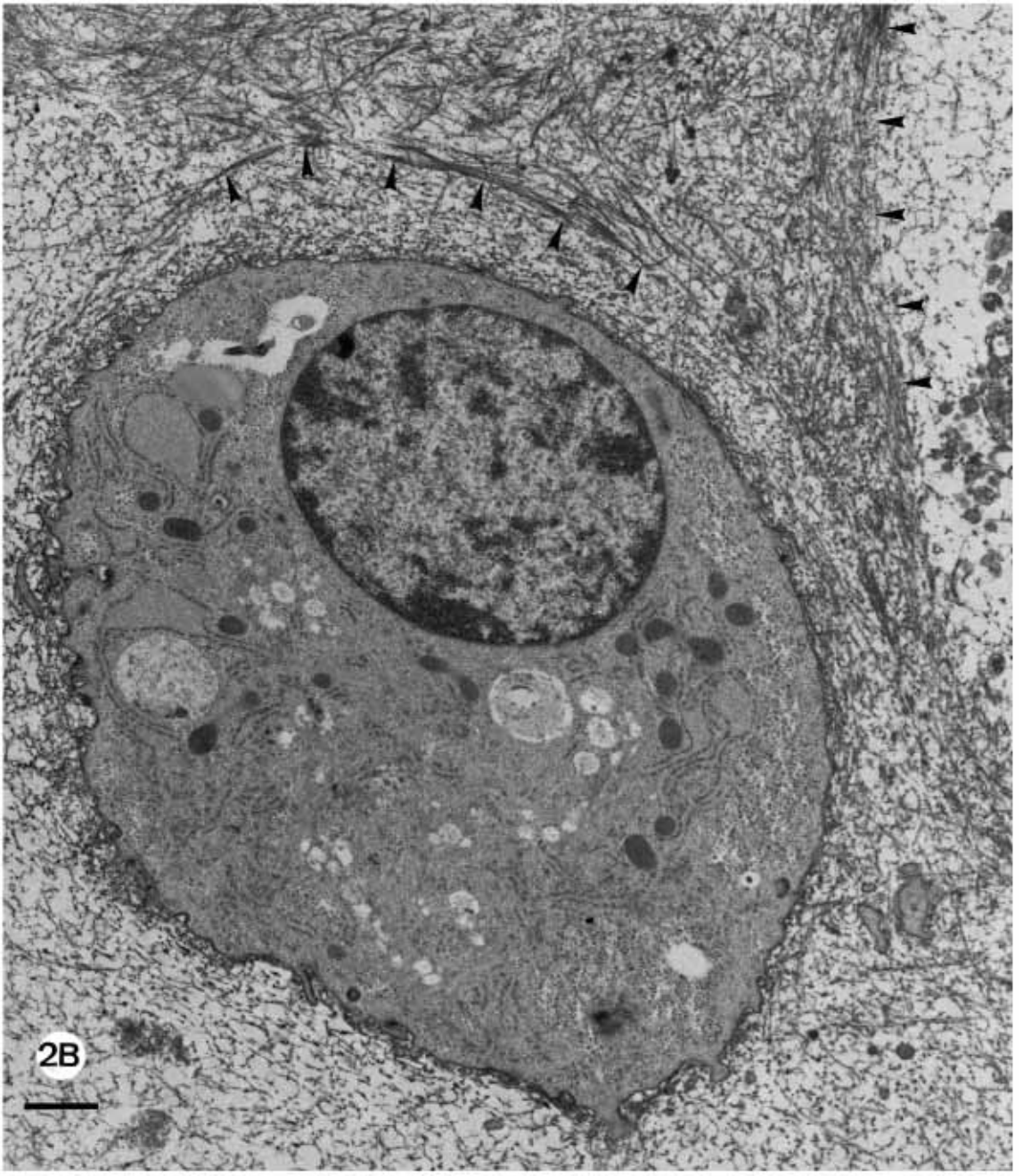


Fig. 2. Electron micrographs of chondrocytes in the peripheral (A) and central (B) region of an alginate bead cultured for 8 months, stained with RHT and prepared as described in Materials and Methods. (A) Two flattened cells at the periphery of the bead. Note the sparse assembly of matrix consisting of proteoglycans and fibrous elements between the cells. In addition please note the cilium (arrowheads). ×3660; bar,1 μm. (B) A round cell in the central portion of the bead. Note the clear separation of the cell-associated matrix from the 'interterritorial' matrix by a

The alginate beads were washed twice with 0.5 ml of the medium; the labeled medium and the washes were combined and are referred to below as the labeled medium fraction. Twenty-five alginate beads then were dissolved by adding 1 ml of 55 mM sodium citrate, 0.15 M NaCl, pH 6.05, at 25°C for 20 minutes. The cells were recovered from the citrate extract by centrifugation (Häuselmann et al., 1992). The pellet consisted predominantly of chondrocytes from the center and periphery of the bead together with their cell-associated matrix; however, we cannot rule out the possibility that other matrix constituents were present in smaller amounts. This pellet was extracted overnight with 1 M NaCl, 50 mM Tris, 5 mM disodium ethylene diamine tetra-acetate (EDTA), 1 mM *N*-ethylmaleimide (NEM) and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5, at 4°C. The cell residue was separated from the NaCl extract by centrifugation. The residue was solubilized in 1% sodium dodecyl sulfate (SDS). The labeled medium, citrate extract, NaCl extract and SDS fractions were dialyzed to remove unincorporated isotope and stored at −20°C.

The [^{3}H]hydroxyproline content of the fractions was measured after acid hydrolysis and separation of the radiolabeled imino acids by HPLC on a cation exchange column. For analysis of collagen type, the samples were incubated overnight at 4°C with pepsin (100 μg/ml) in 0.5 M acetic acid. In each case, the enzyme digestion was stopped



by neutralization with NaOH and the samples were dialyzed. The radiolabeled proteins were separated by SDS-PAGE (Laemmli, 1970) and detected by fluorography (Laskey and Mills, 1975). The ratio of collagen α-chains was measured in each case by laser densitometry using signals integrated by the Hoefer GS365W software (Bonen and Schmid, 1991).

The identification of the radiolabeled collagen chains was confirmed by cyanogen bromide peptide mapping as described by (Barsh et al., 1981) with the modifications of Sokolov et al. (1989).

Quantification of [^{35}S]sulfate-labeled PGs present in different compartments of the alginate beads

After 8 months of culture, the chondrocytes were fed with fresh medium containing [^{35}S]sulfate (final concentration 50 μCi/ml) and all the supplements described above except BAPN-fumarate. After a period of 16 hours, the medium was harvested and stored at 4°C. To release the cells from within the alginate, 25 beads first were dissolved by adding 1 ml of 55 mM sodium citrate and 50 mM EDTA in 150 mM NaCl for 20 minutes at 25°C. The suspension was centrifuged to separate the cells surrounded by cell-associated matrix (the pellet) from components originating predominantly from the 'interterritorial' matrix further removed from the cells (the supernatant) (Häuselmann et al., 1992). The ^{35}S-PGs in each of these two matrix pools were then further extracted at 4°C, using an associative solvent (0.2 M GuHCl) containing protease inhibitors: 5 mM benzamidine HCl, 10 mM NEM and 0.5 mM PMSF. This treatment solubilized all PGs derived predominantly from the 'interterritorial' matrix: no residue was recovered after centrifugation (24,000 ***g***, 15 minutes, 4°C). On the other hand, centrifugation (24,000 ***g***, 15 minutes. 4°C) of the 0.2 M guanidine hydrochloride (GuHCl) extract of the pellet containing PGs derived mostly from the cell-associated matrix yielded a pellet, which was then further extracted at 4°C, using a dissociative solvent

(4 M GuHCl, 0.05 M sodium acetate, pH 6.0) containing protease inhibitors: 5 mM benzamidine · HCl, 10 mM NEM and 0.5 mM PMSF. The radioactivity present in ^{35}S-PGs of associative, dissociative extracts, and labeled media was measured by subjecting a sample of each solution to chromatography on Sephadex G-25 in PD 10 columns, followed by liquid scintillation counting of the material eluting in the void volume (V_o) of the column (Häuselmann et al., 1992).

Chromatography of [^{35}S]sulfate-labeled PGs on Sepharose CL-2B

In preparation for Sepharose CL-2B chromatography, portions of GuHCl extracts and labeled medium were first applied to DEAE-Sephacel ion-exchange chromatography to partially purify the PGs and remove proteases as described elsewhere (Morales et al., 1984). Molecules bound weakly to the DEAE were eluted with 0.15 M NaCl. Radiolabeled PGs were eluted using 1 M NaCl: these ^{35}S-PGs were dialyzed extensively against distilled water and lyophilized. Recovery of ^{35}S-PGs applied to the DEAE column was good (>90%). DEAE-purified PGs from the labeled medium, associative extract of the supernatant, and associative and dissociative extract of the pellet, were reconstituted in 0.05 M sodium acetate, pH 6.0, containing exogenously added hyaluronic acid (40 μg Healon/mg PG) and link protein (80 μg/mg PG) and dialyzed at 4°C for 48 hours against 0.05 M sodium acetate, pH 6.0, in the presence of protease inhibitors. Contents of total PGs were measured using dimethyl-methylene blue (DMB) (Chandrasekhar et al., 1987).

Each sample was then subjected to chromatography on a Sepharose CL-2B column (0.6 cm × 150 cm) eluted with 50 mM sodium acetate, pH 6.0 (Thonar et al., 1986; Häuselmann et al., 1992). Fractions (1.0 ml each) were collected using a flow rate of 5.4 ml/h. The ^{35}S-PGs in each fraction were quantified by scintillation counting (Häuselmann et al., 1992). The void volume (V_o) and total volume (V_t) of the column were determined using ^{35}S-PG aggregates and [^{35}S]sulfate, respectively.

In order to characterize further ^{35}S-PGs present in the labeled medium, a sample of the medium was subjected to equilibrium density gradient ultracentrifugation, using associative conditions (Bayliss and Roughly, 1985; Thonar et al., 1986; Pita and Müller, 1990) and cesium chloride to give a starting density of 1.6 g/ml. At the end of the run, five fractions of equal size were obtained. A sample from each fraction was subjected to chromatography on Sephadex G-25, as described above, to measure ^{35}S-PGs. The 5 fractions were dialyzed first against 50 mM sodium acetate, pH 6.0, then against water and then lyophilized. Radiolabeled PGs in the A1 fraction (density greater or equal to 1.73 g/ml) were reconstituted in 0.05 M sodium acetate and subjected to chromatography on the Sepharose CL-2B column before or after interaction with exogenously added HA and LP as described above.

DEAE-Sephacel and Octyl-Sepharose anion exchange chromatography of small nonaggregating PGs

To characterize and quantify small non-aggregating PGs in the cell-associated matrix, PGs eluting from the Sepharose CL-2B column with partition coefficients (K_d) = 0.6-1.0 were dialyzed for 24 hours at 25°C against several changes of 8 M urea and then purified by DEAE-Sephacel column chromatography in the presence of 8 M urea, using a linear NaCl gradient (0.15 to 1.0 M NaCl) (Yanagishita and Hascall, 1983). Greater than 90% of the ^{35}S-PGs applied to the column were recovered in fractions with molarities between 0.4 and 0.55 M. The purified ^{35}S-PGs then were dialyzed against water, lyophilized, resuspended in 0.2 M GuHCl, 0.1 M sodium acetate, pH 6.3, and subjected to chromatography on Octyl-Sepharose equilibrated with the same buffer (Rosenberg, 1992). After allowing the ^{35}S-PGs to bind to the column for 90 minutes, the column was rinsed with 3 vols of 2 M GuHCl and eluted with a linear gradient of 2 to 6 M GuHCl (Choi et al., 1989). The ^{35}S-PGs did not bind to the Octyl-Sepharose column: they were recovered in the first rinsing step. This fraction was dialyzed at 4°C first against 0.1 M sodium acetate, pH 6.8, then against water and then lyophilized. The ^{35}S-PGs then were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 4% to 8% gradient gel) under reducing conditions (Häuselmann et al., 1992).

High performance liquid chromatography on Superose-12

To measure total keratan sulfate content in ^{35}S-PGs within (i) the A1 fraction of the labeled medium and (ii) the dissociative extract of the cell-associated matrix, the ^{35}S-PGs were treated in each case with Pronase (Block et al., 1991). At the end of the digestion, Pronase was inactivated by boiling for 1 hour. The glycosaminoglycans in the digest were analyzed using a Superose-12 column eluted with 0.45 M sodium acetate and Triton X-100 buffer, pH 7, before and after treatment with glycosidases specific for keratan sulfate (keratanases I and II) and chondroitin sulfate/dermatan sulfate (chondroitinase ABC), using the manufacturers' recommendations. Radioactivity in the fractions was measured by liquid scintillation counting.

Indirect competitive ELISA, using the 1/20/5-D-4 monoclonal antibody against antigenic keratan sulfate

In order to quantify antigenic keratan sulfate, a sample of the A1 fraction of the labeled medium and a dissociative extract of the cell-associated matrix were analyzed by a well-described indirect competitive ELISA, which uses the 1/20/5-D-4 monoclonal antibody specific for a highly sulfated epitope present on the longest keratan sulfate chains (Thonar et al., 1987). The amount of antigenic keratan sulfate present was expressed as equivalents of an international standard of keratan sulfate purified from human costal cartilage (a gift from M. B. Mathews and A. L. Horwitz, The University of Chicago, Chicago, IL) and then related to total PG content measured by the DMB method.

Immunostaining of aggrecan-derived fragments recognized by the 1-C-6 monoclonal antibody

^{35}S-PGs in the A1 fraction from the labeled medium were treated with chondroitinase ABC, keratanase I, and *N*-glycanase alone or in combination and each digest was then separated by SDS-PAGE (4% to 20% gradient gel) using reducing conditions. The gel was blotted onto nitrocellulose (Towbin et al., 1979). The ^{35}S-PGs on the blot were reduced using dithiothreitol (DTT) at 5 mg/ml for 1 hour at pH 8.0, and alkylated using iodoacetamide (IAA) at 7.5 mg/ml for 20 minutes at pH 8.0. Immunostaining was then performed using the 1-C-6 monoclonal antibody specific for an epitope present in both the hyaluronic acid-binding G1 and G2 domains of the aggrecan molecule.

Transmission electron microscopy

Beads cultured for 8 months were rinsed three times in PBS and fixed for 2 hours at room temperature in 0.1 M sodium cacodylate-buffered 1.5% glutaraldehyde (EM grade) and 1% paraformaldehyde, pH 6.7, with 0.4% ruthenium hexammine trichloride (RHT) then rinsed 3× in 0.15 M sodium cacodylate (Hunziker et al., 1982). After post-fixation for 2 hours in 0.1 M sodium cacodylate buffered 1% osmium tetroxide (OsO_4) with 0.4% RHT, pH 6.7, the beads were dehydrated in a series of graded ethanols and embedded in Poly/Bed 812. Thin sections were obtained with a Porter-Blum MT 2-B ultramicrotome and stained with 2% ethanolic uranyl acetate and lead citrate. All electron micrographs were obtained using a JEOL 1200 transmission electron microscope (Häuselmann et al., 1992).

RESULTS

Description of chondrocyte subpopulations

Articular chondrocytes derived from the full-depth of articular

cartilage from the metacarpophalangeal joints of 18-month-old bovine animals were cultured in alginate beads for 8 months. After this extended period in culture, the cells were present as two populations exhibiting marked differences in size, shape and amount of extracellular matrix surrounding them (Figs 1A,B, 2A,B). The first population of chondrocytes was found close to the periphery of the bead and exhibited a flattened appearance reminiscent of cells normally found in the most superficial layer of the tissue (Figs 1A, 2A) (Aydelotte et al., 1988; Hunziker, 1992; Archer et al., 1990). They were arranged in a matrix-poor 1- to 3-cell-thick layer, with the most superficial cell usually directly exposed to the culture medium. The second population was found throughout the remaining depth of the bead. These cells, which were more round in appearance (Figs 1B, 2B), were almost always surrounded by a basket-like meshwork consisting of densely packed fibrils running tangential to the cell surface. This meshwork appeared to separate the cell-associated (pericellular + territorial) matrix poor in fibrils from the further-removed interterritorial matrix rich in randomly arranged fibrils.

Table 1. Accumulation of [^{3}H]proline-labeled proteins in the medium and different matrix pools

Sample	Total [^{3}H]proline (cpm × 10^6)	[^{3}H]OH-proline (% of [^{3}H]proline)	Total [^{3}H]OH-proline (cpm × 10^4)	% of [^{3}H]OH-proline
Medium	1.60	5.6	8.83	33.8
Citrate	0.75	7.3	5.46	20.9
NaCl	1.50	4.1	6.14	23.5
SDS	2.38	2.4	5.68	21.7

Proteins were synthesized during a 16-hour incubation in the presence of [^{3}H]proline and BAPN. For each sample, results are expressed as total [^{3}H]proline in non-dialyzable proteins, % [^{3}H]hydroxyproline ([^{3}H]OH-proline) in the [^{3}H]proline-labeled proteins, total [^{3}H]hydroxyproline-labeled proteins and % of the total [^{3}H]hydroxyproline-labeled proteins synthesized. Citrate denotes the supernatant obtained after citrate solubilization of the beads and mild centrifugation; this is believed to represent the interterritorial matrix pool (Hauselmann et al., 1992). NaCl denotes the 1 M NaCl extract of the pellet recovered by centrifugation, and SDS the solubilized residue remaining after NaCl extraction. The NaCl and SDS fractions are believed to represent principally the cell-associated matrix pool.

Quantitative and qualitative analyses of [^{3}H]collagens

Collagen synthesis was evaluated in the long-term chondrocyte cultures after radiolabeling for 16 hours with [^{3}H]proline. As shown in Table 1, [^{3}H]proline-labeled proteins were recovered in four fractions: the labeled medium, citrate extract, NaCl extract and SDS fraction. The [^{3}H]hydroxyproline content of the fractions revealed that two-thirds of the collagen was retained within the alginate beads during the 16 hours of culture (Table 1). The labeled proteins in each of the fractions were incubated with pepsin, which degrades most noncollagenous proteins and removes nonhelical regions from procollagen molecules. This enzymic digestion simplifies the identification of different collagen types in complex mixtures. A separation of the radiolabeled, pepsin-resistant proteins from each fraction by SDS-PAGE is shown in Fig. 3. The majority of the triple-helical, pepsin-resistant molecules were present in the medium, citrate and NaCl fractions. The fractions contained a prominent band at 95 kDa, which comigrated with collagen α1 chain standards. The relative prominence of α1 chains suggests that the majority of the collagen synthesized by the cultures was type II collagen. Small amounts of α2 chains were detected in the medium and NaCl fractions, suggesting that some type I collagen was synthesized. Densitometric analysis of the fractions yielded α1/α2 ratios of 15 for the medium, 58 for the citrate extract and 19 for the NaCl extract (Table 2). If no type I collagen 'trimer' was present, these values indicate that type I collagen, which becomes a prominent product of chondrocytes cultured as monolayers for longer than a few weeks (Cheung et al., 1976), made up a little bit more than 10% of the total amount of α1 collagen chains synthesized. To test if the majority of the α1 chains were indeed derived from the cartilage-specific type II collagen and not from type I, the samples were first separated by SDS-PAGE as shown in Fig. 3, the entire lane was digested with cyanogen bromide and the resulting peptides were then separated on a second acrylamide gel run perpendicular to the first dimension as described by Barsh et al. (1981). The majority of the material migrating in the α1 chain position yielded in all cases a cyanogen bromide peptide pattern similar to that of α1(II) chains (data not shown), confirming that little type I collagen 'trimer' was present. Besides type II and type I collagen some minor collagen bands were detected. The bands, which ran slightly slower than the α1 chains, were probably derived from type XI. Small amounts of type IX but no type X collagen were detected in the cultures. The citrate fraction (Fig. 3, lane C) and the NaCl fraction (Fig. 3, lane N) did contain another prominent, pepsin-resistant, proline-labeled protein whose chains have a molecular size in excess of 200 kDa. This noncollagenous glycoprotein was resistant to limited pepsin digestion and to bacterial collagenase treatment (Th. M. Schmid et al., unpublished data).

Quantitative and qualitative analyses of ^{35}S-PGs

^{35}S-PGs in the labeled medium

Approximately half the ^{35}S-PGs synthesized by the cells during the 16-hour incubation in the presence of [^{35}S]sulfate were recovered in the labeled medium (Table 3). These PGs were unable to aggregate in the presence of exogenously added hyaluronic acid and link protein; they eluted as a broad peak on Sepharose CL-2B with an average partition coefficient (K_{av}) of 0.70 (Fig. 4A). As only 16% of the ^{35}S-molecules were found in the fraction of lowest density (Table 4), where low buoyant density PGs, i.e. decorin, biglycan and fibromodulin, would be expected to be present, these small ^{35}S-PGs most likely represent degradation products of aggrecan. Forty-one per cent of the ^{35}S-PGs in the medium were recovered in the A1 fraction of the equilibrium density gradient (density greater or equal to 1.73 g/ml) (Table 4). These high-buoyant density molecules had a larger average hydrodynamic size than the other PGs in the medium, but they exhibited as broad a polydispersity (Fig. 5). Chromatography of the Pronase digest of the high-buoyant density ^{35}S-PGs on Superose 12, before and after treatment with specific glycosidases, revealed that 94.6% of the ^{35}S-glycosaminoglycans in the A1 fraction were sensitive to chondroitinase ABC digestion and thus probably represent chondroitin sulfate chains: the remaining glycosaminoglycans were sensitive to digestion with keratanases I+II and were identified as keratan sulfate. These keratan

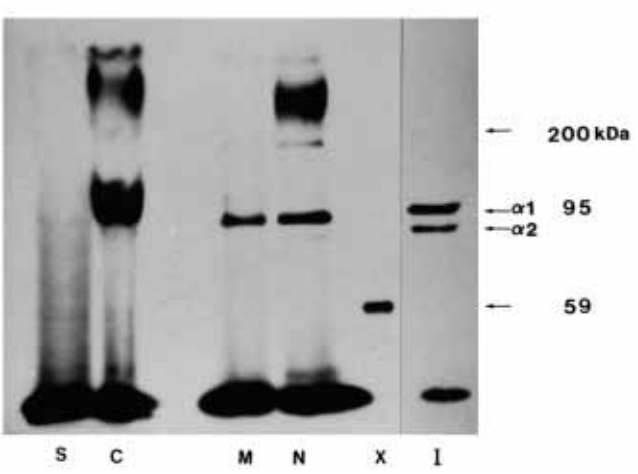


Fig. 3. Fluorogram of pepsin-resistant [^{3}H]proline-labeled proteins in the medium and extracts of beads containing bovine chondrocytes maintained in culture for 8 months and then incubated for 16 hours in the presence of [^{3}H]proline. All lanes were reduced with dithiothreitol. Analysis of the labeled medium (M) and NaCl extract (N) of the cells and their associated matrix (pellet) recovered after citrate solubilization of the beads clearly shows that α1 chains predominate as the main product of collagen synthesis. Lane S, proteins in the SDS extract of cells and their associated matrix after NaCl extraction; lane C, proteins of the 'interterritorial' matrix in the supernatant obtained by centrifugation after citrate solubilization of the beads; lane M, proteins in the labeled medium; lane N, proteins extracted from the cells and their associated matrix by 1 M NaCl, recovered after citrate solubilization of the beads and mild centrifugation (pellet). Lanes X and I, standards for type X and type I collagen.

Table 2. Analysis of type II and type I collagens in the labeled medium and different matrix pools based on densitometric analysis of α1/α2 bands in SDS-PAGE

	α1/α2	Type II (%)	Type I (%)
Medium	15:1	76.7	23.3
Citrate	58:1	94.7	5.3
NaCl	19:1	82.5	17.5

Our calculations of the proportions of type II and type I collagens in the different preparations were based upon the known composition of the type I and type II collagen molecules. Type II, $[\alpha1(II)]_3$; and type I, $[\alpha1(I)]_2\alpha2(I)$ with two α1 chains and one α2 chain. The calculations were also made possible by the fact that our cyanogen bromide analysis did not reveal any significant amounts of type I trimer collagen. Thus a ratio of α1 to α2 chains of 15 to 1 indicates that there are 13 α1(II) chains and 2 α1(I) chains for every α2 (I) chain. To determine the number of molecules of type II and type I in this mixture, one must divide the sum of the type II and I chains by 3 (3 chains per molecule); hence 13:3 = 4.3 and 3:3 = 1. This yields proportions of type II and type I collagens of 4.3 and 1, respectively, indicating that 76.7% of the α1 chains originate from collagen type II and 23.3% from collagen type I in the medium.

Table 3. Percentages of newly synthesized ^{35}S-proteoglycans in labeled medium and different matrix pools

	%
Labeled medium	53.0
Supernatant (0.2 M GuHCl)	20.7
0.2 M GuHCl extract of pellet	8.2
4 M GuHCl extract of pellet	16.1

^{35}S-proteoglycans were synthesized during a 16-hour incubation in the presence of [^{35}S]sulfate. Different matrix pools were recovered after citrate solubilization of the beads and mild centrifugation. The supernatant (representing the interterritorial matrix pool) was made 0.2 M with GuHCl to promote solubilization of the proteoglycans while the pellet (representing the cells and their associated matrix) was extracted first with 0.2 M GuHCl and then 4 M GuHCl. A sample of each pool was then quantified by scintillation counting.

sulfate chains synthesized after 8 months of culture contained significant amounts of the 5-D-4 epitope, although the content of the 5-D-4 epitope was ≈ 5 times lower than in keratan sulfate purified from the cartilage from which the cells were isolated. The core proteins obtained after digestion of the A1-PGs with chondroitinase ABC, keratanase I and *N*-glycanase migrated in SDS-PAGE as a broad band (160-250 kDa), which did not show immunoreactivity (western blot) with the 1-C-6 antibody specific for an epitope present in the G1 and G2 domain of the aggrecan molecule (data not shown). These results strongly suggest that the high buoyant density PGs in the medium represent aggrecan-derived fragments that have lost the hyaluronic acid-binding region.

^{35}S-PGs in different compartments of the alginate gel

Rapid solubilization of the alginate beads with a chelating agent followed by centrifugation provided a supernatant, containing mostly PGs derived from the interterritorial matrix further-removed from the cells, and a pellet, containing the cell-associated matrix (Häuselmann et al., 1992). The supernatant contained 44% of the ^{35}S-PGs in the alginate (Table 3). Chromatography of this sample on Sepharose CL-2B after the addition of hyaluronic acid and link protein revealed the presence of several populations of ^{35}S-molecules differing markedly in size and representing: (i) aggregates eluting in the V_o, (ii) aggrecan subunits and (iii) small nonaggregating PGs (Fig. 4B).

The associative and dissociative extracts of the pellet contained 19% and 37%, respectively, of the ^{35}S-PGs originally present in the beads. The populations of PGs detected in the supernatant also were present in these extracts of the pellet (Fig. 4C,D). Importantly, the associative extract of the pellet contained an unusually high proportion of small nonaggregating PGs eluting with partition coefficients between 0.6 and 1.0 on Sepharose CL-2B (Fig. 4C). These small PGs bound strongly to DEAE-Sephacel in the presence of 8 M urea. They were eluted from the DEAE with 0.5 M NaCl, 8 M urea, and, after dialysis, were chromatographed on Octyl-Sepharose. Surprisingly, they did not bind to this gel, suggesting that they have a different composition than the typical small nonaggregating PGs (biglycan and decorin) which normally bind to this column (Rosenberg, 1992). They migrated as a broad band (4×10^4 to 6×10^4 Da) on SDS-PAGE and after western blotting did not show immunoreactivity with antibodies against the HA-binding region of aggrecan (1-C-6) or a highly sulfated keratan sulfate epitope (5-D-4). These small PGs were not

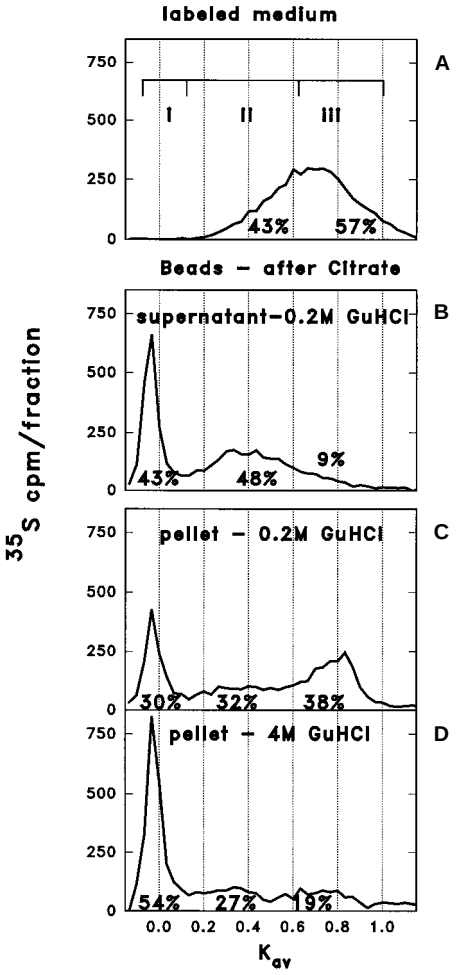


Fig. 4. Sepharose CL-2B sieve chromatography of ^{35}S-PGs in the medium and extracts of alginate beads cultured for 8 months. All ^{35}S-PG-containing samples were partially purified by DEAE-Sephacel anion-exchange chromatography and then interacted with hyaluronan (Healon) and link protein, prior to chromatography on a Sepharose CL-2B column eluted with an associative solvent, as described in Materials and Methods. Analysis of the ^{35}S-PGs in the labeled medium (A) suggests that the aggrecan molecules that diffused into the medium during the 16-hour incubation in the presence of [^{35}S]sulfate had lost their ability to form aggregates. Analysis of GuHCl extracts of the 'interterritorial' matrix (supernatant, B) and cells and their associated matrix (pellet, C and D) recovered after citrate solubilization of the beads shows that proteoglycan aggregates and large nonaggregating proteoglycans predominate as the main product of proteoglycan synthesis. The percentages of aggregates, large nonaggregating proteoglycans and small non-aggregating proteoglycans were calculated by measuring radiolabel present in fractions i, ii and iii. Partition coefficients (K_d) were calculated as described previously (Häuselmann et al., 1992).

found in significant amounts in the subsequent dissociative extract of the pellet (Fig. 4D), which contained predominantly aggregating ^{35}S-PGs bearing chondroitin sulfate (90% of total GAGs) and keratan sulfate (10%).

Table 4. Equilibrium density gradient centrifugation of ^{35}S-proteoglycans in the labeled medium

Gradient fraction	Density (g/ml)	%
A1	1.73	41.2
A2	1.68	16.4
A3	1.60	11.8
A4	1.56	14.7
A5	1.53	15.9

^{35}S-proteoglycans in the labeled medium were subjected to equilibrium density gradient ultracentrifugation, using associative conditions, as described in Materials and Methods. The tube was fractionated into five fractions of equal size (A1-A5). The density and percentage of ^{35}S-proteoglycans in each fraction are given.

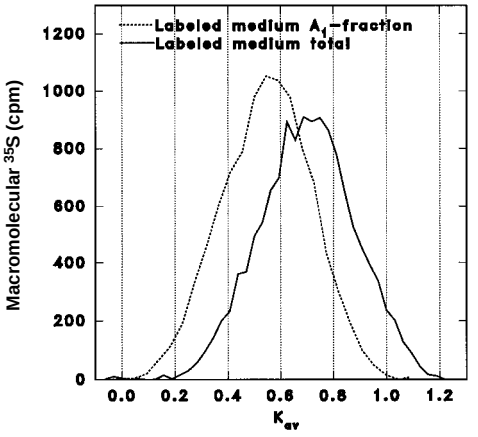


Fig. 5. Sepharose CL-2B sieve chromatography of high buoyant density ^{35}S-PGs from the labeled medium. ^{35}S-PGs from the labeled medium were subjected to equilibrium density gradient ultracentrifugation (associative conditions), as described in Materials and Methods. ^{35}S-PGs with densities equal or greater than 1.73 g/ml (A1 fraction) were then interacted with hyaluronan (Healon) and link protein, and chromatographed on a Sepharose CL-2B column eluted with an associative solvent. The high buoyant density ^{35}S-PGs in the A1 fraction had a larger average hydrodynamic size than ^{35}S-PGs in the total medium, but they exhibited as broad a polydispersity.

DISCUSSION

The results of this study demonstrate that bovine adult articular chondrocytes remain metabolically active when cultured for 8 months in gel beads composed of alginate (Kupchik et al., 1983; Guo et al., 1989; Häuselmann et al., 1992). The cells were shown to have retained the ability to synthesize cartilage-specific molecules such as keratan sulfate-bearing aggrecan and type II collagen. The chondrocytes synthesized much smaller amounts of type I collagen and small nonaggregating PGs, which are expressed in increased amounts by chondrocytes that have become phenotypically unstable in vitro (Benya and Shaffer, 1982; von der Mark, 1986). Aggrecan made up 82% of the ^{35}S-PGs present in the bead at the end of the 16-hour period of labeling: these aggregating PGs were recovered in similar amounts from two distinct pools representing the cell-associated matrix and the matrix further removed from the cells. Sixty-three per cent of all newly synthesized ^{35}S-aggrecan molecules could be fully solubilized without

undergoing denaturation; of these, approximately half were recovered as aggregates. In addition to type II collagen, the cells also synthesized small amounts of type IX collagen, which plays an important role in crosslinking of the collagen network in articular cartilage (Eyre et al., 1992), but no type X collagen, a marker for hypertrophic cartilage undergoing endochondral ossification (Schmid and Linsenmayer, 1987; Bruckner et al., 1989) and for articular chondrocytes that have become hypertrophic in osteoarthritis (von der Mark et al., 1992). The synthesis of type II collagen and aggrecan is down-regulated when chondrocytes are grown as monolayers, especially when the cells are plated at low density (Benya and Shaffer, 1982; Daniel et al., 1984) but it is sustained when the cells are cultured in agarose gel for up to one month (Aydelotte et al., 1992). It is thus not possible at this stage to conclude that the long-term retention of the chondrocytic phenotype in vitro is easier to achieve in alginate, a highly negatively charged milieu (Häuselmann et al., 1992), than in other suspension media that allow the chondrocytes to encapsulate themselves in their own matrix (von der Mark, 1986). Although most of the chondrocytes had retained the round shape typically expressed by chondrocytes that populate the deeper layers of articular cartilage (Aydelotte and Kuettner, 1988), cells lying closer to the surface were flattened. It is important to note that these flattened cells were not present in significant amounts after 3 weeks of culture in alginate beads (Häuselmann et al., 1992). The relationship of the two populations of chondrocytes is not clear at this stage. It is also not known to what extent the two types of cells may differ with respect to the cartilage-specific gene products they produce.

The proportion of ^{35}S-PGs recovered in the medium at the end of the labeling period was much greater than in 8-day-old cultures (53% vs 2% of ^{35}S-PGs; Häuselmann et al., 1992). This was shown to reflect a marked increase in the production of ^{35}S-aggrecan subunits that had lost the HA-binding domain that normally enables these molecules to form aggregates by binding to a strand of HA (Kimura et al., 1980). As flattened cells derived from the superficial layer of bovine steer articular cartilage and cultured in agarose turn over aggrecan more rapidly than cells derived from the deeper layer of the tissue (Aydelotte et al., 1988), it is possible that the superficial flattened cells that were prominent only in the 8-month-old beads may have contributed to this marked increase in limited proteolytic action on the core protein of aggrecan. The relative ease with which one can separate these nonfunctional fragments of aggrecan (which diffuse into the medium) from the larger intact aggrecan subunits and aggregates (which are retained in the bead) should enable investigators to use this culture system to study the turnover of aggrecan, as has been done in the past using cartilage explants (Campbell et al., 1984; Handley et al., 1986; Barone-Varelas et al., 1991) or chondrocytes suspended in agarose (Aydelotte et al., 1986). Importantly, the alginate bead system could for the first time enable investigators to test the hypothesis that the rate of aggrecan turnover is much more rapid in the cell-associated matrix than in further-removed matrix that is no longer under the direct control of the cells (Bayliss, 1992).

The chondrocytes continued to substitute the core protein of aggrecan with a significant number of KS chains. However, these chains contained 5 times less of the 5-D-4 epitope than keratan sulfate chains from the tissue from which the chondrocytes were derived. This suggests that cells suspended in alginate are no more able than cells maintained in other culture systems to sustain over the long term the expression of large amounts of adult-like keratan sulfate rich in the 5-D-4 epitope. As the rate of synthesis of keratan sulfate chains bearing another epitope (MZ15) is influenced profoundly by changing the culture conditions (Zanetti et al., 1985), one should exercise care when interpreting changes in the expression of this marker of the adult chondrocytic phenotype as a manifestation of aging processes.

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