Synthesis of Cartilage Matrix by Mammalian Chondrocytes In Vitro. II. Maintenance of Collagen and Proteoglycan Phenotype

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ABSTRACT The in vitro phenotype of bovine articular chondrocytes is described. Chondrocytes plated at high density in roller-bottle and dish cultures were maintained in vitro. The major matrix macromolecules, collagen and proteoglycan, synthesized by these cells were characterized during the course of the culture period. The chondrocytes synthesized mainly Type II collagen, which was found predominantly in the cell-associated matrix. The media contained a mixture of Type II and Type III collagens. Type I collagen was detectable in neither the medium nor the cell-associated matrix. The proteoglycan monomers found in media and cell-associated matrix had the same hydrodynamic sizes as monomers synthesized by cartilage slices or those extracted from adult articular cartilage. The majority of proteoglycans synthesized by the cells were found in high molecular weight aggregates which were readily recovered from the media and were extractable from cell-associated matrix with low ionic strength buffers. The results demonstrate the long-term in vitro phenotypic stability of the bovine articular chondrocytes. The advantages of the in vitro system as a model for studying the effects of external agents, such as drugs and vitamins, are discussed.

The major collagen type present in hyaline cartilage and a variety of chondrocyte cultures is Type II collagen (3, 4, 9, 10, 20). The production of Type II collagen by mesenchymal cells in culture is generally taken as evidence of expression of the "chondrocytic" phenotype (17). It is thought that phenotypically stable chondrocyte cultures synthesize a small proportion of Type V (AB) collagen (4) and that this collagen type is usually present in hyaline cartilage in a pericellular location (10). This view has been challenged recently by others (1) who view the Type V collagen as a perichondrial contamination. The 1α, 2α, and 3α chains which have been isolated from human and bovine hyaline cartilage migrate on polyacrylamide gels in a manner similar to Type V collagen alpha chains, but they have been shown to be genetically distinct gene products (1, 6). The production of Type I collagen by so called "chondrocytic" cells has been taken as an indication of loss of phenotypic stability and as an indicator of "de-differentiation" in vitro (2, 4, 29). The loss of phenotypic stability has been associated with a variety of culture conditions, especially increasing age in culture, low plating density, and passage of cells (3, 4, 9, 22, 29). Thus, careful analysis of collagen type in chondrocyte cultures has become an essential step in establishing whether such cultures express a true cartilagenous phenotype.

Proteoglycans are responsible for many of the physicochemical properties of hyaline cartilage. Proteoglycan monomers consist of a core protein to which are covalently attached glycosaminoglycans (chondroitin sulfate and keratan sulfate) and two types of oligosaccharides (8, 14, 19, 25, 28). The majority of cartilage proteoglycans are believed to exist as aggregates, in which a large number of proteoglycan monomers interact noncovalently with a long linear strand of hyaluronic acid (12). This interaction is stabilized by link protein, a third component of the aggregate (11).

In the accompanying paper (15) we described the morphological characteristics of bovine articular chondrocytes under conditions which promote the formation of a tissue-like extracellular matrix in vitro. The purpose of this report is to show

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that collagen and proteoglycan synthesized by these chondrocytes in vitro are similar to those isolated from the tissue of origin.

MATERIALS AND METHODS

Materials

Materials and sources used in this study are the following: PD-10, Sepharose CL-2B (Pharmacia Fine Chemicals, Piscataway, NJ); ultrapure guanidinium chloride (GuHCl) (Research Plus Lab, Denville, NJ); cesium chloride (CsCl) (Beckman Instruments, Inc., Palo Alto, CA): pepsin (Millipore Corp., Bedford, MA); [^{3}H]proline (2,3,4,5 ^{3}H; 90 Ci/mmol; ICN, Irvine, CA); $Na_2{}^{35}SO_4$ (840 mCi/mmol) Aquasol II, NCS (New England Nuclear, Boston, MA); PPO and POPOP (Research Products International, Elk Grove, IL); 6-amino hexanoic acid, phenylmethyl sulfonylfluoride (PMSF) (Sigma Chemical Co., St. Louis, MO); benzamidine hydrochloride, glucurolactone, methyl sulfoxide (DMSO), XRP-1 X-Ray Film (Eastman Kodak Co., Rochester, NY); cyanogen bromide (Aldrich Chemical Co., Milwaukee, WI); purified collagenase (Form III) (Advance Biofactors Co., Lynbrook, NY); all chemicals were reagent grade (Fisher Scientific Co., Fairlawn, NJ).

The guanidinium chloride solutions used for proteoglycan isolation and analyses always contained the following proteinase inhibitors at the following concentrations: 0.1 M 6-aminohexanoic acid, 0.01 M Na_2EDTA, 0.005 M benzamidine hydrochloride, and 0.001 M PMSF. The 4 M and 0.5 M guanidinium chloride solutions were buffered with 0.05 M sodium acetate to pH 6.2. They are referred to as the dissociative and associative extraction solvents, respectively. Both 8 M and 1 M guanidinium chloride solutions with 0.05 M sodium acetate and twofold concentration inhibitors were used when proteoglycan from medium was isolated, in order to achieve final concentrations identical to those of either the dissociative or associative solvents. Bovine nasal septum proteoglycan aggregate (A1) and monomer (A1D1)[1] were prepared by CsCl density gradient centrifugation according to the method of Heinegard (13), for use as carrier proteoglycan.

The following macromolecules were generous gifts of several investigators: associatively extracted SWARM rat chondrosarcoma proteoglycan aggregate (aA1) and monomer (aA1D1)[1] from Dr. V. C. Hascall (National Institutes of Health/National; pig dermal fibroblast proteoglycan from Dr. J. Gregory (Rockefeller University) and purified high molecular weight rooster-comb hyaluronic acid from Dr. N. Balazs (Columbia University).

Bovine Type I and II carrier collagens were prepared from calf skin and nasal septum cartilage by mild pepsin digestion, acid extraction, and salt precipitation.

Isolation and Culture Characteristics of Chondrocytes

The complete procedures for the isolation and culture characterization of bovine articular chondrocytes have been described (15). Chondrocytes were plated at high density (2×10^5 cells/cm^2) in plastic dishes or roller bottles. Ham's F-12 medium supplemented with 10% fetal bovine serum, 25 mM HEPES, 50 μg/ml amphotericin B, 50 μg/ml gentamycin, pH 7.2, was used at all times. This supplemented medium is referred to below as medium.

Labeling of Collagen Synthesized by Chondrocyte Cultures

Chondrocytes were exposed to [^{3}H]proline (5 μCi/ml) in the medium for 14 d. Cultures were re-fed with fresh medium containing isotope every other day. Labeled media were collected at each feeding and frozen for subsequent analyses. Both media and cell layers were then separately used for determination of collagen types. Chondrocytes used for the shorter pulse experiments were exposed to 5 μg/ml [^{3}H]proline for 12 h before collagen analysis of the cell layers and media.

Isolation of Collagen from Cell Cultures

The medium was adjusted to 25% saturation with ammonium sulfate, kept for 18 h at 4°C, and centrifuged at 10,000 *g* for 30 min. The medium pellets and the untreated cell layers were mixed with 0.5 M acetic acid with 100 μg/ml pepsin and incubated at 4°C for 48 h. Debris was removed by centrifugation and the supernatants were adjusted to a final 12% NaCl solution. They were kept at 4°C for 18 h, then centrifuged. The resultant pellets of cell-associated matrix and medium-derived collagen precipitates were dissolved in 0.5 M acetic acid, dialyzed against the same solution, and lyophilized before analyses.

Preparation of Cyanogen-Bromide Peptides

Lyophilized samples of the cell-associated matrix collagens were mixed with either Type I or Type II bovine collagen which was used as a marker, then dissolved at a final protein concentration of 1 mg/ml in de-aerated 70% formic acid. The mixture was adjusted to 12 mg/ml with cyanogen-bromide (CNBr) and incubated under nitrogen for 4 h at 26°C. The reaction was terminated by a 20-fold dilution with water, followed by lyophilization.

Collagenase Digestion

Collagenase was used to test for collagenase-susceptible peptides. The lyophilized collagen samples were dissolved in 25 mM Tris, 5 mM calcium acetate, and 0.2 M NaCl pH 7.4, and incubated with 200–300 U of collagenase at 37°C for 4 h. Collagenase digestion was employed to assay for percentage of collagen recovered from the cell-associated matrix by the acetic acid-pepsin extraction. The collagenase assay of Peterskofsky (23) was used. Using this procedure, the percentage of collagen present in the medium and the cell-associated matrix following a 12-h [^{3}H]proline pulse was determined.

SDS Gel Electrophoresis

The procedures of Laemmli (16) were followed using 11 × 0.5 cm–tube gels or 1.5-mm-thick slab gels of 7.5% or 5% acrylamide. Collagen or CNBr peptide samples were dissolved in 6 M urea, 1% SDS ± 1% mercaptoethanol, then heat-denatured at 60°C for 10 min before loading on the gels. After electrophoresis, the gels were stained, destained, and scanned at 580 nm in an ISCO gel scanner. The tube gels were sliced on a Mickle gel slicer into 1-mm sections, dissolved in NCS tissue solubilizer at 60°C for 1 h, then counted in a Packard Model 3333 scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL).

The slab gels were destained and the positions of marker alpha chains were indicated by making small cuts in the gel. These gels were prepared for fluorography by incubating in DMSO for 1 h, followed by 22% PPO in DMSO for 3 h. The gel was washed for 1 h in water, then dried on a Bio-Rad gel dryer (Bio-Rad Laboratories, Richmond, CA). The dried gel was exposed to XRP-1 Film for a period of 1–2 wk at −70°C. For quantification of collagen types at different times in culture, the X-ray fluorogram was placed over the gel, and the regions on the gel corresponding to the dark bands on the x-ray film were excised for ^{3}H counting. The gel slices were solubilized in NCS at 60°C for 1 h, then counted in PPO-POPOP scintillation fluid in a liquid scintillation counter.

Synthesis, Isolation, and Quantification of ^{35}S-Labeled Proteoglycan

It is generally accepted that >95% of the ^{35}S incorporated into macromolecules by chondrocytes grown in tissue culture is present in the glycosaminoglycan moieties of proteoglycans. To quantify the amount of proteoglycan synthesized at various times in culture, short-term ^{35}S pulse-labeling experiments were performed on days 4, 7, 9, 16, and 22 after plating in 35-mm culture dishes. All experiments were performed in triplicate. For each experiment, cells were washed briefly with medium, then cultured in the presence of 1 ml of medium containing $Na_2{}^{35}SO_4$, at 20 μCi/ml, for 15 h. After labeling, the labeled medium was removed, mixed with an equal volume of 8 M GuHCl, and kept at 4°C for 3 h. The cell-associated matrix was extracted with 4 M GuHCl at 4°C for 2 h. After extraction, each cell-associated matrix was treated with 1 ml of papain (100 μg/ml) in 0.05 M sodium acetate, pH 5.8, containing 0.02 M L-cysteine and 0.005 M disodium EDTA at 60°C for 24 h (26). The resultant digest consisted of ^{35}S-labeled glycosaminoglycan bearing peptides which were directly counted for ^{35}S. Samples were either studied immediately or frozen for future analyses. Bovine nasal-septum proteoglycan monomer (A1D1) was added to all sample solutions at a concentration of 1.5 mg/ml to serve as a carrier molecule in subsequent analyses and chromatographic separations. To separate ^{35}S incorporated into macromolecules from unincorporated ^{35}S, 250-μl aliquots of each sample were applied to PD-10 columns (5.5 × 1.8 cm) equilibrated and eluted with 4 M GuHCl. 1-ml fractions were collected in scintillation vials, mixed with 14 ml of Aquasol II and 0.9 ml of 70% ethanol, then counted in a Packard Model 3333 scintillation counter. The excluded volume (Vo) represented ^{35}S incorporated into macromolecules and was used to determine net proteoglycan synthesis at each time point studied.

[1] The abbreviations A1 and A1D1 conform to the nomenclature described by Heinegard (13). The term aA1D1 represents an A1D1 preparation of proteoglycans extracted from cartilage or culture by the associative (a) solvent.

In a similar manner, roller-bottle chondrocytes were cultured in the presence of 25 ml of medium containing $Na_2{}^{35}SO_4$, at 20 μCi/ml. After labeling, cultures were processed the same way as those in the 35-mm dishes.

Slices obtained from bovine articular cartilage at the time of isolation of chondrocytes were incubated at 37°C for 48 h in 2 ml of medium which contained $Na_2{}^{35}SO_4$ at 20 μCi/ml. Slices were washed for 15 min in cold medium, then extracted for 48 h at 4°C with the dissociative solvent. The extract was used for determination of ^{35}S-labeled monomer size.

Determination of Proteoglycan Monomer Size Synthesized by Chondrocytes In Vitro

The hydrodynamic size of the ^{35}S-labeled proteoglycan monomer synthesized by chondrocytes was determined by molecular sieve chromatography for dish and roller-bottle cultures pulse-labeled with $Na_2{}^{35}SO_4$, for 15 h on days 6 and 18. The pooled Vo fractions of the PD-10 columns were re-chromatographed on a Sepharose CL-2B column (120 cm × 0.6 cm) equilibrated in 4 M GuHCl, pH 6.2. The sample was eluted in 4 M GuHCl buffer and collected in 0.5-ml fractions. Each fraction was counted as described in the previous section. The column was precalibrated with a variety of macromolecules, including purified high molecular weight hyaluronic acid, proteoglycan monomers (A1D1) from bovine nasal septum and bovine articular cartilages, SWARM rat chondrosarcoma proteoglycan monomer (aA1D1), fibroblast proteoglycan monomer from pig epidermis, and glucuronolactone. Free ^{35}S sulfate was added to all samples to determine total column volume (Vt). Macromolecules used in the calibration of the column were monitored after fractionation using the technique of Bitter and Muir for uronic acid determination (5).

Determination of the Ability of Newly Synthesized ^{35}S-Labeled Proteoglycan Monomer to Form Aggregate

^{35}S-labeled proteoglycan monomers isolated by dissociative extraction from chondrocyte cultures, that had been pulse-labeled with $Na_2{}^{35}SO_4$ on day 7, were tested for their ability to form aggregates with hyaluronic acid. The pooled Vo fractions (450 μl) from PD-10 columns containing ^{35}S-labeled proteoglycan monomer were combined with 100 μl of a 4 M GuHCl solution containing bovine nasal-septum proteoglycan monomer at 10 mg/ml and high molecular weight hyaluronic acid at 500 μg/ml. This preparation was dialyzed against 0.5 M GuHCl, at 4°C for 2 h, then chromatographed on a Sepharose CL-2B column (120 × 0.6 cm) equilibrated with 0.5 M GuHCl. The sample was eluted in 0.5 M GuHCl, and 0.5-ml fractions were collected and counted as outlined in the previous section. The ^{35}S-labeled proteoglycan which appeared in the excluded volume of this Sepharose CL-2B column was taken as a measure of re-aggregated ^{35}S-labeled proteoglycan.

Determination of ^{35}S-Labeled Proteoglycan Aggregate Formed in Culture

The ability of native ^{35}S-labeled proteoglycan aggregates to be formed in both dish and roller-bottle cultures was investigated using cultures pulse-labeled with $Na_2{}^{35}SO_4$ on day 7. Cultures were labeled as outlined in previous sections. The labeled medium was removed, mixed with an equal volume of 1 M GuHCl, and kept at 4°C for 4 h. The cell-associated matrix was extracted with 0.5 GuHCl for 4 h at 4°C. Samples were then either used immediately for density gradient ultracentrifugation or kept frozen until further processing was performed. Solid CsCl was added to reach a final density of 1.65 g/ml and ultracentrifugation was performed at 40,000 rpm in a Beckman SW 50.1 rotor for 72 h at 4°C. The resultant A1 fractions (>1.70) were dialyzed against several changes of 0.5 M GuHCl at 4°C to remove CsCl. Aliquots from each A1 sample (450 μl) were chromatographed on a Sepharose CL-2B column (120 × 0.6 cm) equilibrated with 0.5 M GuHCl. The column was eluted with 0.5 M GuHCl. Fractions (0.5 ml) were collected and counted as outlined in previous sections. The ^{35}S-labeled proteoglycan which appeared in the excluded volume was taken as a measure of native ^{35}S-labeled proteoglycan aggregate.

Extractability of Cell-associated Matrix with Time

The percentage of ^{35}S-labeled proteoglycan extractable from the cell-associated matrix by associative and dissociative solvents was compared for dish and roller-bottle cultures pulse-labeled with $Na_2{}^{35}SO_4$ on days 5 and 21. Cultures were labeled as outlined in the previous sections. In these experiments the culture medium was removed and the cell-associated matrix was extracted under associative conditions (0.5 GuHCl), followed by extraction under dissociative conditions (4 M GuHCl), as described above. To quantify the unextractable ^{35}S-labeled macromolecules present in the residue of the dissociatively-extracted cell-associated matrix, the latter was treated with papain and counted. The associative and dissociative extracts were chromatographed on PD-10 columns in order to determine the ^{35}S incorporated into proteoglycan, as described above. The total ^{35}S-labeled proteoglycan present in the cell-associated matrix was calculated by summation of ^{35}S-labeled proteoglycan present in the associative and dissociative extracts and in ^{35}S-labeled glycosaminoglycan of the papain-treated residue.

Enzymatic Digestion of ^{35}S-Labeled Proteoglycan

To determine whether dermatan sulfate was present in the major species of proteoglycan synthesized on days 6 and/or 21, aliquots of the A1 fraction and of a pool of the material eluting between 19 and 35 ml in the dissociative Sepharose 2B column run (see Fig. 5) were mixed with bovine nasal A1 proteoglycan carrier, dialyzed against water, lyophilized, and treated with 0.05 M NaOH, 1 M BH_4, for 48 h at 45°C to release free intact glycosaminoglycans (24). After neutralization with acetic acid, the solution was mixed with an equal volume of absolute ethanol and left at −20°C overnight. After centrifugation, the precipitate (free of keratan sulfate), which contained 80–90% of the ^{35}S, was resuspended, dialyzed, lyophilized, and treated with chondroitinase AC or chondroitinase ABC, as described elsewhere (24).

RESULTS

We used collagen-type analysis to confirm the long-term in vitro phenotypic stability of chondrocytes. The results presented for collagen analysis were performed in roller-bottle cultures. Identical results have been obtained in dish cultures but are not reported here. After a pulse of [^{3}H]proline late in the culture period, the total extractable collagen from the cell-associated matrix was analyzed by PAGE. The results of the collagenase assay of the pepsin-solubilized and residual collagen from the cell layers indicated that 75% of the labeled collagen was extracted by our technique. The fluorogram of the collagen extracted from the cell layer indicates that the majority of the collagen is present in one alpha-size chain (Fig. 1 *C*). Another band migrates with a slightly slower mobility (band B) and co-electrophoreses with an isolated α1(V) chain. Both of these chains are sensitive to purified bacterial collagenase (data not shown). This material has not been characterized but can be tentatively identified as α1(V) or 1α chains. Both chains have been isolated from hyaline cartilage and migrate in identical positions in polyacrylamide gels (4, 6).

Long-term labeled cell-associated matrix collagen was sub-

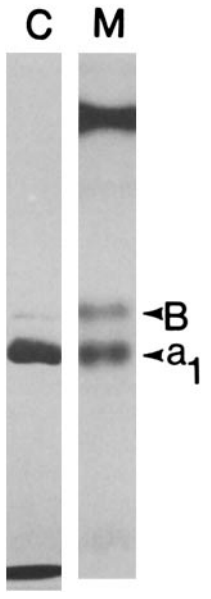

FIGURE 1 Fluorograph of cell-associated matrix and medium collagen from pulses late in the culture period. The pulsed media (*M*) (days 11–14) and cell layer (*C*) (day 14) from separate roller bottles were extracted with pepsin and electrophoresed under nonreducing conditions.

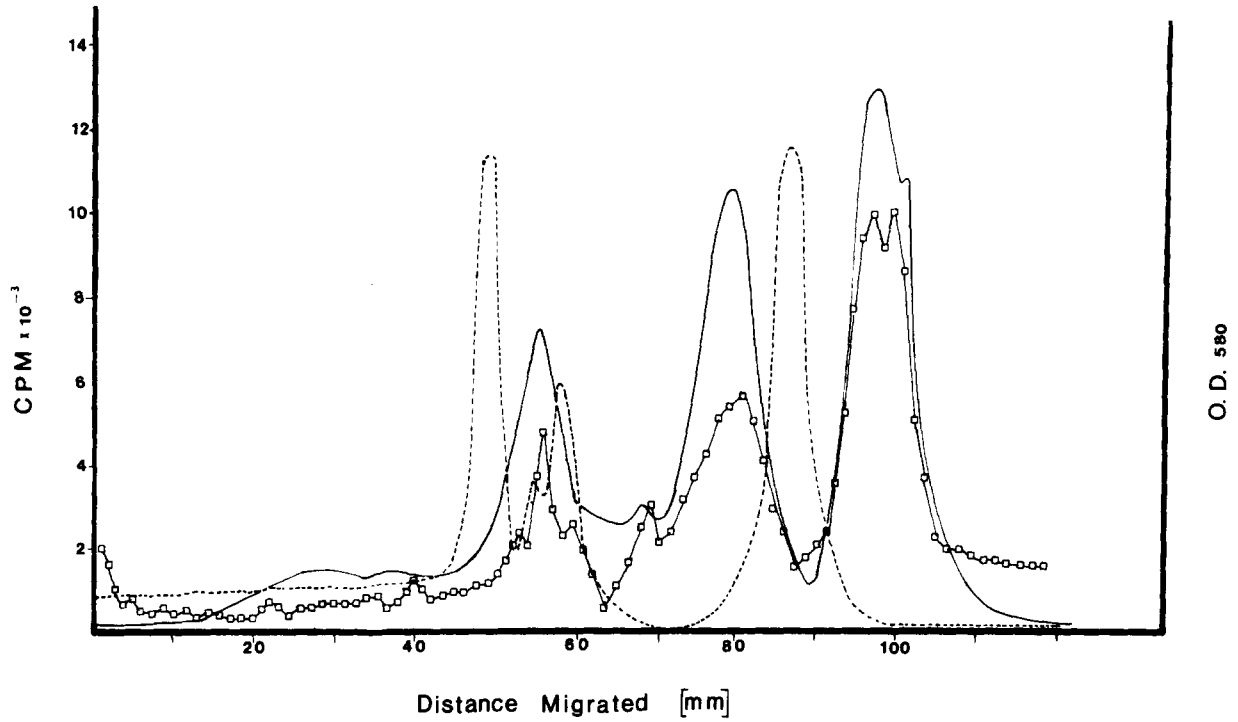

FIGURE 2 Cyanogen bromide digest of [^{3}H]proline-labeled cell-layer collagens. The long-term labeled cell-associated matrix collagen was subjected to CNBr digest in the presence of bovine Type II collagen; Type I collagen was digested in a companion tube. The peptides were electrophoresed on a 7.5% gel and stained before scanning and slicing for counting. Unlabeled Type I peptide (dashed line), unlabeled Type II peptides (straight line), labeled peptides from cell layer (□).

jected to CNBr peptide analysis to confirm the presence of Type II collagen as the major alpha chain species. The long-term labeled collagen isolated from the 14-d cell-associated matrix had a identical polyacrylamide gel profile identical to that of the pulsed material shown in Fig. 1 *C* (data not shown). The extracted collagen was CNBr-digested in the presence of unlabeled bovine Type II or Type I collagen. The results indicated that the majority of labeled collagen in the cell-associated matrix is Type II collagen, since the labeled peptides comigrate with the carrier Type II peptides (Fig. 2). Absence of radioactive peptides in the position of unlabeled Type I peptides indicated a lack of type I collagen synthesis. These results confirm those obtained by the electrophoresis shown in Fig. 1 *C*, which indicated the absence of α2 chains, characteristic of Type I collagen.

The collagens in the media were evaluated over a 2-wk period to see whether there were any changes in chondrocytic expression with time. The continuous labeling of the cell-associated matrix over a 2-wk period and the repeated removal of labeled media made it impossible to compare the amount of labeled collagen present in the matrix with that in the media. A collagenase digest of pulse-labeled media and cell layers on the fifth day of incubation indicated that the media/cell layer collagen ratio was 0.83. This result implies that much of the collagen synthesized by these cells accumulates in the media and is removed from the culture at each feeding. A fluorogram of the pulse-labeled media indicated that there was no qualitative alteration in the collagen expression with time (Fig. 3). This material was further analysed on reducing gels (Fig. 4). It was apparent that the material which just entered the nonreducing gel ran in the alpha-chain position on the reducing gel. This is consistent with its assignment as Type III collagen. The areas corresponding to the bands on the fluorogram (Fig. 3) were excised, and the amount of radioactivity was determined

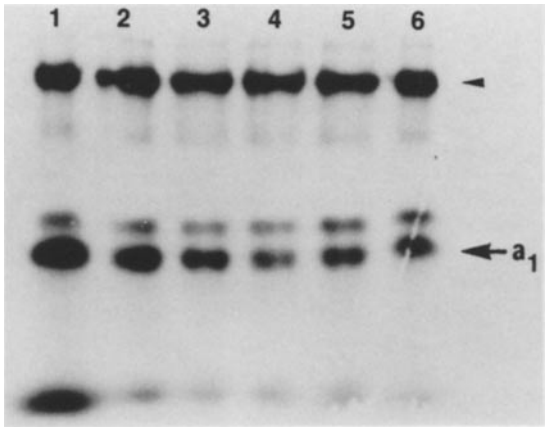

FIGURE 3 Fluorograph of pulse-labeled medium collagens. Collagen was isolated from [^{3}H]proline-labeled media from days 0–2 (*1*), 2–4 (*2*), 4–7 (*3*), 7–9 (*4*), 9–11 (*5*), and 11–14 (*6*). The collagens were electrophoresed under nonreducing conditions on a 5% gel. The arrowhead indicates the position where Type III collagen migrates on this gel.

in a scintillation counter (Table I). Early in the culture period, most of the collagens released into the media were Type III or Type II. The percentage of α(II) chains and B chains slowly decreased with time, while the percentage of Type III gradually increased. The results for the media complement the earlier observation for the cell-associated matrix. There were no α2 chains, confirming the absence of Type I collagen (Figs. 1 *C* and *M*). Type III collagen was confined to the media and was not seen in the cell-associated matrix.

The amount of ^{35}S-labeled proteoglycans decreased progressively during the culture period. Media always contained about twice as much labeled proteoglycan as the dissociative extracts

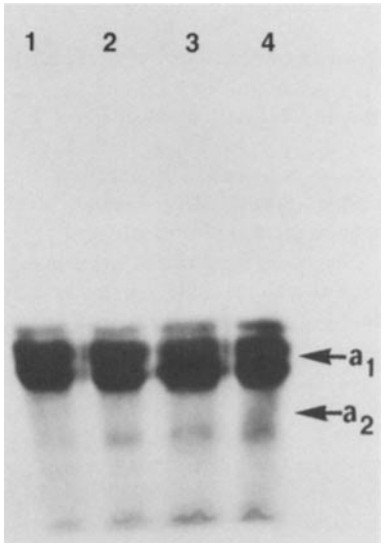

FIGURE 4 Fluorograph of the medium collagens from Fig. 3 electrophoresed under reducing conditions (1% mercaptoethanol). Days 4–7 (*1*), 7–9 (*2*), 9–11 (*3*), and 11–14 (*4*).

TABLE I

% Counts by Region in Medium Obtained from Different Days in Culture

	Day in culture					
Collagen type	2	5	7	9	11	14
Type III	33.5	52.1	56.7	59.5	59.2	67.6
Unknown chain (B)	22.8	11.8	13.1	14.2	14.7	12.1
α1 (II)	42.8	36.0	30.0	26.2	25.7	19.8
α2	0.9	0.1	0.2	0.1	0.4	0.5

of the matrix. The amount of label remaining in the unextractable residue was small (<13% of total ^{35}S). The extractability of ^{35}S-labeled proteoglycan present in the cell-associated matrix of cultures pulse-labeled with $Na_2{}^{35}SO_4$ on days 5 and 21 was assessed to study the degree of organization of the cell-associated matrix with time in culture. The cell-associated matrix of culture dishes and roller bottles was sequentially extracted with the associative solvent, then the dissociative solvent. ^{35}S-labeled proteoglycan present in these two pools and in the unextractable residue was quantificated, as described in Materials and Methods. The percentage of total ^{35}S-labeled proteoglycan, which was extractable with the associative solvent, decreased with time (day 5: 55%; day 21: 36.5%;) (Table II). The percentages obtained for culture dishes and roller bottles were identical. The dissociative solvent was effective in extracting a further 41% (day 5) and 52% (day 21) of the total ^{35}S-labeled proteoglycans. Once again, results were identical for culture dishes and roller bottles. The percentage of ^{35}S-labeled material that remained unextractable was about twice as great on day 21 as on day 5 (Table II).

The size of proteoglycan monomers was assessed by chromatography on Sepharose CL-2B, equilibrated, and eluted with the dissociative solvent. In both dish and roller-bottle cultures, the major species of proteoglycan monomers synthesized on day 6 had average partition coefficients that were practically identical to those of monomers synthesized in cartilage explants and those of proteoglycan monomers in the tissue of origin (Fig. 5). There were no significant differences in average monomer size between medium and cell-associated matrix at either time point (data not shown). On the other hand, a slight difference in monomer size was seen in both dish

TABLE II

Extraction Method: % of Total ^{35}S-Labeled Proteoglycan in Cell-Associated Matrix

	Day 4		Day 21	
	Dish	Roller Bottle	Dish	Roller Bottle
Associative	55.0%	54.8%	36.5%	36.5%
Dissociative	40.0%	39.8%	51.5%	52.1%
Papain	5.0%	5.4%	12.0%	11.4%

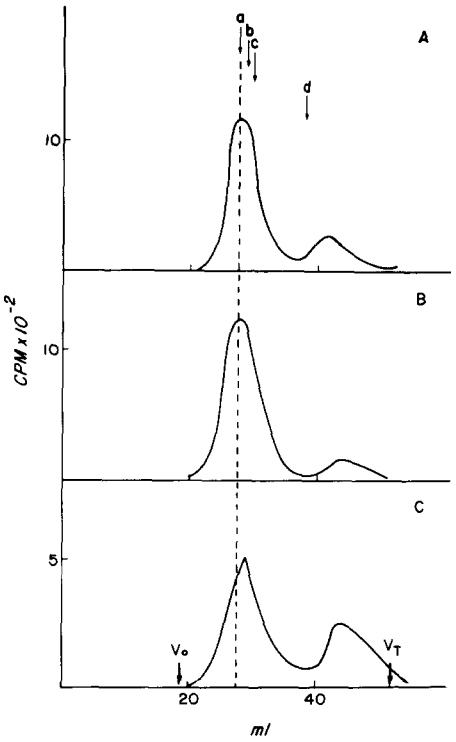

FIGURE 5 Sepharose CL-2B elution profiles of ^{35}S-labeled proteoglycans chromatographed under dissociative conditions. Articular cartilage slices pulse-labeled with $Na_2{}^{35}SO_4$ (*A*) and the cell-associated matrix of chondrocyte cultures pulse-labeled with $Na_2{}^{35}SO_4$ on day 6 (*B*) or on day 21 (*C*) were extracted under dissociative conditions, and an aliquot of the extract was placed on the column. Arrows indicate the locations of the peaks obtained for bovine nasal septum (A1D1) monomer (*a*), bovine articular cartilage (A1D1) monomer (*b*), SWARM rat chondrosarcoma (aA1D1) monomer (*c*), and pig fibroblast monomer (*d*). The excluded volume (V_o) and total volume (V_T) of the column were determined by chromatography of high molecular weight hyaluronic acid and glucuronolactone, respectively.

and roller-bottle cultures between day 6 and day 21. The slightly smaller size seen in the 21-d cultures was still similar to monomer sizes in other cartilagenous tissues, but significantly larger than the size of dermal fibroblast proteoglycans (Fig. 5*A*). Absence of any detectable amounts of dermatan sulfate in the major species of proteoglycan, synthesized by chondrocytes at days 6 and 21, was shown by chondroitinase treatment. Greater than 99% of the ^{35}S-radioactivity, which was present in the chondroitin sulfate-rich fractions, eluted in the total volume of a Sepharose 6B column following digestion.

Cultures were pulse-labeled with ^{35}S on day 7. The media and cell-associated matrix were subjected to associative extraction. Purified proteoglycan was prepared from these extracts for density gradient centrifugation under associative condi-

tions. The A1 fraction, containing proteoglycan aggregates, as well as nonaggregated proteoglycans of high density, were chromatographed on Sepharose CL-2B under associative conditions (Fig. 6*A* and *B*). Native ^{35}S-labeled proteoglycan aggregate represented the majority of ^{35}S-labeled proteoglycan species in both medium (70%) and cell-associated matrix (73%). However, when the dissociative-extracted proteoglycan was incubated with high molecular weight hyaluronic acid before chromatography on Sepharose 2B-CL under associative conditions, the percentage of aggregate reformed was only 32% of the total applied proteoglycan (Fig. 6 *C*).

DISCUSSION

The results of the collagen and proteoglycan analyses indicate that under the conditions used in our study primary cultures of adult bovine articular chondrocytes synthesize matrix-associated structural macromolecules similar to those present in the parent tissue. The results of the characterization of these structural macromolecules indicate that the chondrocytes remain phenotypically stable in primary culture for several weeks. The major matrix-associated collagen synthesized in these cultures is Type II. In addition, the cultures synthesize other collagen chains, which have not been characterized but could be α1(V) or 1α chains. Unlike rabbit and other mammalian chondrocytes in monolayer culture, our cultures show no evidence of Type I collagen biosynthesis (3, 4, 9, 10, 22). Type III collagen has been shown to be synthesized by rabbit articular chondrocytes in monolayer culture (2). It is also synthesized by our bovine articular chondrocytes. The Type III collagen synthesized by our cultures is exclusively secreted into the culture medium and is entirely absent from the cell-associated matrix. With increasing time in culture, the ratio of Type III to Type II collagen in the culture medium increases. The significance of these findings, in terms of phenotypic stability of chondrocytes in monolayer culture, is open to question, especially in light of the fact that no Type I collagen is synthesized even late in culture. The presence of Type III collagen solely in the medium would suggest that this collagen may be present in a pro α1 (III) form or is somehow incompletely processed. This was suggested by Benya et al. (2) for rabbit chondrocytes and is in accordance with the findings of Lichtenstein et al. (18) for fibroblast cultures.

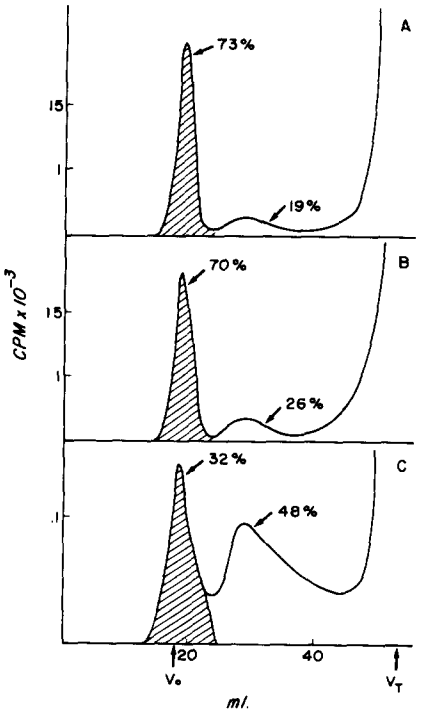

FIGURE 6 Sepharose CL-2B elution profiles of ^{35}S-labeled proteoglycans chromatographed under associative conditions. Roller-bottle cultures were pulse-labeled with $Na_2{}^{35}SO_4$ on day 7 and an aliquot of the ^{35}S-labeled medium (*A*) or the associative extract of the cell-associated matrix (*B*) was applied to the column. *C* represents the elution profile of ^{35}S-labeled proteoglycans extracted from the cell-associated matrix of an identical roller-bottle culture by the dissociative solvent. This extract was dialyzed in the presence of hyaluronic acid to associative conditions (see the text), and an aliquot was applied to the column. Areas under the peaks in the V_0 region were taken as a measure of proteoglycan aggregate present.

The proteoglycans synthesized by these chondrocytes are typical of hyaline cartilage and are indistinguishable from those of bovine articular cartilage. Our results indicate that they are present in the cell-associated matrix and media, largely in aggregate form. It is most probable that the aggregates are link-stabilized. Unpublished results indicate that link proteins are synthesized by the culture and are associated with aggregates isolated from the cell-associated matrix (B. Caterson, personal communication). In addition to the major species of proteoglycan monomer, a small amount of proteoglycan of smaller hydrodynamic size is present. It may represent a degradation product of the large proteoglycan monomer. A neutral proteinase synthesized by chondrocytes cultured in an identical fashion was shown to be capable of degrading proteoglycans in both monomeric and aggregate states (21). However, the possibility that the small peak represents another species of proteoglycan, which is also synthesized by these cultures, cannot as yet be excluded.

With increasing time in culture there is both a decrease in the overall synthesis of proteoglycan and a slight decrease in the hydrodynamic size of the monomer, without any evidence of a change in ability to form aggregate. These findings may reflect an aging phenomenon in culture or may be associated with other factors related to changes in organization of the cell-associated matrix, as the culture period extends.

The long-term stability and relative ease of extraction of native proteoglycan aggregate make this chondrocyte culture an ideal tool for the study of articular-cartilage proteoglycan biosynthesis. It is well established that dissociative conditions are required to extract any significant amount of proteoglycan from adult bovine articular cartilage (27). When the cell-associated matrix is extracted under dissociative conditions and then dialyzed to associative conditions in the presence of excess hyaluronate, only a third of the proteoglycans re-aggregate. On the other hand, we have demonstrated that the majority of proteoglycan synthesized by the cultures can be obtained from the medium and the cell-associated matrix as high molecular weight native aggregates. Using our cell culture system, we will be able to analyze the effects of agents on aggregate formation, size, and stability, a feat not possible with whole tissue or organ culture techniques.

The relative difference in extractability of the cell-associated matrix by the associative solvent on days 5 and 21 is consistent with the morphological observations that the cell-associated matrix undergoes progressive organization with time in culture, such that it resembles the matrix in vivo (15). This decrease in extractability of native aggregate from the cell-associated matrix with time also provides a unique system for the study of

matrix organization and proteoglycan collagen interactions.

In summary, we have described the morphologic (15) and biochemical characteristics of adult articular chondrocyte cultures, which are phenotypically stable during the described culture period. The cultures synthesize proteoglycan, which is present in the matrix and medium predominantly as an aggregate. This native aggregate is readily obtained in large quantities by mild, nondissociative extraction. The morphological and biochemical analyses confirm the ability of isolated articular chondrocytes to produce a cartilagelike matrix in vitro. Such a culture may allow the study of a variety of factors that are capable of effecting alterations in the synthesis, degradation, and organization of cartilage matrix, including vitamins, hormones, drugs, and physical forces. The results of recent experiments have shown that ascorbate has profound effects on matrix synthesis in chondrocyte cultures (7). Future studies are planned to fully characterize the proteoglycans synthesized by these cultures. In addition, we are examining alternative culture methods which might enhance matrix production by cells in vitro. These morphologic and biochemical analyses provide the basis for future research on this unique adult articular chondrocyte culture system.

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