Changes in the Sulfated Proteoglycans Synthesized by "Aging" Chondrocytes

I. DISPERSED CULTURED CHONDROCYTES AND IN VIVO CARTILAGES

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Structural and chemical changes in the sulfated proteoglycans synthesized by "aging" cultures of dispersed chondrocytes were compared to those synthesized by freshly excised, intact cartilage explants in organ culture from chickens of various ages.

In vitro, chondrocytes isolated from embryonic chick vertebral cartilages synthesized a proteoglycan monomer characteristic of cartilage (type IV). Monomers synthesized between 3 days and 6 weeks in culture; (i) showed a decrease in average molecular size, which could be correlated, in part, with a decrease in the average size of the chondroitin sulfate chains; (ii) were able to interact with hyaluronic acid to form proteoglycan aggregates regardless of size; (iii) maintained a relatively constant 6S/4S disaccharide ratio of 2.2 for chondroitin sulfate; and (iv) maintained a constant keratan sulfate composition of 5 to 16%.

Sulfated proteoglycans synthesized by freshly excised embryonic tibia cartilaginous head and by tibial articular cartilages of 1- and 6-year-old chickens were compared. With age: (i) the type IV monomer size decreased; (ii) the chondroitin sulfate chain size decreased; (iii) the type IV monomers retained their ability to interact with hyaluronic acid; (iv) the 6S/4S disaccharide ratio decreased from 1.7 to 0.6; (v) the keratan sulfate composition increased from 7 to 40%.

Although monomer size decreases in both the culture and explant systems, in other respects the differences are considerable. These findings suggest that the changes in sulfated proteoglycans observed in subcultured monolayers of chondrocytes must be interpreted with caution when attempting to relate them to the changes that occur in aging in vivo chondrocytes.

The phenomenon of "aging," particularly on a cellular level, still lacks a widely accepted definition. Some notable exceptions, most experiments on cellular aging have focused on the replicative life spans of cultured cells (see Refs. 1 and 2 for reviews). One of the exceptions has been the delineation of changes in the structural and chemophysical properties of the intercellular matrix in cartilages from young and old animals (3-7). More recently, the change in the synthetic behavior of chondrocytes under different culture conditions has been analyzed and proposed as a model for the aging of chondrocytes in vivo (8, 9).

This report focuses on the question of whether the structural and chemical changes in the type IV-sulfated proteoglycan characteristic of cartilage (10-14) that occurs in vitro are qualitatively equivalent to those known to occur in vivo. This type of study is particularly feasible now, owing to the recent advances in knowledge of the molecular structure of sulfated proteoglycans (15). These molecules characteristic of cartilage consist of a core protein to which are covalently attached ~100 chondroitin sulfate and 30 to 60 keratan sulfate chains with average $M_r = 20,000$ and 5,000, respectively (16). One end of the core protein, which lacks glycosaminoglycan chains, possesses the property of interacting with hyaluronic acid. This allows large numbers of single proteoglycan monomers to form supramolecular aggregates within the cartilage matrix (17, 18).

In this study, we have identified several alterations in the structural features of the sulfated proteoglycans synthesized by dispersed chondrocytes maintained in vitro for several weeks, and in cartilage explants isolated from animals of various ages and maintained in short term organ culture. Our data do not support the notion that the alterations observed in cultured chondrocytes are the same as those observed in cartilages of animals of increasing age. These differences that we and others have found with time in cultures of dispersed chondrocytes may reflect adaptations to the microenvironment in vitro rather than mirror aging in vivo.

**EXPERIMENTAL PROCEDURES**

Materials—Tissue culture supplies were obtained from Grand Island Biological Co.; ultrapure guanidinium chloride from Schwarz/Mann; 6-aminohexanoic acid, benzamidine, and 3,5-diaminobenzoic acid from Aldrich; chondroitinase ABC (Proteus vulgaris) from Miles Laboratories; papain (2 X crystallized) and hyaluronic acid from Sigma; Sepharose 2B and 6B from Pharmacia; Bio-Gel P-30 (200 to 400 mesh) from Bio-Rad; carrier-free $^{35}$S]sulfate as the sodium salt (~750 mCi/mmole of sulfur) from Amersham; and Aquasol from New England Nuclear.

Cell Cultures— Cultures consisting of over 98% functional chondrocytes were obtained by trypsinizing 11-day-old embryonic chick vertebral cartilages as described elsewhere (11). Primary floating chondrocytes were harvested on Day 4 by gently aspirating the medium with a Pasteur pipette. The few fibroblasts in these primary cultures...
remained attached to the substrate. The floating cells were concentrated by centrifugation, washed with Eagle's minimal essential medium, resuspended in 0.25% trypsin for 10 min, and then plated at 5–10^5 cells/100-mm dish as secondary subcultures. For further subculturing, the cells were washed with Eagle's minimal essential medium, treated for 3 min with a trypsin-EDTA solution, and plated at 3000 cells/fibroblast dish. Fibroblast cultures were prepared from subdermal tissues of 11-day-old chick embryos and processed, as were the dispersed chondrocytes (19).

Other experiments involved brief organ cultures of intact pieces of cartilage. Vertebral cartilages and entire cartilage heads from 11-day-old embryos, and tibial articular cartilages from 1- and 6-year-old chickens were removed free of adhering connective tissues, cut into small 1-mm² pieces, and organ cultured. By Day 11, during chick embryo development, tibial epiphysial heads are entirely cartilaginous. During further development, the cartilaginous model is replaced by bone, except at the site of the articular cartilage. These intact pieces of cartilage were incubated and analyzed in the same manner as that used for the dispersed chondrocytes.

DNA contents of cultured, dispersed chondrocytes and intact organ-cultured vertebral cartilages were determined by the fluorometric assay of Hinegardner (20) using calf thymus DNA as a standard.

Analytical Procedures—Linear sucrose gradients were prepared by using a Buchler apparatus. Analytical Sepharose 2B and Sepharose 6B gel filtration columns (105 × 0.6 cm) were prepared and eluted with 0.5 M sodium acetate, pH 7.0. Average running time was 24 h. Effluent fractions were assayed for hexuronic acid with an automated carbazole procedure (21). For radioactivity measurements, aliquots of samples were mixed with 1 volume of water and 10 ml of Aquasol. Samples were counted with a Beckman LS 3155 scintillation counter. Quenching was determined by the external standard method.

Proteoglycan Extraction and Characterization on Sucrose Gradients— Cultures were incubated for 6 h in 4 ml of fresh medium containing 10 to 30 µCi/ml of ³⁵S[sulfate]. Proteoglycan extraction was carried out essentially as described previously (10, 11). Radioactive medium was removed from two 100-mm dishes and any floating cells removed by centrifugation. Proteoglycans in the medium were precipitated by the addition of ethanol and potassium acetate (pH 6.5) and then centrifuged for 2 h at 27,000 rpm. 31 fractions of 0.5 ml were collected from the bottom of the gradient.

The first 17 fractions were mixed together when type IV-sulfated proteoglycans were isolated from cultured chondrocytes or organ-cultured cartilages; Fractions 10 through 20 were mixed when type III macromolecules were isolated (see Fig. 3 below). Pooled fractions were dialyzed against three changes of 0.5 M sodium acetate, pH 7.0, containing 20 mM sodium sulfate, and then against three changes of distilled water. Five milligrams of carrier bovine nasal proteoglycans (A1-D1 fraction) (25) were added to the labeled material prepared from two 100-mm dishes and the mixture was freeze-dried. Carrier proteoglycans are in vast excess in comparison to the labeled proteoglycans; consequently, the hexuronic acid analyses described below refer almost exclusively to the carrier proteoglycans.

Chromatographic Characterization of Sulfated Proteoglycans— Sepharose 2B and Sepharose 6B columns (105 × 0.6 cm) were prepared and eluted with 0.5 M sodium acetate, pH 7.0. Freeze-dried samples (0.5 mg) were each solubilized in 0.15 ml of 0.5 M sodium acetate buffer, pH 7.0, and applied to the appropriate column. Fractions of 0.52 ml were collected and aliquots of each (0.12 ml) were analyzed for hexuronic acid with an automated carbazole procedure (21) and then for radioactivity.

When assayed for ability to bind to hyaluronic acid, 0.5 mg of each freeze-dried sample was dissolved in 0.15 ml of 0.5 M sodium acetate buffer, pH 7.0. Then, 5 to 10 µg of hyaluronic acid were added and 12 to 16 h later, the mixture was applied to a Sepharose 2B column (12). Fractions of 0.52 ml were collected and analyzed as described above.

Enzymic Treatments—Portions of freeze-dried samples (0.5 mg) were digested with 100 µg of papain at 65°C for 4 to 5 h in 190 mM sodium acetate, pH 7.8, 5 mM EDTA, and 5 mM cysteine hydrochloride in a total volume of 0.2 ml. Samples were chromatographed on a Sepharose 6B column immediately or stored frozen until used (24). Fractions of 0.52 ml were collected and analyzed as described above.

Lympholized samples were dissolved in 50 µl of Tris buffer, and chondroitinase ABC digestion (0.1 unit/0.5 mg of sample) was done as described elsewhere (11). Briefly, floating chondrocytes were transferred onto Whatman No. 1 paper, and paper chromatograms developed for 20 to 22 h with 1-butanol/acetate/1 N ammonium (2:3:1). The paper chromatograms were air-dried, 1.0-cm strips were cut, and each strip eluted in 0.5 ml of 10 mM HCl. Radioactivity contents in the eluted material were determined.

Keratan sulfate chains were analyzed as follows. Portions of the lympholized samples were dissolved in Tris buffer and treated with chondroitinase ABC followed by alkaline borohydride mixture (26): 0.05 M sodium hydrosulfite, 1 M sodium borohydride for 24 h at 45°C. After neutralization with acetic acid, samples were applied to a Bio-Gel P-30 column (110 × 0.6 cm).

Electron Microscopy—Cultured cells were fixed in 3% glutaraldehyde in 0.25 M cacodylate buffer, pH 7.4, for 2 h at room temperature, and postfixed for 1 h in 1% osmium tetroxide. Fixed cells were then dehydrated in a gradient series of ethanol and embedded in Epon 812. Ultrathin sections were stained with 0.5% uranyl acetate and 1% lead citrate. Sections were examined on a JEOL electron microscope.

RESULTS

Cultured Chondrocytes—Pure primary populations of chick embryo chondrocytes were obtained from vertebral cartilages, as described elsewhere (11). Briefly, floating chondrocytes which have accumulated matrix components on their peripheries were selectively harvested from the medium of primary cultures. The floating chondrocytes from 2- to 3-day-old chick embryo chondrocytes were obtained from vertebral cartilages, as described elsewhere (11). Briefly, floating chondrocytes which have accumulated matrix components on their peripheries were selectively harvested from the medium of primary cultures. The floating chondrocytes from 2- to 3-day-old chick embryo chondrocytes were obtained from vertebral cartilages, as described elsewhere (11).
cultures were transferred to secondary cultures after treatment with proteolytic enzymes. As a consequence of this treatment, many chondrocytes become attached to the plastic substrate, whereas the remaining ones still float in the medium. The number of floating chondrocytes normally decreases with the age of the secondary culture: by Day 2 and 3, ~50%, and by Day 4 and 5, ~90% of the chondrocytes adhere to the culture dish-forming characteristic epithelioid colonies of polygonal cells. The chondrocytes were always subcultured just before reaching confluency.

For the present study, chondrocytes were grown for a total period of 6 weeks by means of six successive subcultures. After six subcultures (Fig. 1), the great majority of the chondrocytes maintained their gross cytological characteristics: polygonal morphology, lack of overlapping, lack of motility, and a metachromatic extracellular matrix after staining with methylene blue (27). Only a small percentage (~1 to 2%) of “giant” cells and other fibroblast-like cells were observed. However, there are striking differences in the rough endoplasmic reticulum between early and late passed chondrocytes (Fig. 2, A and B). Cells subcultured two to three times display many prominent, parallel arrays of rough endoplasmic reticulum. By the 5th or 6th passage, these parallel arrays have invariably disappeared. Instead, the numerous cisternae appear to be randomly distributed and distended, containing a relatively homogeneously stained material.

$[^35]S$Sulfate Incorporation—Secondary cultures were prepared. At each time point, floating and substrate-bound chondrocytes were separated and pulsed independently with $[^35]S$ulfate for 6 h. Table I shows the result of one typical experiment. The level of total $[^35]S$ulfate incorporation/cell, as estimated by DNA content, was usually maximal in the early days of a given culture, dropping as that culture aged. In tertiary cultures, the level of incorporation was slightly lower than that of late secondary substrate-bound chondrocytes (Table I). Similar decreases in the level of incorporation were observed in subsequent subcultures, as shown in Table I. In all cases, ~90% of the total $[^35]S$-labeled macromolecules were digested by chondroitinase ABC (see Table II below).

Pattern of $[^35]S$-labeled Proteoglycans on Sucrose Gradients—The $[^35]S$-labeled proteoglycans synthesized by chondrocytes can be separated into two size classes on a linear sucrose gradient in 4 M guanidine. The larger proteoglycan, operationally termed type IV monomer, is characteristic for cartilages and cultured chondrocytes. With the techniques used, the smaller type I $[^35]S$-labeled proteoglycan fraction appears to be synthesized by many kinds of cells: fibroblasts, myotubes, spinal cord, etc. (see also, Ref. 14).

Fig. 3A illustrates the patterns of sulfated proteoglycans synthesized by secondary floating chondrocytes as compared to secondary substrate-bound chondrocytes after a 3-day growth period in vitro. A profile for proteoglycans from organ-cultured, intact vertebral cartilages of the same embryonic age is shown for comparison. The figure demonstrates that the type IV monomers synthesized by both these dispersed cultured cell populations (i.e. floaters and substrate-bound chondrocytes) migrate on sucrose gradients more slowly than those synthesized by organ-cultured embryonic vertebral cartilages. Clearly, the monomers synthesized by cultured chondrocytes have smaller average molecular sizes than those synthesized by the intact vertebral cartilages. The figure shows also that the type IV monomer in floating chondrocytes has a larger molecular size than that of substrate-bound chondrocytes. In both cell populations, however, the overall proportion between type IV and type I sulfated proteoglycans is similar to that present in organ-cultured, intact vertebral cartilages.

The decrease in the molecular size of type IV monomers was more evident at later stages of chondrocyte growth in culture. Figure 3, B and C, shows results for chondrocytes grown for 6 and 10 days, respectively, in secondary cultures. The decrease of the molecular size of the monomers is related to the age of the culture. The decrease is faster in substrate-bound chondrocytes and the relative proportion between type IV and type I proteoglycan changes, with the proportion of type I greatly increased in 10-day-old substrate-bound chondrocytes.

Secondary chondrocytes were subcultured after trypsinization and grown for 6 days in tertiary cultures. The pattern of migration of the sulfated proteoglycans synthesized by these cells is shown in Fig. 3D. There is no significant further decrease of the average molecular size of the type IV monomers synthesized in these tertiary as compared with the secondary cultures. On the other hand, the ratio between the modified type IV and type I sulfated proteoglycans is now greatly decreased.

When the proteoglycans synthesized by chondrocytes subcultured three more times, i.e. grown for a total of 6 weeks in culture, were extracted and analyzed in the same manner, they appeared essentially identical with those synthesized by tertiary chondrocytes.

Interaction of Type IV Monomers with Hyaluronic Acid—Type IV proteoglycan monomers interact specifically with hyaluronic acid under the experimental conditions used. Neither type III, II, nor I obtained from normal dermal fibroblasts or muscle cells or chondrocytes treated with phorbol-12-myristate-13-acetate or 5′-bromodeoxyuridine (10, 19) bind to hyaluronic acid under the conditions used here (data not shown). The ability of type IV monomers to interact with hyaluronic acid was tested. $[^35]S$-labeled type IV proteoglycan monomers from different cultures were collected after sepa-
ration on sucrose gradients, and dialyzed and lyophilized in the presence of carrier bovine nasal cartilage proteoglycan. Carrier proteoglycans were used to facilitate the recovery of the limited amounts of material synthesized by cultured chondrocytes and to provide an internal standard for a direct comparison with the $^{35}$S-labeled proteoglycans.

Portions of the lyophilized materials were dissolved in the appropriate buffer and then chromatographed on Sepharose 2B columns. The elution of the carrier was monitored by analysis for hexuronic acid, whereas the elution patterns of the experimental samples were monitored by radioactivity. As shown in Figs. 4, A, C, and E, type IV monomers from both vertebral cartilages and from cultured condrocytes (secondary or 6th passage) are included in the column. The vertebral cartilage monomers eluted earlier ($K_{av} = 0.18$) than the bovine nasal cartilage carrier ($K_{av} = 0.21$), whereas type IV monomers

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**Fig. 2.** Electron micrographs of dispersed cultures of chondrocytes. A, 2nd passage chondroblasts ($\times 24,000$); B, 6th passage chondroblasts ($\times 16,000$).
Fluorometric assay (20).

Macromolecules were extracted from cell layers and medium as described under "Experimental Procedures." An aliquot of the cell homogenate was used to determine the DNA content by means of a fluorometric assay (20).

| Stage of growth          | Days in culture | dpm/µg of DNA |
|--------------------------|-----------------|---------------|
| Secondary floating chondrocytes | 3              | 230,000       |
| Secondary floating chondrocytes | 6              | 140,000       |
| Secondary SB-chondrocytes | 10             | 100,000       |
| Secondary SB-chondrocytes | 3              | 200,000       |
| Secondary SB-chondrocytes | 6              | 65,000        |
| Secondary SB-chondrocytes | 10             | 39,000        |
| Tertiary SB-chondrocytes  | 4              | 42,000        |
| Tertiary SB-chondrocytes  | 8              | 36,000        |
| Quarternary SB-chondrocytes | 6              | 26,000        |

Chondrocytes were pulsed for 6 h with radioactive sulfate. Labeled macromolecules were extracted and each labeled sample was independently run on a sucrose gradient (24).

Chondrocytes at different stages of growth in vitro were pulsed for 6 h with [35S]sulfate. Labeled proteoglycans were run simultaneously and their profiles are included on each graph for comparison. See text for details.

The elution profiles in the presence of hyaluronic acid are shown in Fig. 4, B, D, and F. They demonstrate that the great majority of the 35S-labeled monomers synthesized by organ-cultured vertebral cartilages and by the cultured chondrocytes, as well as the carrier proteoglycan, eluted in the excluded volume, indicating that specific interactions with hyaluronic acid had occurred.

Structural Characteristics of the Type IV Monomers—The decrease in the molecular weight of type IV monomers can be caused by several different structural changes in the macromolecules. One such change could be due to alteration of the average molecular size of the polysaccharide chains.

Portions of the 35S-labeled type IV monomers with carriers were digested with papain to release the polysaccharide chains. The digests were directly chromatographed on Sepharose 2B columns in 0.5 M sodium acetate to obtain an estimate of the average molecular weight of the released polysaccharide chains (24). The profiles obtained on Sepharose 2B columns in 0.5 M sodium acetate are of larger hydrodynamic size (Kav = 0.42 and 0.46; M, = ~31,000 and ~25,000, respectively) than those present in bovine nasal proteoglycan (Kav = 0.52; M, = ~18,500) (24). It is clear that the chondroitin sulfate chains of...
the type IV monomers synthesized by cultured chondrocytes are shorter than those of the monomers synthesized by vertebral cartilages in organ culture. This structural change is even more evident in the type IV monomers from 10-day-old secondary chondrocytes (Fig. 5C), since their chains have a significantly lower molecular size ($K_v = 0.56; M_r = \sim 15,500$). Unexpectedly, the chondroitin sulfate chains synthesized by 6-wk-old chondrocytes appeared to have a slightly larger molecular size in this particular sample (Fig. 5D).

We then determined the relative ratios of the 6S and 4S disaccharides in the isolated proteoglycans. The macromolecules were digested with chondroitinase ABC and digests were analyzed by paper chromatography. Table II shows that: (i) the 6S/4S disaccharide ratio remains practically unchanged throughout the entire series of chondroblast subcultures; (ii) the ratios are the same for the organ-cultured vertebral cartilage proteoglycans; and (iii) 90 to 95% of the radioactivity in any sample was digested by the chondroitinase ABC.

The constant presence of undigested material, which comprised 5 to 10% of the total sample radioactivity, gives a reasonably accurate estimation of the relative amount of keratan sulfate to chondroitin sulfate in the samples. We can therefore conclude that the type IV monomers synthesized at each chondrocyte subculture contained a constant ratio of keratan sulfate to chondroitin sulfate.

![Fig. 5. Sepharose 6B elution profiles of 35S-labeled chondroitin sulfate chains.](image)

### Fig. 5. Sepharose 6B elution profiles of 35S-labeled chondroitin sulfate chains. Isolated type IV sulfated proteoglycans, as described in the legend to Fig. 3, were digested with papain and then applied to the column. A, vertebral cartilage; B, 3-day-old secondary floating chondrocytes; C, 10-day-old secondary substrate-bound chondrocytes; D, 6-week-old chondrocytes (six successive subcultures).

#### TABLE II

**Relative amounts of 6S and 4S disaccharides in the isolated proteoglycans**

| Sample            | Undigested | 6D-4S | 4D-4S | 6S/4S | Ratio 6S/4S |
|-------------------|------------|-------|-------|-------|-------------|
| Embryonic vertebral cartilage | 6S          | 61    | 32    | 1.9    |              |
| 2-passage chondrocytes | 10          | 63    | 27    | 2.3    |              |
| 3-passage chondrocytes | 7           | 63    | 29    | 2.2    |              |
| 4-passage chondrocytes | 6           | 65    | 29    | 2.2    |              |
| 6-passage chondrocytes | 9           | 61    | 30    | 2.0    |              |

*Data are expressed as the percentage of the total radioactivity recovered.

Embryonic and Adult Cartilages—Identical experiments were done for the 35S-labeled proteoglycans synthesized by short term organ cultures of explants of embryonic tibial heads and articular cartilages directly isolated from chick embryos and adult chickens.

Cartilaginous tibial heads from a 11-day-old chick embryo and articular cartilages from 1- and 6-year-old chickens were dissected and labeled immediately in *vitro* for 6 h with [35S]sulfate. Labeled proteoglycans were extracted and analyzed on linear sucrose gradients. The patterns obtained, shown in Fig. 6, demonstrate that: (i) the proteoglycans synthesized by embryonic tibial cartilages have nearly the same molecular sizes as those observed in embryonic vertebral cartilages; (ii) the type IV monomers synthesized by the 6-year-old chicken articular cartilages have much smaller sizes than those synthesized by the embryonic cartilage; and (iii) the overall proportion between the type IV and type I classes of 35S-labeled proteoglycans is greatly different in embryonic as compared to adult cartilages.

Type IV monomers were collected from the sucrose gradients, dialyzed, and lyophilized in the presence of carrier bovine nasal proteoglycans. The isolated monomers were then tested for the ability to bind to hyaluronic acid and for glycosaminoglycan chain sizes. Fig. 7 shows that type IV monomers synthesized by both embryonic cartilages and adult chicken articular cartilages are able to bind to hyaluronic acid, and, therefore, the decrease of the molecular size observed for labeled adult chicken cartilage monomers ($K_v = 0.31$) compared with embryonic cartilage ($K_v = 0.16$) does not affect their characteristic property to bind to hyaluronic acid.

The Sepharose 6B elution profiles of papain digest of type IV monomers shown in Fig. 8 demonstrate that chondroitin sulfate chains synthesized by embryonic cartilages ($K_v = 0.41; M_r = \sim 32,000$) are longer than those present in the bovine nasal carrier ($K_v = 0.52; M_r = \sim 18,500$). Conversely, in 1-year-old chicken articular cartilage, the average molecular size of the newly synthesized polysaccharide chains is \sim 21,500 ($K_v = 0.48$; data not shown), and decreases to \sim 12,500 ($K_v = 0.59$) in 6-year-old articular cartilages (Fig. 8B).

Since almost 40% of the polysaccharides present in the 35S-labeled type IV monomers of the 6-year-old articular cartilages are keratan sulfate (see Fig. 9), the pattern reported in Fig. 8B contains a mixture of \sim 60% chondroitin sulfate and 40% keratan sulfate chains.

Aliquots of the isolated type IV monomers were then digested with chondroitinase ABC, and the digests were resolved on chromatographic paper. Table III shows that 95% of the type IV monomer radioactivity from embryonic tibial cartilaginous heads was digested by the enzyme, and that the relative proportion of 6S and 4S disaccharides is similar to
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FIG. 7. Sepharose 2B elution profiles of $^{35}$S-labeled type IV proteoglycans before and after interaction with hyaluronic acid. A, embryonic cartilage; B, as in A, but preincubated with hyaluronic acid (1 to 2% carrier proteoglycans, w/w); C, 6-year-old articular cartilage; D, as in C, but preincubated with hyaluronic acid.

FIG. 8. Sepharose 6B elution profiles of $^{35}$S-labeled glycoaminoglycans prepared from type IV monomers by papain digestion. A, embryonic cartilage; B, 6-year-old articular cartilage.

that found in embryonic vertebral cartilage monomers. On the contrary, only 77 and 60% of the monomer radioactivity, from 1- and 6-year-old articular cartilages, respectively, were digested by chondroitinase ABC, of which ~35% migrated as 6S disaccharide and 65% as 4S disaccharide. These findings demonstrate that the amount of keratan sulfate increases from 5 to 10% in proteoglycans synthesized by embryonic cartilage to 20 to 40% in proteoglycans synthesized by adult articular cartilages.

Aliquots of the isolated type IV monomers were treated with chondroitinase ABC and then with alkaline borohydride to β-eliminate the keratan sulfate chains. The samples were then applied to a P-30 column (28). About 10% of the total radioactivity from embryonic cartilage monomers eluted as a peak ($K_v = 0.32$) in the column, whereas the remaining 90% eluted with the total column volume (Fig. 9). Conversely, ~45% of the total radioactivity from 6-year-old articular cartilage monomers eluted as a peak ($K_v = 0.40$) in the column.

The percentages of radioactivity included in the column and eluted with the total column volume give accurate estimations of the keratan sulfate and the chondroitin sulfate, respectively, in the isolated monomers (28-29). These data confirm that the relative proportion of keratan sulfate is much higher in adult articular cartilage proteoglycans than in embryonic cartilage proteoglycans.

DISCUSSION

Modifications of proteoglycan structure have been reported to occur in cartilages from different species as they age, although the relationship of such modifications to the issue of aging is still problematical (3, 5-7, 30). For example, Hjertquist and Wasteson (30) reported a shortening of the chondroitin sulfate chains in articular cartilages from normal, older human individuals, a change that also occurs precociously in individuals affected by osteoarthritis. Inerot et al. (7) reported a progressively increased keratan sulfate content and a decreased chondroitin sulfate content in normal hip articular cartilages from dogs of increasing age; however, they did not observe a concomitant change in the average molecular size of the chondroitin sulfate chains. These and other experimental observations demonstrate that the structural modification of proteoglycans associated with aging cartilages in vivo may vary modestly from species to species. Changes similar to those described by these investigators, which were based on chemical analysis of proteoglycans, also occur in chick cartilages as described above. Moreover, by using freshly excised cartilage for short term organ culture, we have in this paper shown that the changes in proteoglycan structures such as decreased monomer size with time, reflect changes in the biosynthesis of proteoglycans. Thus, the changes in chondroitin sulfate chain size (30) and in the relative amounts of keratan sulfate (7), which characterize

| Tissue type                  | Undigested | ADi-6S | ADi-4S | 6S/4S Ratio |
|-----------------------------|------------|--------|--------|-------------|
| Embryonic tibial cartilage  | 7          | 59     | 35     | 1.7         |
| 1-year-old articular cartilage | 23        | 27 (35) | 51 (65) | 0.5         |
| 6-year-old articular cartilage | 40        | 22 (37)| 38 (62)| 0.6         |

* Data are expressed as the percentage of the total radioactivity recovered.

* In parentheses, percentages of total 6S plus 4S radioactivity recovered.

TABLE III

Relative amounts of 6S and 4S disaccharides in the isolated proteoglycans
Proteoglycans extracted from the cartilage matrix of aging animals, may be due primarily to changes in their biosynthesis (Figs. 8 and 9). Clearly, we cannot conclusively rule out the alternative, although very unlikely possibility, that the structural changes in the newly synthesized proteoglycans occur after the proteoglycans are released into the extracellular environment.

Structural modifications occurring with time, such as the reduction of the average monomer weight and the shortening of the chondroitin sulfate chains, are common to sulfated proteoglycan monomers synthesized by in vitro chondrocytes, as well as to those synthesized by freshly excised cartilage. However, there exist two main qualitative structural alterations; namely, the decrease of the chondroitin sulfate/keratan sulfate ratio and the decrease of the 6S/4S disaccharide ratio, which are expressed only in adult and aging cartilages in vivo; these alterations are not observed in “old” or frequently subcultured chick chondrocytes. Consequently, although cultured chondrocytes are useful for many types of studies relating to chondrogenesis, they, nevertheless, may be a poor model for the study of aging as it occurs in the animal. Alternatively, cultured chondrocytes may prove to be useful for the study of specific aspects of aging chondrocytes; for example, the mechanism of shortening the chondroitin sulfate chains. In this context, it will be important to compare the kinds of sulfated proteoglycans synthesized in vitro by chondrocytes derived from embryos with those derived from old animals.

Under the culture conditions used in this study, a reasonably stable cartilage phenotype can be maintained for many cell doublings, at least in terms of cell morphology and the continued synthesis of a modified type IV sulfated proteoglycan. Alternatively, simply by adding embryo extract, S-bromo-mesoxyuridine, fibronectin, or phorbol-12-myristate-13 acetate, cultured chondroblasts can be induced to dedifferentiate rapidly (31). Such dedifferentiated chondrocytes promptly cease synthesizing their type IV monomer, inhibit their synthesis of collagen, and initiate the synthesis of other forms of collagen chains, and initiate the synthesis of type II collagen chains, and initiate the synthesis of type II collagen chains (19, 32-35). Similarly, Benya et al. (9) and von der Mark et al. (36) have shown that the types of collagen chains may vary as normal chondrocytes are subcultured. At present, it is not possible to relate precisely which of these changes are uniquely associated with dedifferentiation in vitro and which are characteristic for aging in vivo. It is not even clear whether: (i) the modest numbers of aging and dying chondrocytes that appear as part of the normal developmental program of embryonic vertebral and limb bud cartilages, or (ii) the changes displayed by the aging and dying hypertrophying chondrocytes at the epiphyseal plates of young and growing animals undergo the same changes that occur in aged animals.

The systematic qualitative changes in the sulfated proteoglycans, or even in collagen chains of aging cells in culture do not fulfill the notion of the accumulation of “faulty molecules” predicted by Orgel’s aging hypothesis (37). On the other hand, the accumulation of systematically altered complex molecules, such as sulfated proteoglycans, which require sequential enzymatic additions, may be more prone to systematic changes in entire regulatory pathways than in the straightforward translation of a simple protein.

Pendling more detailed comparisons of the same terminally differentiated cells in vitro and in vivo, claims regarding aging or “senescence” (8) in the former must be interpreted with considerable caution. Possible causes for these differences could be the very different microenvironments of the chondrocytes in vitro and in vivo. One very conspicuous difference is the higher rate of replication in cultured chondrocytes as compared to chondrocytes in vivo. The chondrocytes in culture double in number approximately every 48 h. For the most part, the definitive chondrocytes in vivo rarely replicate. The constantly dying chondrocytes in vivo are replaced by the generation of new cells in the perichondrium (38, 39). In vitro, it is primarily the upper surface of the chondrocyte that is covered with matrix; the undersurface adhering to the plastic substrate generally has very little matrix (27). This contrasts with the chondrocyte in vivo which is totally surrounded by a sizeable mass of matrix. That some of these factors may be involved in the differences in the activity of chondrocytes growing as replicating monolayers and their activity in older animals is suggested by the results obtained when sizeable pieces of intact vertebral columns are organ-cultured for periods up to 10 days (see following paper, Ref. 40).

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