Synthesis of Cartilage Matrix by Mammalian Chondrocytes In Vitro. III. Effects of Ascorbate

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ABSTRACT Chondrocytes isolated from bovine articular cartilage were plated at high density and grown in the presence or absence of ascorbate. Collagen and proteoglycans, the major matrix macromolecules synthesized by these cells, were isolated at times during the course of the culture period and characterized. In both control and ascorbate-treated cultures, type II collagen and cartilage proteoglycans accumulated in the cell-associated matrix. Control cells secreted proteoglycans and type II collagen into the medium, whereas with time in culture, ascorbate-treated cells secreted an increasing proportion of types I and III collagens into the medium. The ascorbate-treated cells did not incorporate type I collagen into the cell-associated matrix, but continued to accumulate type II collagen in this compartment. Upon removal of ascorbate, the cells ceased to synthesize type I collagen.

Morphological examination of ascorbate-treated and control chondrocyte culture revealed that both collagen and proteoglycans were deposited into the extracellular matrix. The ascorbate-treated cells accumulated a more extensive matrix that was rich in collagen fibrils and ruthenium red-positive proteoglycans. This study demonstrated that although ascorbate facilitates the formation of an extracellular matrix in chondrocyte cultures, it can also cause a reversible alteration in the phenotypic expression of those cells in vitro.

Embryonic chick chondrocytes easily modulate or "dedifferentiate" in culture (19). They tend to lose their characteristic extracellular matrix, become motile, and assume a spindle shape similar to that of fibroblasts (18). These fibroblastlike chick chondrocytes synthesize predominantly hyaluronic acid and small amounts of chondroitin sulfate-containing proteoglycans. The shift from a polygonal to a spindle-shaped chondrocyte is also accompanied by a change in synthesis from the cartilage-specific type II collagen to the typical interstitial type I collagen (19, 32). Thus, the switching of collagen gene expression is accompanied by alterations in both cell morphology and matrix biosynthesis.

The present study was undertaken to examine the effects of ascorbate on long-term chondrocyte cultures. We used bovine articular chondrocytes, cultured under conditions that have been shown to preserve the phenotype of these cells for long periods of time in vitro (13, 14). In the absence of ascorbate, these chondrocytes synthesize and accumulate a metachromatic matrix (14) that contains type II collagen, as well as monomeric and aggregated proteoglycan species characteristic of the parent cartilage (13). In the presence of ascorbate, the extracellular matrix showed significantly increased amounts of collagen and ruthenium red-positive proteoglycans. Biochemical data indicate that ascorbate-treated chondrocytes synthesize collagens of types I, II, and III. Type II collagen was incorporated into the matrix, and collagens of types I and III were released into the culture medium. Type I collagen synthesis ceased when ascorbate is removed from the medium. Proteoglycan synthesis was stimulated by ascorbate, yet the proteoglycan hydrodynamic size was not altered and remained identical to that of the parent cartilage.

MATERIALS AND METHODS

Materials used in this study and their sources were as follows: PD-10 Sepharose CL-2B (Pharmacia Fine Chemicals, Piscataway, NJ); ultrapure guanidine HCl (GdnHCl) (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 Biochemicals, Irvine, CA); $Na_2{}^{35}SO_4$ (840 mCi/mmol), Aquasol II, and NCS (New England Nuclear, Boston, MA); 2,5-diphenyloxazole and 1,4-bis[2-(5-phenoxazoyl)]benzene (Research Products International, Elk Grove, IL); 6-aminohexanoic acid and phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO); benzamidine hydrochloride, glucaronolactone, DMSO, and XRP-1 x-ray film (Eastman Kodak Co., Roch-

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ester, NY); cyanogen bromide (Aldrich Chemical Co.,, Milwaukee, WI); purified collagenase (Form III) (Advance Biofactors Co., Lynbrook, NY); hyaluronidase (Worthington Biochemical Corp., Freehold, NJ); all other chemicals were reagent grade (Fisher Scientific Co., Lynbrook, NY). Associatively extracted Swarm rat chondrosarcoma proteoglycan aggregate and monomer were generous gifts from Dr. J. Kimura, Rush Medical College, Chicago, IL; monomeric pig dermal fibroblast proteoglycans from Dr. J. Gregory, Rockefeller University, New York; and purified high-molecular-weight rooster comb hyaluronic acid from Dr. A. Balazs, Columbia University, New York.

The GdnHCl solutions used for the isolation and characterization of proteoglycans were buffered with 0.05 M sodium acetate to pH 6.2. They routinely contained the following proteinase inhibitors at the concentrations indicated: 0.01 M 6-aminohexanoic acid, 0.01 M Na_2EDTA, 0.005 M benzamidine hydrochloride, and 0.001 M phenylmethylsulfonyl fluoride. The 0.5 and 4 M GdnHCl solutions are referred to as the associative and dissociative extraction solutions, respectively. When proteoglycans were isolated from medium, equal volumes of a double concentration of the associative or dissociative solutions were added. Proteoglycan aggregates (A1) and monomers (A1D1) from bovine nasal septum were prepared by CsCl density gradient centrifugation, according to the method of Sajdera and Hascall (26) and used as carrier proteoglycans. Bovine types I and II carrier collagens were prepared from calf skin and nasal septum cartilage, respectively, by mild pepsin digestion, acid extraction, and salt precipitation (22). Type V collagen was extracted from 14-d-old chick embryos, and purified by differential salt precipitation (31).

Isolation and Culture Characteristics of Chondrocytes: Procedures for the isolation and characterization of bovine articular chondrocytes have been described in detail elsewhere (13). Cells were plated at high density (2×10^5 cells/cm^2) in 35- or 60-mm culture dishes, 75-cm^2 flasks, or in roller bottles, and were grown for various periods before labeling or fixation. The growth medium was Ham's F-12, supplemented with 10% fetal bovine serum, 25 mM HEPES (pH 7.2), 50 μg/ml gentamycin, and 50 μg/ml amphotericin B. In the experimental series, ascorbate was added at a concentration of 50 μg/ml. Cultures were refed with fresh medium every 48 h.

Morphology: Chondrocytes were grown for 14 d in roller bottles or on Thermanox coverslips that had been placed into the bottoms of 8-well Multiplate dishes (Lux Scientific, Inc., Thousand Oaks, CA). Cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, dehydrated, and embedded in Epon, as described in detail elsewhere (14). Some cultures were exposed to 0.1% ruthenium red during pre- and postfixation. Sections were cut on a LKB 8810A ultramicrotome (LKB Instruments, Inc., Rockville, MD) with glass or diamond knives. Thick sections were stained with toluidine blue, thin sections with uranyl acetate followed by lead citrate. Thin sections were examined in a Philips EM-301 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Determination of ^{35}S-labeled Proteoglycans Synthesized in Culture: The formation of ^{35}S-labeled proteoglycan aggregated in vitro was investigated, using 35-mm dish cultures that were pulse labeled with $Na_2{}^{35}SO_4$ (20 μCi/ml for 15 h) on days 5 and 14 of incubation. The medium and the associatively extracted (4°C, 4 h) matrix proteoglycans were frozen at −70°C for further analysis. The remaining matrix proteoglycans were dissociatively extracted (4°C, 2 h). The unextractable residue was digested with papain (100 μg/ml, in 0.05 M sodium acetate buffer, pH 5.8, containing 20 mM L-cysteine and 5 mM disodium EDTA (60°C, 24 h). The papain digest contained freed ^{35}S-labeled glycosaminoglycans, which were counted for ^{35}S. Proteoglycans were analyzed from medium fraction after addition of an equal volume of 8 M GdnHCl (4°C, 4 h). In order to separate ^{35}S incorporated into macromolecules from incorporated ^{35}S in the medium and matrix extracts, 250-μl aliquots of each sample were applied to PD-10 columns (5.5 × 11.8 cm), and equilibrated and eluted with 4 M GdnHCl (12). Unlabeled proteoglycan monomer (A1D1; 1.5 mg/ml) from bovine nasal septum was added to all sample solutions to serve as carrier in subsequent analyses and chromatographic separations. 1-ml fractions were collected in scintillation vials, mixed with 0.9 ml of 70% ethanol and 14 ml of Aquasol II, then counted in a Packard model 3333 scintillation counter (Packard Instrument Co., Downers Grove, IL). The excluded volume (V_0), which contained ^{35}S-labeled macromolecules, was used to determine net proteoglycan synthesis.

Determination of Proteoglycan Monomer Size Synthesized by Chondrocytes In Vitro: Dish cultures were pulse labeled with $Na_2{}^{35}SO_4$ for 15 h on days 5 and 14. After dissociative extraction, the hydrodynamic size of ^{35}S-labeled proteoglycan monomers was determined by molecular sieve chromatography. Portions of the pooled V_0 fractions from PD-10 columns were chromatographed on a Sepharose CL-2B column (120 × 0.6 cm), equilibrated in 4 M GdnHCl. The sample was eluted in 4 M GdnHCl 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: (*a*) purified high-molecular-weight hyaluronic acid; (*b*) proteoglycan monomers (A1D1) from bovine nasal septum and bovine articular cartilage; (*c*) Swarm rat chondrosarcoma proteoglycan monomer (A1D1); (*d*) fibroblast proteoglycan monomer from pig dermis; and (*e*) glucaronolactone. Free $^{35}SO_4$ was added to all samples in order to determine total column volume (V_t). Macromolecules used in the calibration of the column were monitored for uronic acid after fractionation, according to the technique of Bitter and Muir (4). Proteoglycan aggregates of the associatively extracted cell-associated matrix were examined, utilizing a separate CL-2B column and 0.5 M GdnHCl as a eluent. Fractions were collected and the radioactivity was determined as above.

Labeling of Collagen Synthesized by Chondrocyte Cultures: Chondrocytes grown in flasks or roller bottles were exposed for 14 days to medium containing 5 μmCi/ml [^{3}H]proline. Labeled media were collected every 48 h and frozen for subsequent analyses. The long-term labeled (14 d) cell layers were scraped from the dishes or bottles and suspended in cold 0.5 M acetic acid solution (pH adjusted to 2.5 with 0.1 N HCl). In some experiments, [^{3}H]proline (5 μCi/ml) was administered to previously unlabeled cultures for a 12-h period. The media were removed and the cell layers treated as outlined above. In pulse- or long-term labeled, ascorbate-treated cultures, fresh ascorbate was added at each feeding.

Isolation of Collagen from Cell Cultures: Media from labeling experiments were adjusted to 30% saturation with ammonium sulfate and kept at 4°C for 18 h. The precipitated collagen was collected by centrifugation at 10,000 *g* for 30 min, then dissolved in 0.5 M acetic acid. After removal of undissolved material by centrifugation, the acetic acid solution was brought to 100 μg/ml pepsin and incubated at 4°C for 18 h. The digest was adjusted to 2 M NaCl and kept at 4°C for 18 h in order to reprecipitate all collagen types (22). The salt-precipitated media collagens were collected by centrifugation, dissolved in 5 ml of 0.5 M acetic acid, dialyzed against the same solution for 48 h at 4°C, and then lyophilized.

Cell layers from short- and long-term labeling experiments were extracted with 0.5 M acetic acid containing 100 μg/ml pepsin at 4°C for 48 h. The cell layer residue was further extracted with 50 mM Tris-HCl buffer, pH 7.5, 1 M NaCl for 24 h at 4°C. The extractions were combined and neutralized, and collagen was selectively precipitated from solution with a final 4.4 M NaCl concentration. After centrifugation, the precipitated collagens were resuspended in 5 ml of 0.5 M acetic acid, dialyzed against the same solution, and lyophilized.

Determination of Total Protein and Collagen: Chondrocytes, grown in the presence or absence of ascorbate for 5 d, were pulse-labeled with 5 μCi/ml [^{3}H]proline for 18 h. Total protein and collagen in the medium and cell layer were determined separately for each of three dishes, according to a modification of the "bacterial collagenase digestion procedure" of Peterkofsky (25). Trichloroacetic acid pellets from the media and cell-associated matrix were pretreated with bovine testicular hyaluronidase (50 μg/ml in 0.5 M phosphate buffer, pH 5.5, 0.1 M NaCl) for 1 h at 37°C. The hyaluronidase digests were trichloroacetic acid precipitated, then subjected to the collagenase digestion procedure of Peterkofsky (25). Collagenase digestion was started with an excess of enzyme, and additional enzyme was added half way through the incubation to insure complete digestion.

SDS Gel Electrophoresis: The procedures of Laemmli (15) were followed, using either 11 × 0.5-cm tube gels or 1.5-mm-thick slab gels of 6% polyacrylamide. Collagen samples were dissolved in 6 M urea containing 1% SDS ± 1% mercaptoethanol, and were heat denatured at 60°C for 10 min. After electrophoresis, gels were stained with Coomassie Blue, destained, and scanned in an ISCO gel scanner (ISCO Inc., Lincoln, NE) at 580 nm. Tube gels were sliced into 1-mm sections on a Mickle gel slicer, dissolved in NCS tissue solubilizer at 60°C for 1 h, and then counted in a Packard model 3333 scintillation counter.

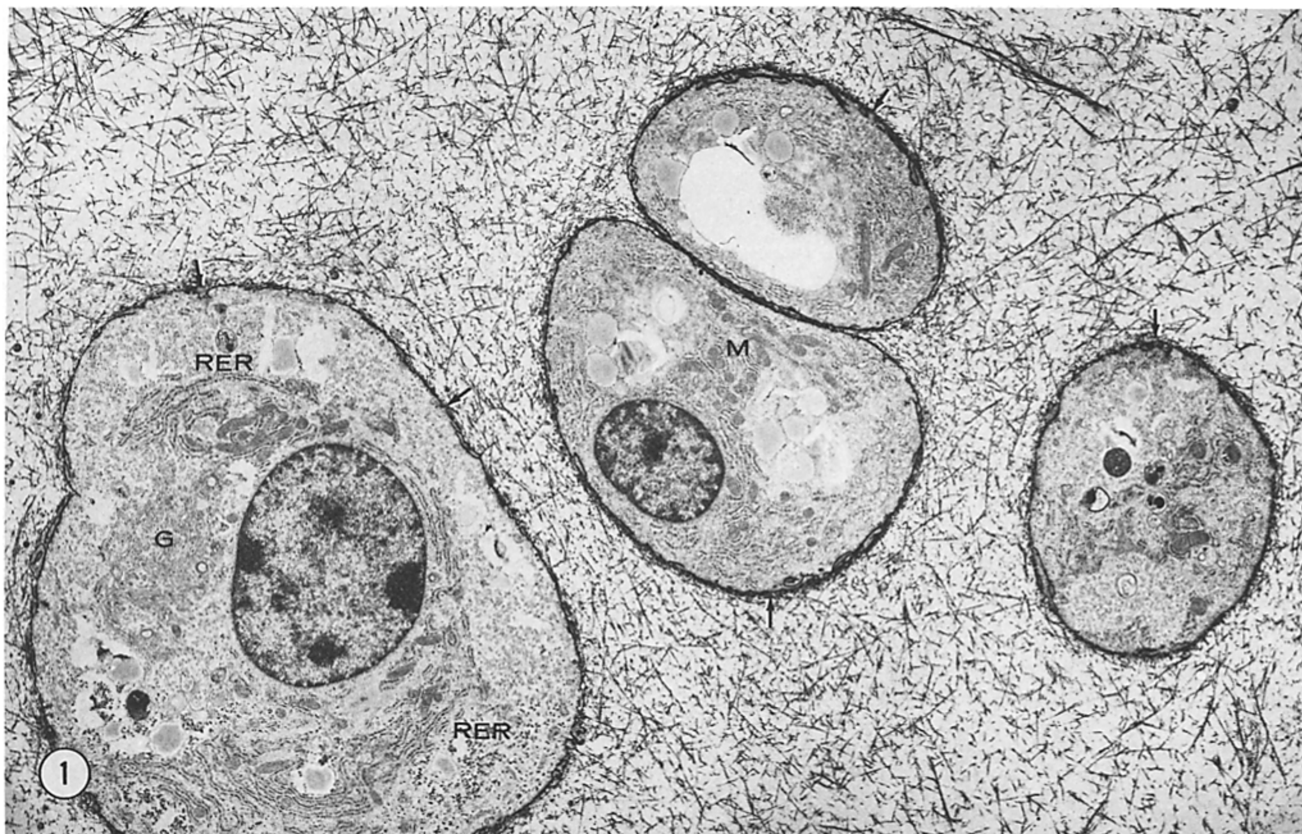
After destaining the slab gels and after indicating the positions of the α-chains, gels were prepared for fluorography, according to the techniques of Bonner and Laskey (6). Dried gels were exposed to XRP-1 film for a period of 1–2 wk at −70°C. For quantitation of collagen types synthesized over various time periods in culture, x-ray fluorograms were placed over gels, and the regions on the gels corresponding to the dark bands on the x-ray fluorograms film were excised for scintillation counting. The gel slices were solubilized in NCS at 60°C for 1 h, and then counted in a liquid scintillation counter.

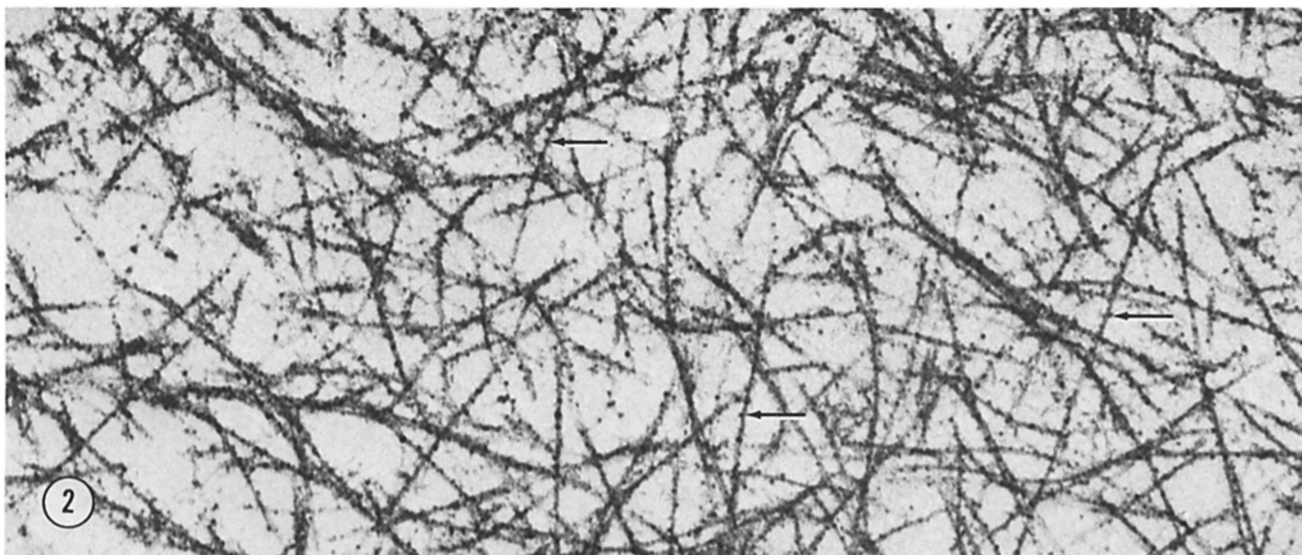
RESULTS

Chondrocytes, grown in the presence of ascorbate, formed multilayers in dishes, and monolayers alternating with random streaks (100 × 3 × 1 mm) in roller bottles. Multilayers and streaks contained several layers of cells interspersed in an abundant extracellular matrix. Superficial cells were surrounded by territorial matrix, which was reduced at the lateral surfaces, yet prevented direct contact between cells. Deeper

within the cultures, cells were sparse, and were surrounded by dense, ruthenium red-positive rims of territorial matrix (Fig. 1). The extraterritorial matrix was abundant and consisted of polydispersed, ruthenium red-positive proteoglycans that had precipitated along the dense fibrillar network of collagen (Figs. 1 and 2). Cells displayed well-developed strands of round endoplasmic reticulum and Golgi complex, numerous polysomes, clusters of mitochondria, and a few bundles of perinuclear filaments.

Chondrocyte cultures that were not exposed to ascorbate produced significantly less extracellular matrix (Fig. 3). The density of the matrix framework was decreased, relative to ascorbate-treated cultures, and there was little ruthenium red-positive material. The chondrocytes displayed dilated cisternae of rough endoplasmic reticulum, which contained a finely granular electron-dense material (Fig. 4).



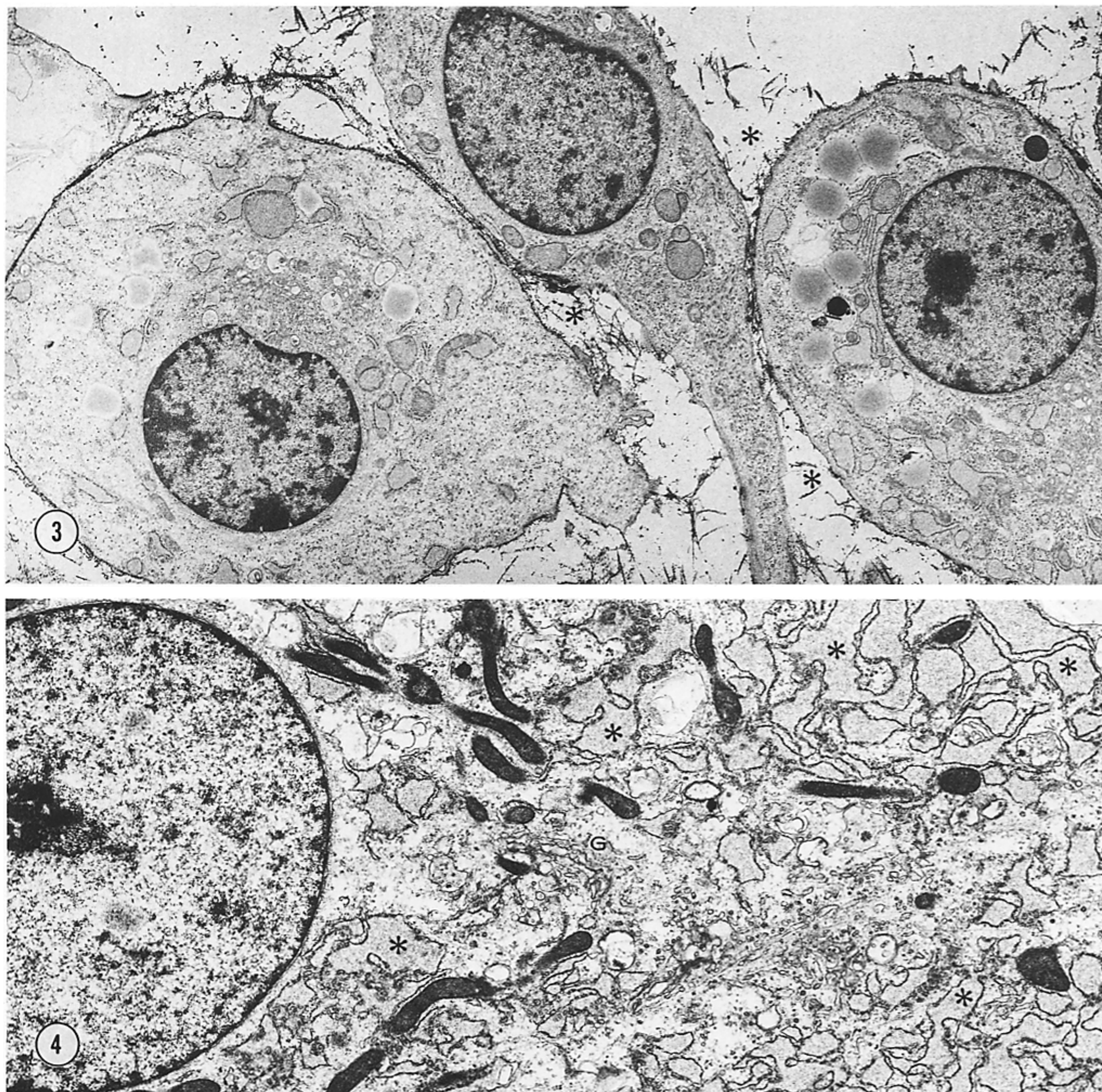


FIGURES 1 and 2 Fig. 1: Bovine articular chondrocytes, grown in the presence of ascorbate for 14 d, display well-developed strands of rough endoplasmic reticulum (*RER*) and Golgi complex (*G*). Chondrocytes are surrounded by dense rims of ruthenium red-positive territorial matrix (*arrow*). The extraterritorial matrix is abundant and consists of dense fibrillar collagen. Ruthenium red, × 4,500. Fig. 2: The extraterritorial matrix of a 14-d-old, ascorbate-treated chondrocyte culture consists of a rather dense network of collagen fibrils. Globules of ruthenium red-positive proteoglycans are present at regular intervals along the collagen fibrils (*arrow*). Ruthenium red, × 27,000.

Effects of Ascorbate on Proteoglycans

Ascorbate induced a twofold increase in the amount of ^{35}S incorporation into matrix macromolecules of chondrocyte cultures at both days 5 and 14 of incubation (Table I). In both ascorbate-treated and control cultures, ^{35}S incorporation was significantly higher at day 5 than at day 14 of incubation, yet the relative amounts of proteoglycans extracted under associative (proteoglycan aggregates) and dissociative (proteoglycan monomers) conditions were identical. The percent of associatively extracted matrix proteoglycans was lower in ascorbate-treated cultures than in controls. The exposure of chondrocytes to ascorbate had little effect upon the hydrodynamic size of associatively extracted matrix proteoglycan monomers (Fig. 5). The elution profiles were indistinguishable from those of controls, and the average size of the matrix proteoglycan monomers was nearly identical to those of monomers from bovine nasal septum and articular cartilage. In both ascorbate-treated and control cultures, normal proteoglycan aggregates were extracted under associative conditions (Fig. 5).

The distribution of proteoglycans between cell-associated matrix and media was approximately equal in each experiment for ascorbate-treated and control cultures. In order to compare sizes of proteoglycan monomers released by control and ascorbate-treated chondrocyte cultures, media from ^{35}S



FIGURES 3 and 4 Fig. 3: Bovine articular chondrocytes, grown in the absence of ascorbate for 14 d, produce significantly less extracellular matrix (*asterisks*) than those of ascorbate-treated cultures (cf. Fig. 1). The collagen network is sparse and loose, and there is little ruthenium red-positive material. Ruthenium red, × 5,500. Fig. 4: Chondrocyte of 14-d-old control culture displays dilated cisternae of rough endoplasmic reticulum that contain finely granular, electron-dense material (*asterisks*). The Golgi complex (G) is moderately well-developed. × 9,100.

TABLE I
Matrix Proteoglycans in 5- and 14-d Cultures

	Control				Ascorbate-treated			
	cpm extracted		% extracted		cpm extracted		% extracted	
	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14
	cpm		%		*cpm*		%	
Associative extract (0.5 M GdnHCl)	39,046	24,235	59.5	57.9	65,339	37,953	45.7	46.2
Dissociative extract (4.0 M GdnHCl)	19,711	11,720	30.0	28.0	60,083	38,178	42.0	46.5
Papain extract	6,920	5,928	10.5	14.1	17,694	5,977	12.3	7.3
Total	65,677	41,883			143,116	82,108		

Ascorbate:control total ratio = 2.0. *cpm*, counts per minute.

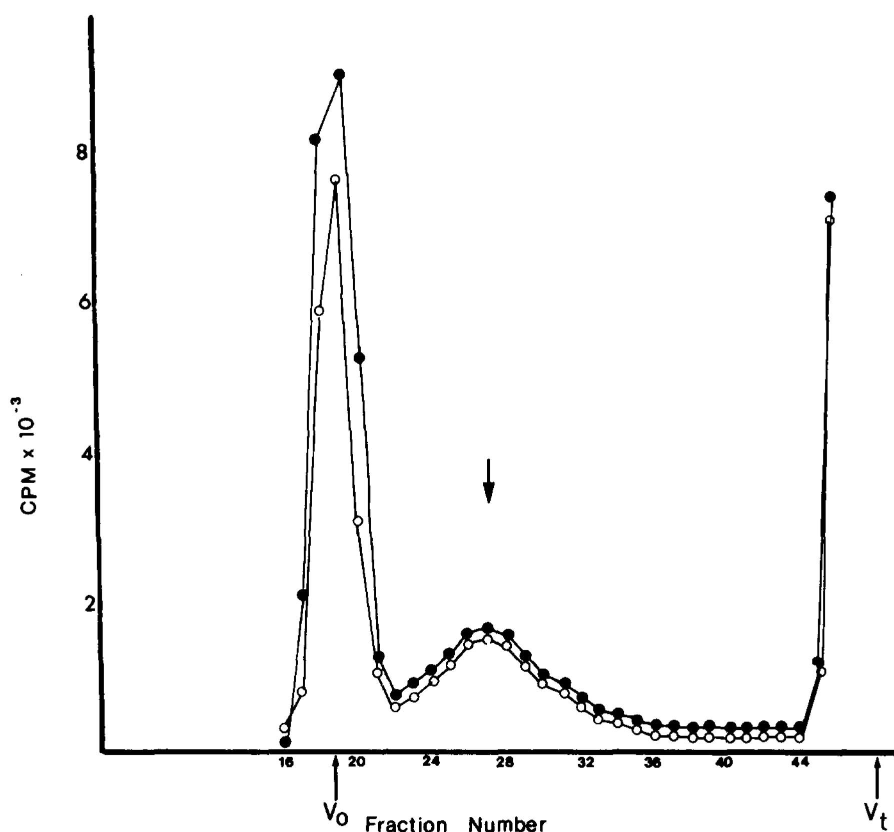


FIGURE 5 Sepharose CL-2B elution profiles of ^{35}S-labeled proteoglycans, chromatographed under associative conditions. Chondrocyte cultures are pulse labeled with $Na_2{}^{35}SO_4$ on d 14, and the cell layers extracted in 0.5 M GdnHCl. Aliquots of the extracts from ascorbate-treated (*filled circles*) and control (*empty circles*) cultures are placed on the column (the arrow indicates the location of the peak obtained from bovine nasal septum monomer [A1D1]).

pulse-labeled cultures were dissociatively extracted and chromatographed on a Sepharose CL-2B column (Fig. 6). The major species of medium proteoglycan monomer had an average partition coefficient that was practically identical to those of monomers in cartilage explants and in parent tissues.

Collagen Biosynthesis

A collagenase digestion experiment was performed to determine the influence of ascorbate on total protein and collagen synthesis and the distribution of labeled proteins between the cell layer and the medium (Table II). Ascorbate treatment stimulated [^{3}H]proline incorporation into protein. Labeling ratios of ascorbate-treated to control cultures were 2.6 for the cell layer and 1.6 for the medium. Corresponding ratios for collagenase-sensitive proteins were 2.7 for the cell layer and 13.2 for the medium, indicating a marked stimulation of collagen released into the media of ascorbate-treated cells.

Collagen type analysis was used to monitor the phenotypic expression of ascorbate-exposed chondrocytes in long-term culture. Collagens were analyzed after limited pepsinization and neutral salt extraction to ensure maximum recovery of collagen from the cell layers and to remove noncollagenous peptides, as well as to cleave procollagen peptides. Successive extractions with acid and neutral salt removed 97% of the total collagenase susceptible counts from the cell-associated matrices. The majority of the cell-associated matrix collagens in long-term labeled, ascorbate-treated chondrocyte cultures was present in a band that co-migrated with the α_1-(I) carrier. A minor collagen fraction migrated with a slightly slower mobility, and co-electrophoresed with α_1-(V) chains (Fig. 7). There was a trace amount of collagen that co-migrated with α_2-(I) chains. Pulse-labeled cell-associated matrix collagens from control cultures were also electrophoresed for a shorter time to facilitate visualization of low-molecular-weight bands (Fig. 8). Under this condition the α_1 and B bands showed little separation. Both the α_1 and B bands were sensitive to bacterial collagenase, whereas all of the low-molecular-weight species were insensitive. The control cell-associated matrix did not exhibit an α_2 component, indicating a complete lack of type I collagen biosynthesis, although we can not rule out

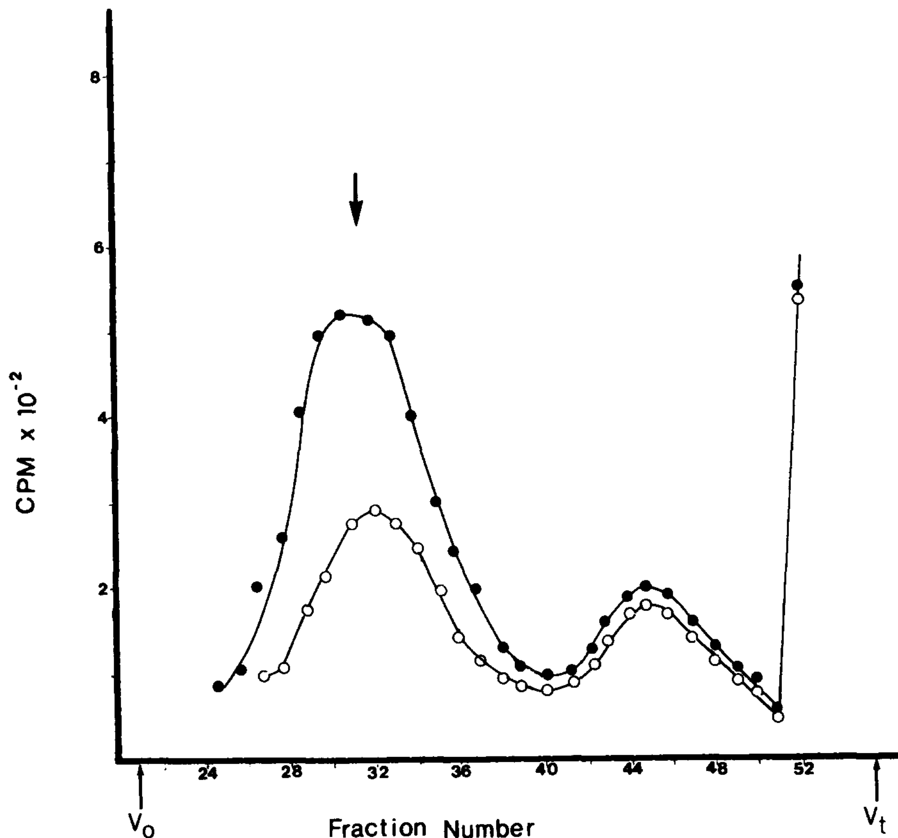


FIGURE 6 Sepharose CL-2B elution profiles of ^{35}S-labeled proteoglycans, chromatographed under dissociative conditions. Dish cultures are pulse labeled with $Na_2{}^{35}SO_4$ on day 14, and the medium adjusted to 4M GdnHCl using 8 M GdnHCl. Aliquots of the ascorbate-treated (filled circles) and control (empty circles) culture extracts are placed on the column (the arrow indicates the location of the peak obtained from bovine nasal septum monomer [A1D1]).

TABLE II

Total Protein and Collagen Synthesis in 5-d Chondrocyte Cultures

	Cell layer			Media		
	Total protein*	Collagen‡	Percent collagen§	Total protein	Collagen	Percent collagen
	cpm		%	*cpm*		%
Control	27,918 ± 2,906	7,150 ± 1,238	4.5	33,285 ± 3,551	3,297 ± 205	1.8
Ascorbate	71,378 ± 4,670	19,185 ± 4,276	4.7	52,283 ± 691	43,444 ± 2,122	13
Ascorbate:control	2.6	2.7		1.6	13.2	

* Total counts per minute (*cpm*) of proline incorporated into trichloroacetic acid-precipitable protein after hyaluroidase treatment. Mean and SD of three determinations.

‡ Collagenase-released cpm. Mean and SD of three determinations.

§ Calculated according to the formula: (collagen cpm × 100)/(noncollagen cpm × *F*) + collagen cpm, where *F* = 5.4 to reflect the amount of enrichment of proline in collagen with respect to the average protein (25).

synthesis of type I trimer.

Over a 2-wk period control chondrocytes synthesized and released types II and III collagens, and an uncharacterized chain, which co-migrated with α_1-(V). α_2-Chains were not observed (see reference 13). However, ascorbate-treated cells released α_2-chains into the medium (Fig. 9). Appropriate bands were excised from the gels and subjected to scintillation counting. Control medium contained no radioactivity in the α_2 region, even after 2 wk of culture (Table III). In the medium of ascorbate-treated cultures, however, the α_1:α_2 ratios fell from 3.6 at day 5 to 2.7 at day 14. These data indicated that increasing amounts of type I collagen were released into the culture medium with time. Upon reduction, the collagenase-sensitive material, which originally ran near the top of the gel (Fig. 9), migrated as an α-sized chain (Fig. 10). Such behavior is characteristic of type III collagen, although further analysis will be required to verify the identity of this material.

Cells were pulse labeled with [^{3}H]proline on day 14, to characterize the collagens that were synthesized late in the culture period and retained in the extracellular matrix (Fig. 11). Both ascorbate-treated and control matrices contained α_1-chains, and chains that co-migrated with α_1 (V), but neither contained α_2-chains, nor putative type III chains. Ascorbate-treated, pulse-labeled matrix collagens were further quantitated to confirm the fluorographic data. Labeled collagens, along with unlabeled carrier collagens, were electrophoresed in tube gels, which were stained, sliced into 1-mm sections, and the slices counted in a scintillation counter (Fig. 12). The complete lack of α_2-chains confirmed that there was no type I collagen retained in the matrix, even at a time when the α_1:α_2 ratio in the medium was 2.5:1.

The reversibility of the ascorbate effect was tested by growing chondrocytes in ascorbate-supplemented medium for 12 d, followed by growth in ascorbate-free medium for 5 d. After

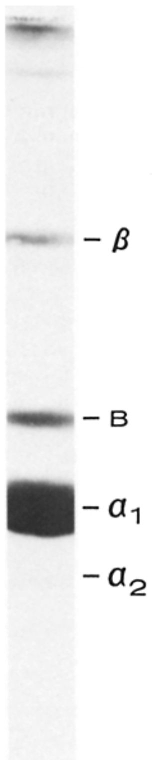


FIGURE 7 Fluorograph of ^{3}H-labeled collagens from cell layer. Ascorbate-treated chondrocyte cultures are labeled with 5 μCi/ml ^{3}H[proline] for 14 d and the collagens extracted as described in Materials and Methods. The collagens are electrophoresed on a 6% gel and fluorographed (only the upper portion of the gel is shown; the lower portion does not reveal any collagenase-sensitive bands). The positions of α_1 (I) chains, α_2 (I) chains, and β-chains are indicated. *B* designates the position of an uncharacterized collagen, which migrates in the position of α_1 (V).

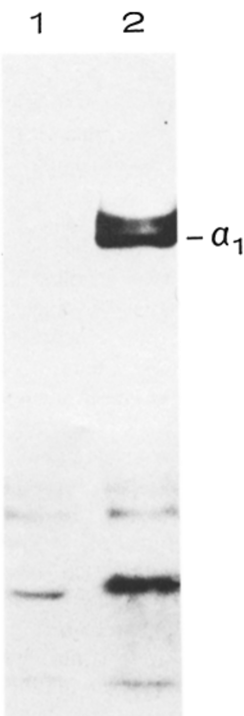


FIGURE 8 Fluorograph of ^{3}H[proline], pulse-labeled collagens from control cell-associated matrix. Control chondrocytes, grown in 75-cm^2 flasks for 5 d, are pulse labeled for 18 h with 5 μCi/ml ^{3}H[proline]. The collagens are extracted, electrophoresed on a 6% polyacrylamide gel, and fluorographed. In this and subsequent gels, approximately equal amounts of radioactive protein was added to each channel. Channel *1* is exposed to purified bacterial collagenase before electrophoresis. Channel *2* is untreated. The position of α_1 (I) chains is indicated (the entire gel is shown).

2 d in ascorbate-free medium the α_2-chains, previously synthesized and released by these cells, were no longer evident (Fig. 10).

DISCUSSION

In the present study, we examined the influence of ascorbate on the phenotypic expression of bovine articular chondrocytes in vitro. In the presence of ascorbate, these chondrocytes displayed prominent rough endoplasmic reticulum, Golgi complex, and polysomes. Territorial and extraterritorial matrices were abundant and consisted of dense networks of collagen fibrils, interdispersed with ruthenium red-positive proteoglycans. In the absence of ascorbate, bovine articular chondrocytes showed dilated cisternae of rough endoplasmic reticulum and sparse extracellular matrix. These findings were similar to those reported for chick embryonic chondrocytes in vitro (21). Chick chondrocytes, grown in the absence of ascorbate, displayed extremely dilated cisternae of rough endoplasmic reticulum, a condition that was rapidly reversed by addition of ascorbate to the culture medium. In the same system, the typical 65-nm cross-banding pattern of collagen fibrils was only observed in ascorbate-treated cultures.

The proteoglycans synthesized by bovine articular chondrocytes were characteristic of hyaline cartilage. Their hydrodynamic size was indistinguishable from that of proteoglycans extracted from slices of bovine articular cartilage, from which

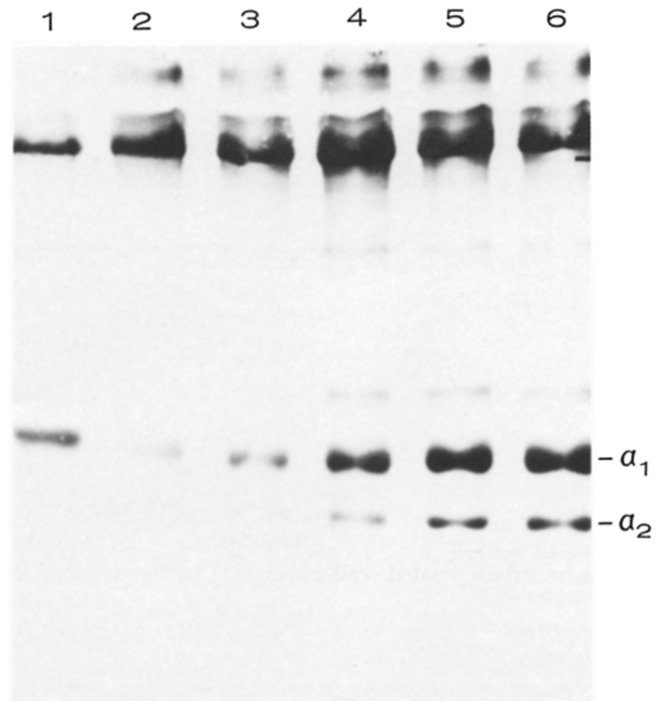


FIGURE 9 Fluorograph of ^{3}H-labeled collagens isolated from ascorbate-treated medium. The isolated collagens are electrophoresed on 6% polyacrylamide gels and fluorographed. The media are collected from the roller bottles after cultures have been labeled from days 0 to 2 (*1*), from days 2 to 4 (*2*), from days 4 to 7 (*3*), from days 7 to 9 (*4*), from days 9 to 11 (*5*), and from days 11 to 14 (*6*). The positions of α_1 (I) and α_2 (I) chains are indicated.

TABLE III

Quantitation of Collagen Types in Media of Ascorbate-treated and Control Cultures at Days 5 and 14 of Incubation

	Control		Ascorbate-treated	
Collagen type	Day 5	Day 14	Day 5	Day 14
	% cpm		*% cpm*	
Type III	52	68	87	46
Unknown chain (B)	12	11.5	1.5	6
α_1	36	20	9	35
α_2	0.1	0.5	2.5	13
α_1:α_2	360	40	3.6	2.7

Values are given as the percentage of counts per minute per collagen region.

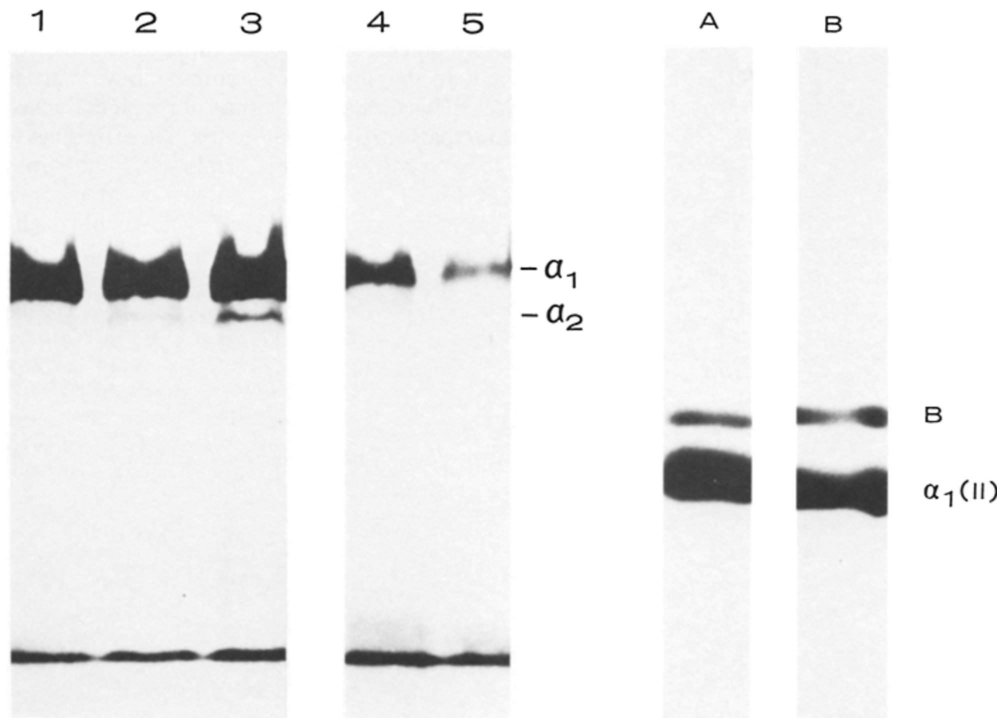


FIGURE 11 Fluorograph of ^{3}H[proline], pulse-labeled cell-layer collagens. Roller bottles of ascorbate-treated and control cultures are pulse labeled for 12 h with 5 μCi/ml ^{3}H-[proline] on day 14 of culture. Channel *A* shows the collagen extracted from the control cell layer, and *B* shows the collagen extracted from the ascorbate-treated cell layer. The upper portion of the gel, containing the α-chains, has been magnified. The lower portion does not reveal any collagenase-sensitive bands.

FIGURE 10 Fluorographs of ^{3}H-labeled collagens isolated from ascorbate-treated and post-ascorbate-treated cell culture media. The media are collected from the roller bottles after labeling the cultures with ^{3}H[proline] from days 2 to 4 (*1*), from days 4 to 7 (*2*), and from days 7 to 9 (*3*). Ascorbate is removed on day 12 and the cells are exposed to ascorbate-free medium. Ascorbate-free cultures are labeled with ^{3}H[proline] from days 13 to 14 (*4*) and from days 14 to 17 (*5*). After reduction with 1% β-mercaptoethanol, the media collagens are electrophoresed on a 6% polyacrylamide gel.

the chondrocytes had been isolated. There was a twofold increase in the amount of ^{35}S-labeled proteoglycan retained by the ascorbate-treated, cell-associated matrix, relative to controls, at both days 5 and 14. The ascorbate-treated cell-associated proteoglycans were less extractable under associative conditions than were those of control cultures. This phenomenon was observed in both short-term (5-d) and long-term (14-d) cultures. The difference in extractability might reflect a higher molecular organization of the matrix in ascorbate-treated cultures.

Despite these differences in extractability of proteoglycan aggregates, control and ascorbate-treated cultures were similar in the following ways: (*a*) both ascorbate-treated and control cells produced abundant extracellular matrix, which was rich in cartilage proteoglycans; (*b*) proteoglycans synthesized by bovine articular chondrocytes and released into the medium, represented about one-half of the total proteoglycan elaborated; (*c*) the molecular sizes of proteoglycan monomers from matrix and medium were identical in ascorbate-treated and control cultures. Proteoglycans of small hydrodynamic size, characteristic of fibroblasts, were not observed.

The matrix collagens of control and ascorbate-treated cultures were similar, with type II collagen predominating. Although we did not directly examine for the presence of type 1 trimer, a CnBr peptide analysis of the collagen control cell layers confirmed the presence of type II collagen with no detectable type I peptides (13). The media of both ascorbate-treated and control cultures contained a high-molecular-weight collagen. After reduction, this collagen migrated as an α-sized chain, and was probably type III collagen. The predominance of this type III-like collagen in the media might indicate that this collagen is incompletely processed or present in a pro-α_1 (III) form. This has been suggested for rabbit chondrocyte (2) as well as for fibroblast cultures (17). It should also be noted from studies by Benya and Nimni (1) that long-term cultures of cartilage slices contained up to 8% type III collagen. The significance of high levels of type III collagen synthesis by our chondrocyte cultures is unclear.

In addition the media of ascorbate-treated cultures contained α_2-chains, which were indicative of type I collagen. The α_2 chain of type I collagen was evident 2 d after initiation of ascorbate treatment. The ratio α_1:α_2 ratio approached that of type I collagen by 9 d in culture, and remained stable thereafter. The ascorbate effect was reversible, as α_2-chains disappeared from the medium after removal of the vitamin. Ascorbate-treated cells, which were pulse labeled with [^{3}H]-proline at day 14, synthesized type II collagen, some of which was incorporated into the matrix. There was no indication of α_2-chains in the matrix. Thus, the cells seemed to concurrently synthesize a variety of collagen types, some of which were selectively incorporated into the matrix. The synthesis and release of type I collagen into the medium of ascorbate-treated cultures may be due to the presence of two distinct cell populations. One cell population may require ascorbate to produce type I collagen, while the other may remain insensitive to the vitamin.

Our findings are at variance with those of Capasso et al. (7), who found no type I collagen in ascorbate-treated cultures of chick embryonic chondrocytes. The reason for this discrepancy may be a different ascorbate-treatment schedule. Although the ascorbate concentrations were similar in both studies, Capasso et al. (7) added fresh ascorbate every 3–4 d, as contrasted to every 2 d in our studies. Given the short half-life of this vitamin in media and the rapid reversal of the ascorbate effect, maintenance of a minimal ascorbate level may be critical for type I collagen biosynthesis. In no case did

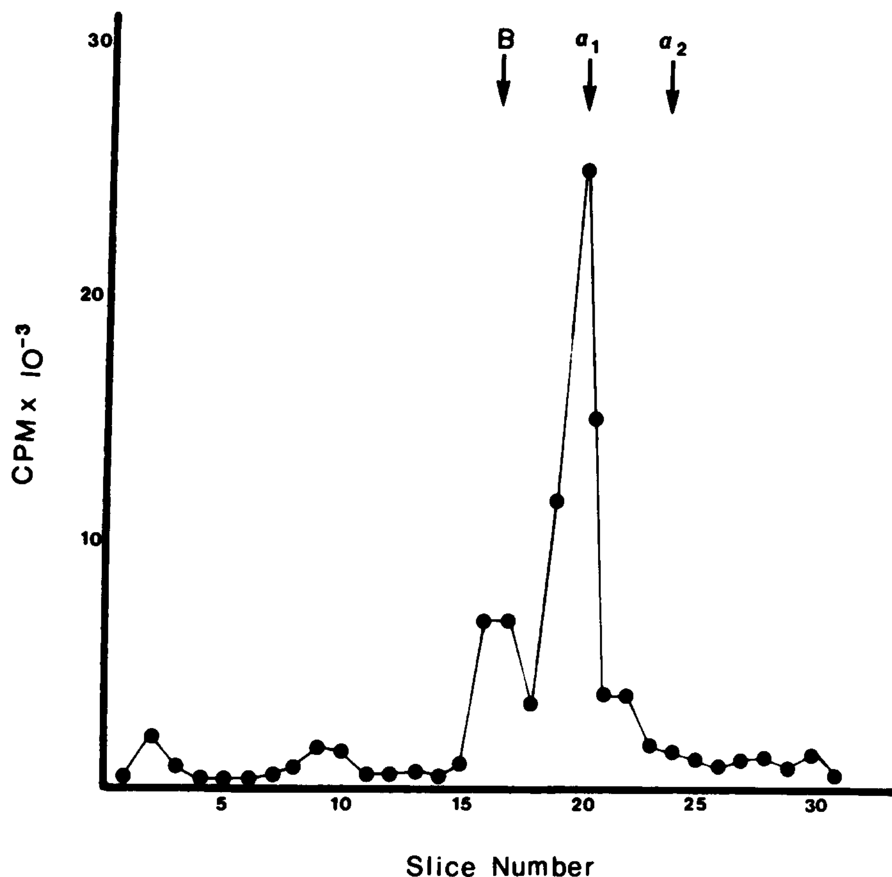


FIGURE 12 Tube gel of ascorbate-treated cell layer collagens (Fig. 11) is cut into 1-mm sections, dissolved in NCS, and counted for radioactivity. The arrows indicate migration distances of α_1, α_2, and the uncharacterized *B* chains.

we observe the short chain collagen which has been observed in other systems (28). However, we have observed this short chain collagen in control and ascorbate-treated chick chondrocyte cultures (Daniel, J. C., manuscript in preparation).

There is extensive literature on the effects of ascorbate on connective tisssue cells in vitro. It is generally acknowledged that ascorbate acts as a co-factor for the lysyl- and prolyl-hydroxylases (5). Hydroxylation of proline to hydroxyproline stabilizes the collagen triple helix and is necessary for normal collagen secretion (11, 24). Alternatively, it has been suggested that ascorbate can influence the phenotypic expression of fibroblasts with respect to collagen synthesis (23). Ascorbate may regulate collagen production by independently controlling collagen polypeptide synthesis, posttranslational hydroxylations, and the activities of the two hydroxylases. A 4-d exposure of fibroblasts to ascorbate caused a twofold increase in the amount of procollagen mRNA, yet no change in the amount of noncollagenous mRNA was observed (30). Bovine smooth muscle cells required ascorbate in their growth medium in order to maintain their morphology (29). In this system, ascorbate increased protein synthesis, but decreased cell-doubling times. Other investigators, using rabbit smooth muscle cells, have noted that ascorbate decreased the amount of insoluble elastin that was synthesized and incorporated into the extracellular matrix (10). These differences may be related to ascorbate-induced alterations in both the hydroxylation of proline and the cross-linking of lysine.

It has long been known that agents, such as chick embryo extract (27), BudR (20), and high concentrations of potassium (8), induce phenotypic instability in chondrocyte cultures. Repeated cell passage also causes a modulation of phenotype sometimes referred to as "dedifferentiation," which has been shown to be reversible under specific culture conditions (3). In each case, cell morphology, as well as collagen and proteoglycan biosynthesis, are altered. In the present experiments, only collagen expression is altered, whereas proteoglycan hydrodynamic size is unaffected. Recent immunohistochemical studies have shown that polygonal, as well as fibroblastlike chick chondrocytes in monolayer culture can synthesize type I collagen (9, 32). These data suggest that the various phenotypic traits of chondrocytes are not necessarily coordinately expressed. Several different phenotypic properties should be examined whenever one is attempting to characterize the effects of any agent upon chondrocytes in vitro. In light of our results, caution must be exercised when interpreting data from ascorbate-exposed cultures. The failure to see normal type II collagen from long-term monolayer cultures of articular chondrocytes may be explained by the routine use of ascorbate in the culture medium (16) or the analysis of combined cell layers and media.

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