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); [3H]-proline 2,3,4,5$^3$H; 90 Ci/mmol; ICN Biochemicals, Irvine, CA); Na$_2$35SO$_4$ (840 mCi/mmole), Amersham, Arlington Heights, IL); Aquasol II, and NCS (New England Nuclear, Boston, MA); 2,5-diphenyloxazole and 1,4-bis[2-(5-phenoxazoyl)]benzene; 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., Rochester, MN).
ester, NY); cyanogen bromide (Aldrich Chemical Co., Milwaukee, WI); purified collagen (Bachem Advanced Biochemicals, Lynbrook, NY); hyaluronic acid (Warthington Biochemical Corp., Freehold, NJ); all other chemicals were reagent grade (Fisher Scientific Co., Lynbrook, NY). Associatively extracted Swarrm rat chondrosarcoma proteoglycan aggregate and monomer were generous gifts from Dr. J. Kimura, Rush Medical College, Chicago, IL; monomeric pig dermal fibrillod proteoglycans from Dr. J. Gregory, Rockefeller University, New York; and purified high-molecular-weight rodeur comb hyaluronic acid from Dr. A. Bajaj, Columbia University, New York. The GDHCl 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 NaN$_3$, 0.005 M benzamidine hydrochloride, and 0.001 M phenylmethylsulfonyl fluoride. The 0.5 and 4 M GdnHCl solutions were 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 CaCl$_2$ density gradient centrifugation, according to the method of Sajdera and Hascall (26) and used as carrier proteoglycans. Both A1D1 and A1D1 monomers were prepared from calf skin and nasal septum cartilage, respectively, by mild papain 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 10×10 cm dishes, and 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 ug/ml gentamycin, and 50 ug/ml amphotericin B. In the experimental series, ascorbate was added at a concentration of 50 mg/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 (Nalgen 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 post-fixation. Sections were cut on a LKB 88 10A 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 vitrro was investigated, using 35-mm dish cultures that were pulse labeled with Na$_2$35SO$_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, 4 h) with 0.5 M sodium acetate buffer, pH 5.8, containing 20 mM L-Cysteine, 100 mM Na$_2$EDTA, 0.01 M 6-aminohexanoic acid, 0.01 M Na$_2$EDTA, 0.005 M benzamidine hydrochloride, and 0.001 M phenylmethylsulfonyl fluoride. After electrophoresis, gels were stained with Coomassie Blue, destained, and treated with SDS + 1% mercaptoethanol, and were heat denatured at 60°C for 10 min. The gel slices were excised for scintillation counting. The gel slices were solubilized in NCS 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 e- and gels, gels were sliced into 1-mm sections on a Mic1de gel slicer, dissolved in NCS tissue solubilizer at 60°C for 1 h, and then counted in a Packard model 3333 scintillation counter. 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 decontaminating the slab gels and after indicating the positions of the e- and gels, gels were stained for fluorography, according to the techniques of Bonner and Laskey (6). Dried gels were exposed to XR-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 DANIEL ET AL. Chondrocytic Phenotype and Ascorbate 1961
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 polydisperse, 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

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**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                  | cpm extracted                   |
|                      | Day 5  | Day 14 | % extracted | Day 5  | Day 14 | % extracted |
| Associative extract (0.5 M GdnHCl) | 39,046 | 24,235 | 59.5  | 57.9  |
| Dissociative extract (4.0 M GdnHCl) | 19,711 | 11,720 | 30.0  | 28.0  |
| Papain extract       | 6,920  | 5,928  | 10.5  | 14.1  |
| Total                | 65,677 | 41,883 |        |        |

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

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 [3H]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 α1(I) carrier. A minor collagen fraction migrated with a slightly slower mobility, and co-electrophoresed with α1(IV) chains (Fig. 7). There was a trace amount of collagen that co-migrated with α2(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 α1 and B bands showed little separation. Both the α1 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 α2 component, indicating a complete lack of type I collagen biosynthesis, although we can not rule out

FIGURE 5 Sepharose CL-2B elution profiles of 35S-labeled proteoglycans, chromatographed under associative conditions. Chondrocyte cultures are pulse labeled with Na235SO4 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]).
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 \( \alpha_1(V) \). \( \alpha_2 \)-Chains were not observed (see reference 13). However, ascorbate-treated cells released \( \alpha_2 \)-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 \( \alpha_2 \) region, even after 2 wk of culture (Table III). In the medium of ascorbate-treated cultures, however, the \( \alpha_1:\alpha_2 \) ratio 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 \( \alpha \)-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 \( \alpha_1 \)-chains, and chains that co-migrated with \( \alpha_1(V) \), but neither contained \( \alpha_2 \)-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 \( \alpha_2 \)-chains confirmed that there was no type I collagen retained in the matrix, even at a time when the \( \alpha_1:\alpha_2 \) 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

| TABLE II |
|----------------|----------------|
| **Total Protein and Collagen Synthesis in 5-d Chondrocyte Cultures** |
| **Cell layer** | **Media** |
| **Percent collagen** | **Percent collagen** |
| **Total protein* cpm** | **Collagen* cpm** | **Total protein cpm** | **Collagen cpm** | **Percent collagen** |
| 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 hyaluronidase treatment. Mean and SD of three determinations.

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

* Calculated according to the formula: (collagen cpm x 100)/(noncollagen cpm x F) + collagen cpm, where F = 5.4 to reflect the amount of enrichment of proline in collagen with respect to the average protein (25).
FIGURE 7 Fluorograph of 3H-labeled collagens from cell layer. Ascorbate-treated chondrocyte cultures are labeled with 5 μCi/ml 3H[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 α1 (I) chains, α2 (I) chains, and β-chains are indicated. B designates the position of an uncharacterized collagen, which migrates in the position of α1 (V).

2 d in ascorbate-free medium the α2-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 8 Fluorograph of 3H[proline], pulse-labeled collagens from control cell-associated matrix. Control chondrocytes, grown in 75-cm² flasks for 5 d, are pulse labeled for 18 h with 5 μCi/ml 3H[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 α1 (I) chains is indicated (the entire gel is shown).

FIGURE 9 Fluorograph of 3H-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 α1 (I) and α2 (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

| Collagen type          | Control Day 5 | Control Day 14 | Ascorbate-treated Day 5 | Ascorbate-treated Day 14 |
|------------------------|---------------|----------------|-------------------------|-------------------------|
| Type III               | 52            | 68             | 87                      | 46                      |
| Unknown chain (B)      | 12            | 11.5           | 1.5                     | 6                       |
| α1                     | 36            | 20             | 9                       | 35                      |
| α2                     | 0.1           | 0.5            | 2.5                     | 13                      |
| α1; α2                 | 360           | 40             | 3.6                     | 2.7                     |

Values are given as the percentage of counts per minute per collagen region.
the chondrocytes had been isolated. There was a twofold increase in the amount of 35S-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 I 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-α1 (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 α2-chains, which were indicative of type I collagen. The α1 chain of type I collagen was evident 2 d after initiation of ascorbate treatment. The ratio α1:α2 ratio approached that of type I collagen by 9 d in culture, and remained stable thereafter. The ascorbate effect was reversible, as α2-chains disappeared from the medium after removal of the vitamin. Ascorbate-treated cells, which were pulse labeled with [3H]-proline at day 14, synthesized type II collagen, some of which was incorporated into the matrix. There was no indication of α2-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
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 tissue 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 fibroblast-like 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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REFERENCES
1. Benya, P. D., and M. E. Nimni. 1979. The stability of the collagen phenotype during stimulated collagen, glycosaminoglycan, and DNA synthesis by articular cartilage organ slices. Arch. Biochem. Biophys. 192:327-335.
2. Benya, P. D., S. R. Padilla, and M. E. Nimni. 1977. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer but not type II; verification by cyanogen bromide peptide analysis. Biochemistry. 16:865-872.
3. Benya, P. D., and J. D. Shaffer. 1982. Dedifferentiated chondrocytes reexpress the differentiated phenotype when cultured in agarose. Cell. 20:215-224.
4. Bitter, T., and H. Muir. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. 4:320-334.
5. Blanck, T. J. J., and B. Peterkofsky. 1975. The stimulation of collagen secretion by ascorbate as a result of increased proline hydroxylation in chick embryo fibroblasts. Arch. Biochem. Biophys. 171:250-261.
6. Bosser, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.
7. Capasso, O., E. Giotti, G. Pontarelli, F. S. Ambesi-Imp佐bato, L. Nitch, G. Tajana, and R. Cancedda. 1982. The culture of chick embryo chondrocytes and the control of their differentiated function in vitro. *Exp. Cell Res.* 142:197-206.

8. Daniel, J. C., R. A. Kosher, J. E. Hamos, and J. W. Lash. 1974. Influence of external potassium on the synthesis and deposition of matrix components by chondrocytes in vitro. *J. Cell Biol.* 63:843-854.

9. Domrann, A., B. M. Verteil, and N. B. Schwartz. 1980. Immunological methods in the study of chondroitin sulfate proteoglycans. *Curr. Top. Dev. Biol.* 14:169-198.

10. Dunn, D. M., and C. Franzblau. 1982. Effects of ascorbate on insoluble elastin accumulation and cross-link formation in rabbit pulmonary artery smooth muscle cultures. *Biochemistry.* 21:4195-4202.

11. Fessler, J. H., and L. I. Fessler. 1978. Biosynthesis of procollagen. *Annu. Rev. Biochem.* 47:129-162.

12. Kimura, J. H., C. B. Caputo, and V. C. Hascall. 1981. The effect of cycloheximide on synthesis of proteoglycans by cultured cells from the Swarm rat chondrosarcoma. *J. Biol. Chem.* 256:589-594.

13. Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of head of bacteriophage T4. *Nature (Lond.)* 227:680-685.

14. Mayne, R., J. R. Schlitz, and H. Holtzer. 1973. Some overt and covert properties of chondrogenic cells. In *Biology of the Fibroblast.* E. Kulonen and J. Pikkarainen, editors. Academic Press, Inc., New York. 61-68.

15. Mayne, R., M. S. Vail, P. M. Mayne, and E. J. Miller. 1976. Changes in type of collagen synthesized as clones of chick chondrocytes grow and eventually lose division capacity. *Proc. Natl. Acad. Sci. USA* 72:1674-1678.

16. Peterkofsky, B. 1972. Regulation of collagen secretion by ascorbic acid in 3T3 and chick embryo fibroblasts. *Biochem. Biophys. Res. Commun.* 49:1343-1350.

17. Peterkofsky, B. 1982. Cytodifferentiation in chondrocytes. *Methods Enzymol.* 82:453-471.

18. Peterkofsky, B. 1982. Bacterial collagenase. *Methods Enzymol.* 82:632-637.

19. Peterkofsky, B. 1982. Bacterial collagenase. *Methods Enzymol.* 82:453-471.

20. Peterkofsky, B. 1982. Bacterial collagenase. *Methods Enzymol.* 82:632-637.

21. Sajdera, S. W., and V. C. Hascall. 1969. Protein-polysaccharide complex from bovine nasal cartilage. *J. Biol. Chem.* 244:77-87.

22. Schlitz, J. R., and H. E. Conrad. 1982. A unique type molecular collagen secreted by cultured chick embryo chondrocytes. *J. Cell Biol.* 92:462-470.

23. Von der Mark, H., and K. Von der Mark. 1979. Isolation and characterization of collagen A and B chains from chick embryos. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 99:101-105.

24. Von der Mark, H., and K. Von der Mark. 1979. Isolation and characterization of collagen A and B chains from chick embryos. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 99:101-105.

25. Von der Mark, K. V. Gaus, J. Von der Mark, and P. Muller. 1977. Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. *Nature (Lond.)* 267:531-532.