Selective Emergence of Differentiated Chondrocytes during Serum-free Culture of Cells Derived from Fetal Rat Calvaria

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ABSTRACT Cells dispersed from the chondrocranial portions of fetal rat calvaria proliferated and performed specialized functions during primary culture in a chemically defined medium. Mature cultures were typified by multilayered clusters of redifferentiating cartilage cells. Flattened cells that lacked distinguishing features occupied areas between the clusters. Alkaline phosphatase–enriched, ultrastructurally typical chondrocytes within the clusters were encased in a dense extracellular matrix that stained prominently for chondroitin sulfate proteoglycans. This matrix contained fibrils measuring 19 nm in diameter, which were associated with proteoglycan granules that preferentially bound ruthenium red. A progressive increase in the number of cells indicated the proliferation of certain elements in the primary culture. The cells in primary culture were biochemically as well as morphologically heterogeneous since they were found to synthesize type I and type II collagens. Homogeneous populations of redifferentiated chondrocytes were recovered as floating cells and were shown to express the chondrocyte phenotype in secondary culture. Subcultured cells synthesized type II collagen and its precursors almost exclusively and incorporated $^{35}SO_4$ into proteoglycan monomer and aggregates to a greater degree than the cells in primary culture. The pattern of proteoglycan monomer and aggregate labeling resembled that of intact cartilage segments and bovine articular chondrocytes. Skin fibroblasts harvested from the same rat fetuses failed to proliferate when maintained under identical conditions. Hence, exogenous hormones, growth factors, and protein are not required for chondrocyte growth and maturation.

Improved methods of cell culture and the use of specific markers for cell specialization have combined to advance our knowledge of chondrocyte growth and differentiation. Much of our information derives from two in vitro approaches: the *de novo* differentiation of cartilage cells from cultured mesodermal elements, and the reappearance of specialized functions among chondrocytes cultured from mature cartilage tissue. Elaboration of type II collagen and of specific sulfated proteoglycans has been used to gauge expressions of the differentiated state.

Successful primary culture of nontransformed animal cells, including cartilage cells, has required in most instances the use of culture media fortified with serum or with ill-defined protein additives (1–3, 10–13, 17, 18, 23, 26–31, 33, 37, 43, 47–52, 59). Hazards of microbiological contamination, variation in composition among different lots, inclusion of poorly defined

growth modifiers (stimulators and inhibitors), and difficulties in analysis and in purification of liberated substances are among the drawbacks encountered with the use of such media. Recently, Kato et al. (25) reported that chondrocytes maintained in the absence of serum proliferate in the presence but not in the absence of added multiplication-stimulating activity (MSA). Successful culture, however, required an initial period of exposure to a serum-supplemented incubation medium. We have found that cells isolated from the calvaria of rat fetuses proliferate and specialize when cultured in a medium which is serum-free and, at least initially, chemically defined (7, 8). Although these cells possessed morphological and biochemical characteristics reminiscent of osteoblasts (40, 41), subsequent studies suggested that chondrocytes, presumably derived from the chondrocranium, grew and matured in this system (8). The present experiments were undertaken in order to define the

morphological, ultrastructural, and functional characteristics of these cells in primary and secondary culture. We now report that chondrocytes emerge and specialize preferentially in the previously described culture medium.

MATERIALS AND METHODS

Calvaria used for cell isolation were obtained by sharp dissection from 19- to 20-d-old fetal rats and trimmed of loose connective tissue and periosteum (7, 8, 39). The cartilage plaques (chondrocranium) of these calvaria extend laterally from the junction of the sagittal and lambdoid (occipital) sutures to the outermost extremities of the lambdoid suture. To isolate cells, ~70–100 chondrocyte-enriched posterior portions (backs) of calvaria were placed into 25-ml siliconized Erlenmeyer flasks containing 15 ml of dissociating medium consisting of 120 mM NaCl, 1 mM CaCl$_2$·H$_2$O, 3 mM KH$_2$PO$_4$, 1 mM MgSO$_4$·7H$_2$O, 20 mM Tris-HCl (pH 7.3) in 30 mM mannitol, 2 mg/ml highly purified collagenase (CLS; Worthington Biochemical Corp., Freehold, N. J.) and 50 μg/ml gentamycin (Sigma Chemical Co., St. Louis, Mo.). The tissue was incubated with shaking for 45–50 min at 37°C. The resultant cell suspensions were filtered through tne supporting screens of 13-mm metal swinney filters (Millipore Corp., Bedford, Mass.) to remove debris and washed three times in serum-free medium (7) supplemented with MCDB 104 trace elements (32) (formula 79-5219; Grand Island Biological Co., Grand Island, N. Y.). This medium was subsequently used for incubation of the cells as well. Calvarium cells were then seeded into 35-mm plastic petri dishes (Corning Glass Works, Corning, N. Y.), 1 × 10^6 cells/dish, and incubated at 37°C in a humidified atmosphere of 2.5% CO$_2$ and 97.5% air. Cell viability, as determined by trypan blue exclusion, was 95%. The medium was replaced 18h after culture initiation and every 3rd D thereafter with serum-free medium containing 25 μg/ml ascorbic acid.

Subcultures

Subcultures were prepared by removing round, refractile, floating cells from 5-d cultures incubated in the absence of ascorbic acid. Cells and media were collected from several dishes, pooled, washed, counted in a hemacytometer, and plated at 1.8 × 10^5 cells/35-mm dish in conditioned medium obtained from primary cultures or in nonconditioned medium containing 10% (vol/vol) fetal calf serum (Flow Laboratories, McLean, Va.). The serum-supplemented medium was removed after 4 h, and the cell layers were washed three times with culture medium and then incubated in serum-free nonconditioned medium. The medium was replaced twice per week thereafter with serum-free medium containing 25 μg/ml ascorbic acid.

Cell Counts

The cells were removed from the culture plates with a mixture of highly purified collagenase (1% wt/vol; Worthington Biochemical Corp.) and trypsin (0.4% wt/vol; Difco, Detroit, Mich.) in Ca, Mg-free phosphate-buffered saline (PBS). Cells were then counted in a hemacytometer.

Skin Fibroblasts

Skin fibroblasts were prepared from the back skin of the same fetuses used for calvarium cell isolation. The tissue was minced and digested as described for calvaria and seeded at 1 × 10^6 cells/35-mm dish in serum-free medium.

Electron Microscopy

Cell cultures were prepared for electron microscopy by a modification of the method of Bunge et al. (6). Cultures were fixed in a mixture of 2% glutaraldehyde (Sigma Chemical Co.) in serum-free medium with 1% sucrose (adjusted to 270 mosmol, pH 7.3) and 0.5% ruthenium red (Polysciences, Inc., Warrington, Pa.). Fixation proceeded at room temperature for 1.5 h, then at 4°C for 0.5 h. After rinses in cold serum-free medium containing 4% sucrose, the cultures were postfixed for 1.5 h at 4°C in 1% OsO$_4$ in serum-free medium with 1% sucrose and 0.05% ruthenium red. The cultures were then stained en bloc with uranyl acetate, dehydrated through graded alcohols, and embedded in Epon. In some cases, ruthenium red was omitted from fixation.

Some cultures were exposed to bovine testicular hyaluronidase (Sigma Chemical Co.) when it was desirable to remove electron microscopically detectable proteoglycans (19, 21). Briefly, cultures were treated with 1% hyaluronidase in serum-free medium for 60 min at 37°C. After treatment with the enzyme, both treated and control cultures were rinsed with serum-free medium and fixed as described above.

Areas of differentiation were selected by microscopic examination, sawed out,

and glued to Epon studs parallel to the culture surface for en face sectioning. Thin sections were cut with a diamond knife on an LKB Ultratome (LKB Instruments, Inc., Rockville, Md.), picked up on Formvar-coated grids, counterstained with uranyl acetate and lead citrate, and examined in a Philips 201 electron microscope.

Light Microscopy

Cultures were fixed with 2% glutaraldehyde for 1.5 h or absolute alcohol for 20 min. The extracellular matrix was visualized by one of the following staining methods: (a) alcian blue 8GX (14, 45), (b) toluidine blue (28), or (c) safranin 0 (44). Alkaline phosphatase cytochemistry was performed using Sigma Kit No. 85L-1 (Sigma Chemical Co.). Living cultures were observed with a Lietz Dialux inverted phase-contrast microscope.

Collagen Labeling and Analysis

Primary or secondary chondrocyte cultures were incubated for 4 h at 37°C in 1 ml of Dulbecco's minimum essential medium (DMEM) (Grand Island Biological Co.) containing 50 μg/ml of L-ascorbic acid and 25 μg/ml of β-aminopropionitrile. [2,3,4,5,^{3}H-(N)]Proline, 50 μCi/ml (sp act 100 Ci/mmol; New England Nuclear, Boston, Mass.), was then added to the culture, and the incubation continued for 20 h at 37°C. After incubation, the media from cultures were pooled, and the cell layers were washed twice with 3 ml of 0.15 M NaCl/0.05 M Tris-HCl, pH 7.5. The washings were combined with the media, and the solutions were cooled in an ice bath to 4°C and supplemented with 20 mM disodium EDTA, 20 mM N-ethylmaleimide, and 0.300 mM phenylmethylsulfonyl fluoride. The cell layers and adhering extracellular matrix were suspended in 5.0 ml of 0.4 M NaCl/0.1 M Tris-HCl, pH 7.5, containing the same protease inhibitors as described above, and were sonicated at 60 Hz for 30 s in an ice bath. Aliquots of the media and cell fractions were then dialyzed against distilled water for 24 h, hydrolyzed in 6.0 M HCl, and assayed for [^{3}H]hydroxyproline and ^{3}H-protein.

To estimate the proportions of genetically distinct types of newly synthesized collagens, aliquots of the media and cell fractions were dialyzed against 0.5 M acetic acid at 4°C. Pepsin, 100 μg/ml, was added and the samples were incubated at 4°C for 15 h. After incubation, the pepsin was inactivated by raising the pH of samples to 8.5, and the samples were dialyzed against 0.4 M NaCl/0.1 M Tris-HCl, pH 7.5, at 4°C. The pepsin-resistant ^{3}H-proteins were recovered by sequential salt precipitation using 2.6, 3.2, and 4.0 M NaCl. The precipitated fractions were redissolved in 0.4 M NaCl/0.1 M Tris-HCl, pH 7.5, and the solubilized proteins were reprecipitated with the corresponding salt concentrations. Samples of the original pepsin digests, as well as of the fractions precipitated with different salt concentrations, were treated with SDS, as described elsewhere (56). The ^{3}H-polypeptides were electrophoresed on 6% polyacrylamide slab gels with delayed reduction of the disulfide bonds and analyzed as previously described (6, 56).

Cyanogen bromide (CNBr) peptide analysis of the collagen types was performed as described by Benya et al. (4) and compared with standards isolated from rat skin and sternum (35, 36, 42, 56).

Proteoglycan Labeling and Analysis

Analysis of proteoglycan monomer and aggregate synthesized by organ culture, primary cultures, and subcultured chondrocytes was carried out as follows (20): 24-h organ cultures and 14-d primary and secondary cultures were refed with fresh serum-free medium, 1 ml/35-mm dish, containing Na$_2$^{35}SO$_4$, 10 μCi/ml (sp act 800 mCi/mmol of sulfur; New England Nuclear), for 12–18 h at 37°C. Media and cell layers were separated and extracted with 4 M guanidinium hydrochloride (Sigma Chemical Co.) supplemented with protease inhibitors, 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine (Eastman Kodak Co., Rochester, N. Y.), and 0.05 M EDTA, for 4 h at 4°C, under constant agitation. 250-μl aliquots of each sample were then chromatographed on PD-10 columns (Pharmacia Fine Chemicals, Piscataway, N. J.) under dissociative conditions (4 M guanidinium hydrochloride). The ^{35}SO$_4$-labeled macromolecules represented synthesized proteoglycans eluted in the excluded volume (V$_0$) of these columns. These V$_0$ fractions were collected and counted with 14 ml of Aquasol II (New England Nuclear) containing 0.9 ml of 95% ethanol in a Beckman model LS7000 scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.).

The separated ^{35}SO$_4$-labeled proteoglycans were rechromatographed on Sepharose 2B-CL (Pharmacia Fine Chemicals) in 4 M guanidinium hydrochloride containing the same protease inhibitors as described above. Proteoglycan monomer size was determined by scintillation counting of each fraction (0.9 ml each) and compared with standards including high molecular weight umbilical-cord hyaluronic acid (V$_0$ marker) (a generous gift of Dr. J. Kimura, National Institute of Dental Research, National Institutes of Health), glucuronolactone (V$_T$ marker) (Sigma Chemical Co.), and bovine articular cartilage monomer.

Cell-associated matrices of subcultured chondrocytes were extracted under associative conditions (0.5 M guanidinium hydrochloride with protease inhibitors)

at 4°C for 16 h. Aggregate (A₁) samples were then prepared by associative CsCl density gradient ultracentrifugation in a Beckman ultracentrifuge utilizing an SW 50.0 rotor (initial density 1.65 g/ml; 40,000 rpm; 10°C; 47 h). Portions of A₁ fractions were then chromatographed on Sepharose 2B-Cl under associative conditions. Proteoglycan aggregate eluted in the excluded volume (V₀) separate from monomer.

RESULTS

Morphological Characteristics of Developing Primary Cultures and Subcultures

The freshly isolated cells attached to the culture surfaces within 5–10 min of plating. After 1–2 d, the cultures contained a mixture of flattened, triangular-shaped cells and a few round, more highly refractile cells. The round refractile cells appeared to increase in number and formed multilayered clusters in which they elaborated an extracellular matrix. Subsequently, each cluster expanded and emerged as a nodule sitting upon a layer of flattened cells. By 1–2 wk in culture, the nodules had become increasingly refractile and dense (Fig. 1), and were separated by flattened cells or by cell-free expanses of the culture plate surface. By the 4th wk, many of the cells in the nodules contained large, translucent cytoplasmic vacuoles.

Although putative chondrocytes ultimately dominated the primary cultures, the persistence of cells that did not exhibit the chondrocyte phenotype prompted us to undertake the preparation of subcultures that were more clearly free of non-chondrocytic elements. For this purpose, we used floating cells from cultures incubated in the absence of ascorbic acid. These cells were a homogeneous population of chondrocytes, as previously described (11, 30, 31). When seeded into chemically defined medium, the floaters did not attach to the supporting surface of the culture dishes, even at high densities (e.g., 5 × 10⁵ cells/35-mm dish).

Consequently, we tested the effects of alternate incubation media that might facilitate attachment: conditioned medium (ascorbic acid–supplemented [25 μg/ml] medium collected from primary cultures over a 6-d period [days 6–12]) and chemically defined medium supplemented with 10% fetal calf serum. Prolonged exposure of the floating chondrocytes to conditioned medium or a brief (4-h) initial exposure to serum-supplemented medium followed by incubation in uncondi-

tioned, chemically defined medium permitted attachment, growth, and redifferentiation of the chondrocytes. Rather than clustering in discrete nodules, as in the primary cultures, chondrocytes elaborating extracellular matrix proliferated to dominate the entire culture surface (Fig. 2). Flattened cells were not apparent in confluent secondary cultures.

Staining Characteristics of the Developing Primary Cultures and Subcultures

Virtually all of the cells within the nodules stained intensely for cytoplasmic alkaline phosphatase (not shown), whereas the flattened cells either failed to stain or stained lightly. The subcultured cells likewise stained for alkaline phosphatase. The extracellular matrix between and surrounding the cultured cells stained intensely with safranin 0 and alcian blue, indicating the presence of proteoglycans (not shown). The extracellular matrix of both the primary culture nodules and the entire subcultured cell population stained metachromatically (red) with toluidine blue, indicating the presence of proteoglycans (not shown).

Evidence for Cell Proliferation in Primary Cultures and Subcultures

The seeding efficiency of the primary cultures was found to be 40% in cultures derived from the chondrocyte-enriched back portion of calvaria as judged by enumeration of the attached cells at 24 h after culture initiation. Subsequent cell counts performed over a 14-d period provided evidence for cellular proliferation. The number of cells per culture doubled, rising from 6 × 10⁵ cells/35-mm dish on day 1 to 1.32 × 10⁶ cells/35-mm dish on day 14. The subcultured cells likewise proliferated over a 16-d period, rising from 1.4 × 10⁵ cells/35-mm dish on day 1 to 4.27 × 10⁵ cells/35-mm dish on day 16. Primary cultures of skin fibroblasts failed to proliferate in this system.

Electron Microscopy of Differentiated Nodules and Subcultured Cells

Within 7 d of culture initiation, clusters were found to contain metachromatic extracellular matrix. This matrix con-
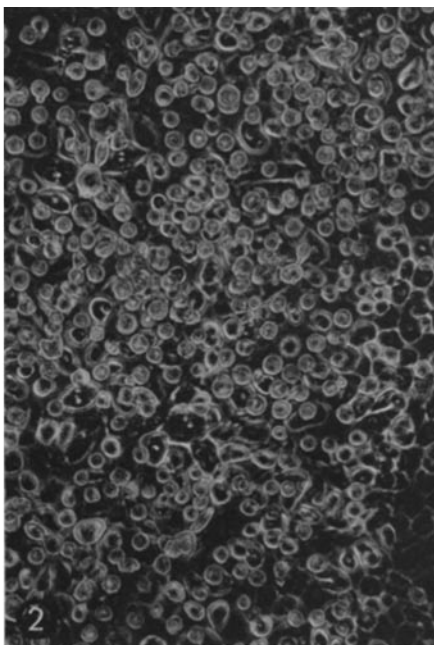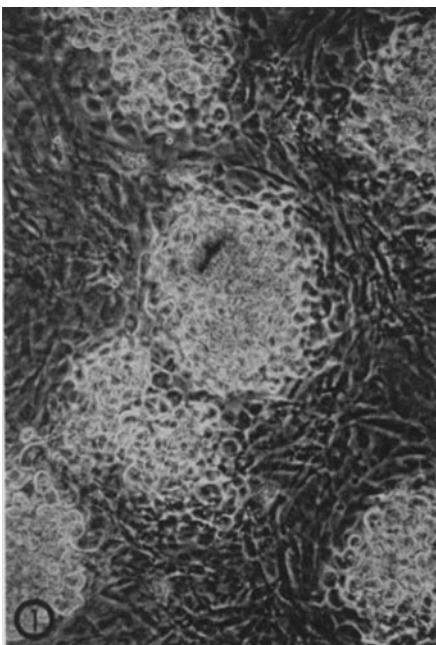

FIGURE 1 A primary culture, 1 wk after initiation, containing several well-formed nodules interspersed among flattened, fibroblastlike cells. ×100.

FIGURE 2 A homogeneous population of chondrocytes 2 wk after subculture. Nearly all the cells at this time exhibit the round, refractile characteristics of cells present in the nodule of primary cultures. × 100.

sisted of a loose meshwork of faintly banded collagen fibrils that were 19 nM in diameter. Ruthenium red–stained granules (putative proteoglycans) (2, 15, 16, 19, 21, 33, 34, 38, 46, 53, 55) 20–30 nM in diameter were associated with many of the collagen fibrils (Fig. 3) and with the cell surfaces. Ruthenium red staining additionally revealed thin, delicate, nonstriated fibrils (4–5 nM in diameter), which connected the proteoglycan granules (Fig. 3). These fibrils seemed to interconnect with the striated fibrils as well. To determine whether the ruthenium red–stained granules represented proteoglycans, cultures were exposed to hyaluronidase before preparation for electron microscopy. Hyaluronidase digestion eliminated the granules as well as the interconnecting thin fibrils but not the banded fibrils (Fig. 4) (19, 21). The cells contained extensive rough endoplasmic reticulum filled with an amorphous electron-dense material, and a well-developed Golgi apparatus associated with numerous coated vesicles and secretory vesicles containing proteoglycan-like granules. The cell surfaces exhibited numerous coated pits and vesicles. In some cases, channels ending in coated pits penetrated into the cell. The channels also appeared to be coated.

The spatial organization of the cells in the nodules of 7-d cultures was characterized by wide intercellular expanses filled with a loosely knit extracellular matrix. In some regions, the thin, striated fibrils were coalesced to form thicker fibrils with a diameter of 41 nm.

Examination of the nodules after 30 d in culture revealed extensive differentiation reminiscent of in vivo cartilage. The endoplasmic reticulum, but not the Golgi apparatus, was reduced in amount. Many coated vesicles could be seen in the area of the Golgi apparatus and near the plasma membrane.

The cells occupied lacunae in what had become a strikingly dense extracellular matrix (Fig. 5). They appeared polarized, with smooth surfaces oriented toward the outer portion of the nodule and rough surfaces, composed of projections from the cell surface, oriented toward the interior. The nodules were surrounded by two to three layers of elongated fibroblastlike

cells giving the appearance of perichondrium (Fig. 6). Additionally, the cells in the nodules closely resembled the various levels of maturation of chondrocytes found in the intact calvaria. Elongated cells were on the outermost portion of the nodules, with hypertrophic cells present in the innermost portion and resting chondrocytes intermediary in position. Some of the cells contained lipid inclusions which have been reported to be present in mitotically active chondrocytes (29).

Confluent cultures of the purified chondrocytes in secondary cultures differed in several respects from their primary counterparts. First, the chondrocytes, surrounded by their copious intervening matrix, were evenly distributed across the entire culture plate. They did not form nodules as occurred in the primary culture. However, after 2 wk in culture, the chondrocytes differentiated into hypertrophiclike cells in which much of the cytoplasm was displaced to the periphery of the cell by a large vacuole containing structurally amorphous material (Fig. 7). The nucleus was surrounded by a "stalk" of cytoplasm containing active endoplasmic reticulum studded with ribosomes and filled with an electron-dense amorphous material. The Golgi apparatus was also present in a juxtanuclear position and formed many secretory vesicles filled with proteoglycan-like granules. Second, the extracellular matrix surrounded individual chondrocytes and formed a territorial matrix. The matrix of each cell formed discrete zones about the cell and did not appear to expand into any neighboring matrix territories. Thus each cell was embedded in its own discrete matrix milieu. In some instances the proteoglycan granules were closely associated with collagen fibrils, as was the case in the primary cultures. However, in other instances, the proteoglycans enmeshed a chondrocyte, filling the extracellular space from the surface of the cells to the periphery of the matrix territory (Fig. 8). Possibly, in these preparations, the fixation process did not totally collapse the proteoglycans into small spheres but rather partially collapsed the structure, forming long, large, branching cylindrical structures that interconnected to neighboring aggregates (19). Collagen fibrils could still be
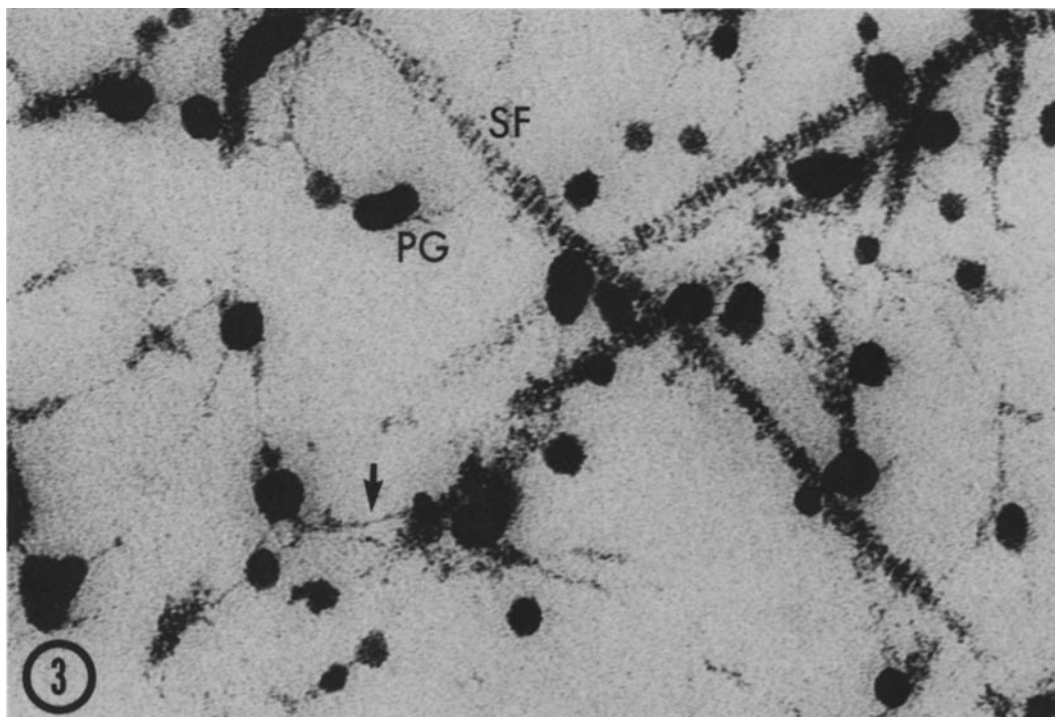

FIGURE 3 A high-power magnification showing striated collagen fibrils (*SF*) associated with proteoglycan granules (*PG*) in a 7-d primary culture. Thin, nonstriated microfibrils (arrow) are seen to connect to striated fibrils and proteoglycan granules in the extracellular matrix. Fixed with glutaraldehyde, osmium tetroxide, and ruthenium red. × 180,000.
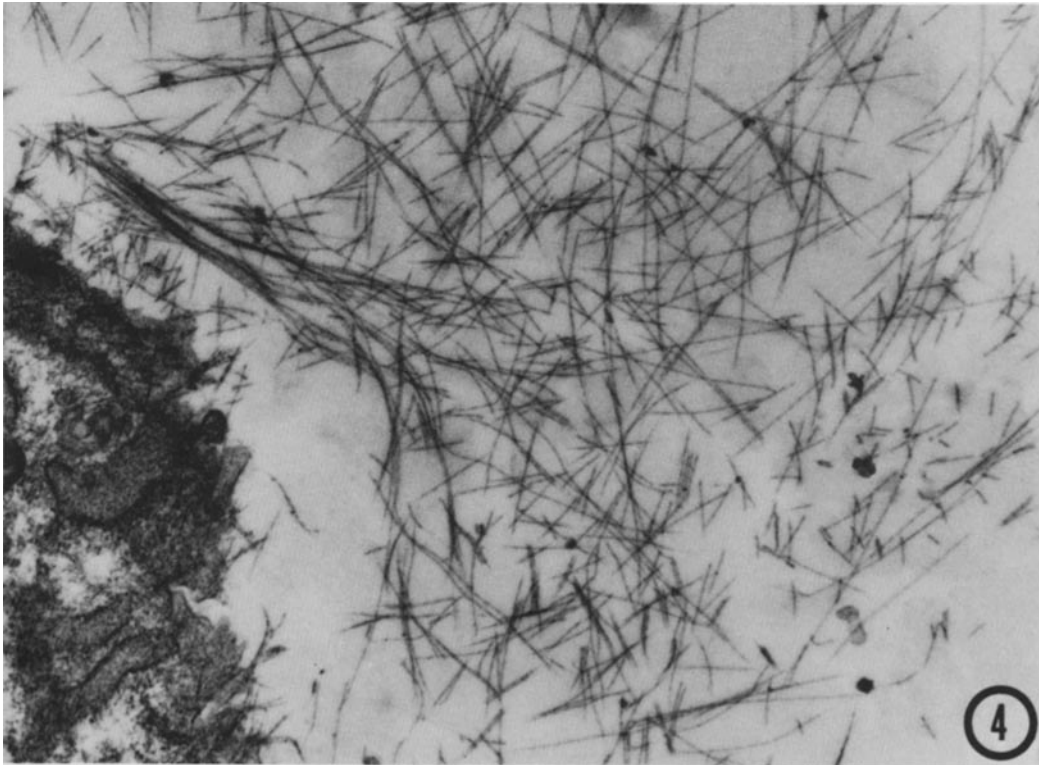
FIGURE 4 The extracellular matrix of cartilage nodules after exposure to hyaluronidase. The proteoglycan aggregates are absent, as are the thin interconnecting fibrils. The banding pattern of the collagen fibrils can be seen more clearly. 7-d primary culture. Fixed with glutaraldehyde, osmium tetroxide, and ruthenium red. X 31,000.
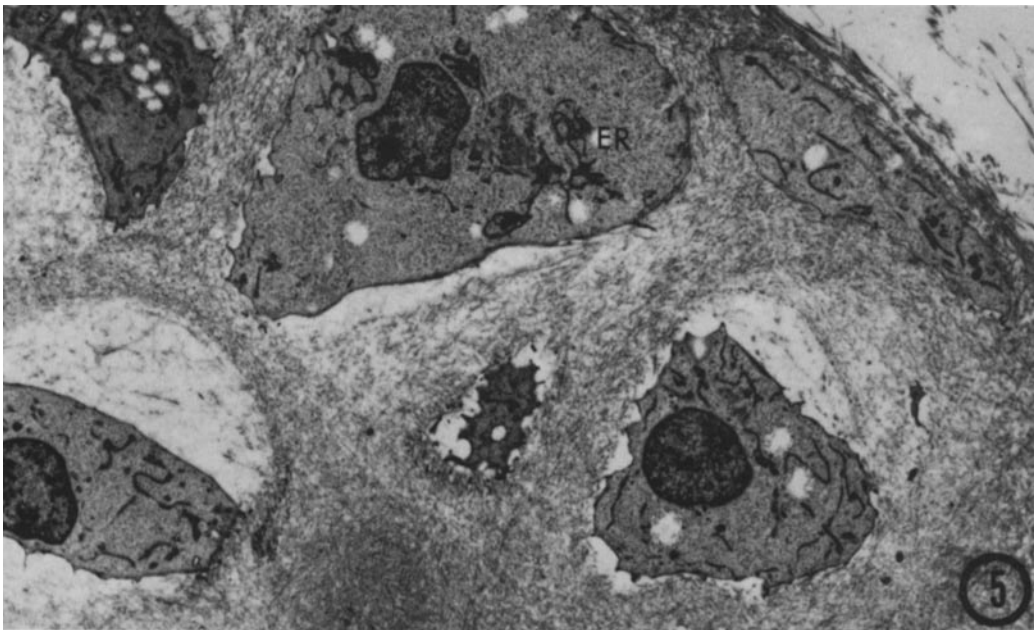

FIGURE 5 Nodules in a 30-d culture contain a very dense extracellular matrix forming lacunae surrounding the chondrocytes. There is a reduced amount of endoplasmic reticulum (ER) in these cells. Fixed with glutaraldehyde and osmium tetroxide. X 2,500.

seen crisscrossing through the intricate network, and numerous thin fibrils interconnected the large proteoglycan aggregates. The large granules had an average length of 217 nm and an average diameter of 32 nm.

Collagen Synthesis by Primary and Subcultured Cells Cultured in Defined Medium

Cells actively incorporated radioactive proline into protein, and a significant portion of [^{3}H]proline was converted to [^{3}H]hydroxyproline (Table I). Separate examination of cells from the chondrocranium-rich posterior segments revealed that ~7% of the radioactive proline was converted to hydroxypro-

line. In subsequent experiments the relative distribution of newly synthesized [^{3}H]hydroxyproline-containing macromolecules in the medium and cell layer was determined. In cultures prepared from the chondrocranial segments of calvaria, ~50% of the hydroxyproline was found in the medium (Table II).

To examine the distribution of the genetically distinct collagens formed by the cultures, newly synthesized proteins extracted from the incubation medium and from the cell layers were subjected to limited pepsin proteolysis under nondenaturing conditions. Almost equal distributions of [^{3}H]hydroxyproline were found in the 2.6 M and 3.2 M NaCl precipitates when the medium fraction was examined after pepsin digestion (Table III). Less radioactively labeled hydroxyproline was
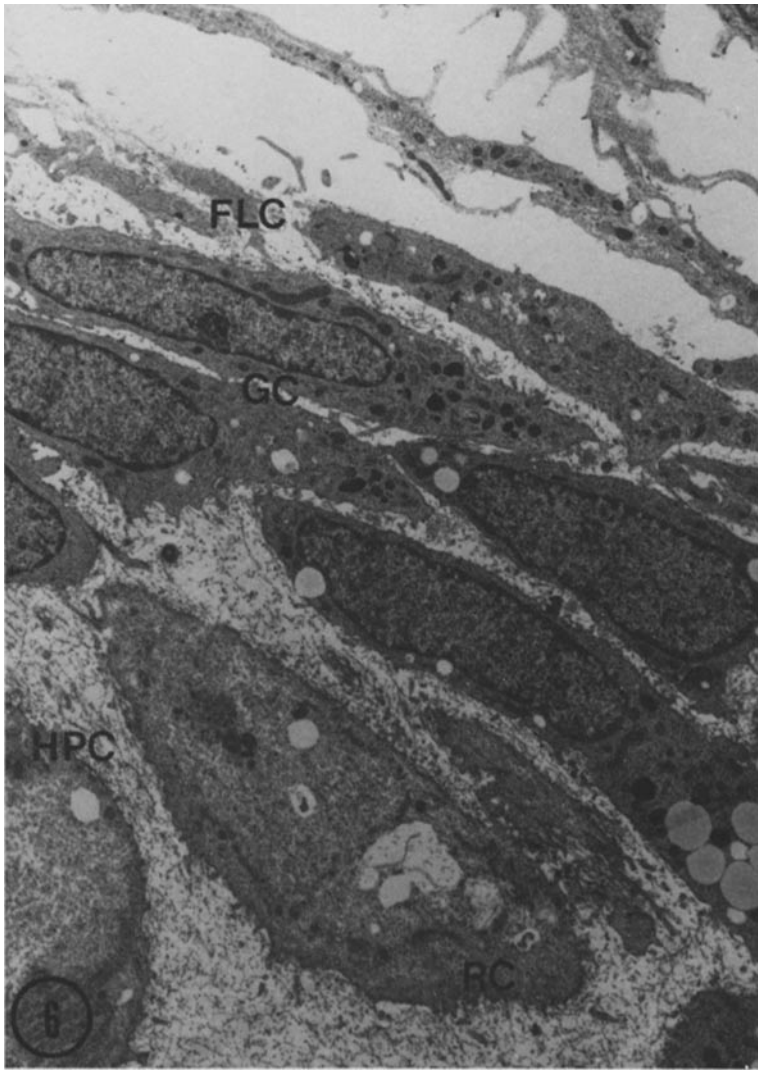
FIGURE 6 A nodule from a primary culture after a 30-d incubation. A hypertrophic cell (*HPC*) is present in the center, with resting cells (*RC*) in the middle and growing cells (*GC*) outermost. The nodule is surrounded by long fibroblastlike cells (*FLC*) giving the appearance of a perichondrium. Fixed with glutaraldehyde and osmium tetroxide. × 4,900.

recovered in 4.0 M NaCl precipitate. The NaCl precipitations recovered ~90% of the total, nondialyzable [³H]hydroxyproline.

Examination of the 2.6 M NaCl precipitate by SDS PAGE indicated that most of the radioactive polypeptides migrated in the positions of $\alpha_1(I)$ and α_2 chains of type I collagen (Fig. 9A). These bands were present in an ~2:1 ratio of α_1 to α_2, indicating that they were derived from type I collagen. No evidence of $\alpha_1(III)$ chains of type III collagen or αB chains of A-B collagen were noted in this fraction. Examination of the 3.2 M NaCl precipitate by the same technique revealed that the region in which $\alpha_1(I)$ chains migrated was markedly accentuated and largely consisted of polypeptides which had a mobility about the same as that of $\alpha_1(I)$ (Fig. 9B). The ratio of α_1 to α_2 chains varied between 9.6:1 and 14.1:1 in three separate experiments. The polypeptide chains migrating in the $\alpha_1(I)$ position were not linked by interchain disulfide bonds since they were unaffected by the delayed reduction of disulfides with 2-mercaptoethanol. Because these chains were soluble in 2.6 M NaCl but could be precipitated with 3.2 M NaCl and had the same mobility in SDS PAGE as did $\alpha_1(I)$, they possibly represented type II collagen.

The identification of these chains as $\alpha_1(II)$ was achieved by CNBr peptide mapping (4, 5). Separation by SDS PAGE of marker peptides $\alpha_1(I)$-CB6 and $\alpha_1(II)$-CB10,5 representing type I and type II collagens are shown in Fig. 10A and C, respec-

tively. The profile of CNBr peptides from α_1 chains in the 2.6 M NaCl fraction (Fig. 10B) was similar to the $\alpha_1(I)$ standard, thus confirming that most of the collagen in this fraction is type I. By contrast, the peptide map derived from α_1 chains in 3.2 M NaCl fraction (Fig. 10D) resembled the pattern obtained with the $\alpha_1(II)$ standard, indicating that the majority of collagen in this fraction was type II. A small peak in the position of $\alpha_1(I)$-CB6 was also noted, suggesting the presence of type I or type I-trimer collagens (4, 56, 57). However, the ratio of $\alpha_1(II)$-CB10,5 to $\alpha_1(I)$-CB6 indicated that >85% of the collagen in the 3.2 M NaCl fraction consisted of type II collagen. The small amount of radioactivity recovered in the 4.0 M NaCl precipitate and supernatant consisted mostly of low molecular weight fragments; it was not possible to identify them by CNBr peptide mapping.

Assay of [³H]hydroxyproline from subcultures revealed ~11% of the [³H]prolyl residues incorporated into the newly synthesized polypeptides to be hydroxylated, and ~72% of the [³H]hydroxyproline was found in the medium fraction. Examination of the ³H-labeled polypeptides in the medium by SDS PAGE revealed three major bands larger than α_1 chains of type II collagen (Fig. 11). A major band of radioactivity was also present in the $\alpha_1(II)$ position. The electrophoretic mobilities of the polypeptides larger than α chains were identical to pro-α, pc-α, and pn-α chains, representing intact and partially modified type II procollagens, respectively (58). These polypeptides
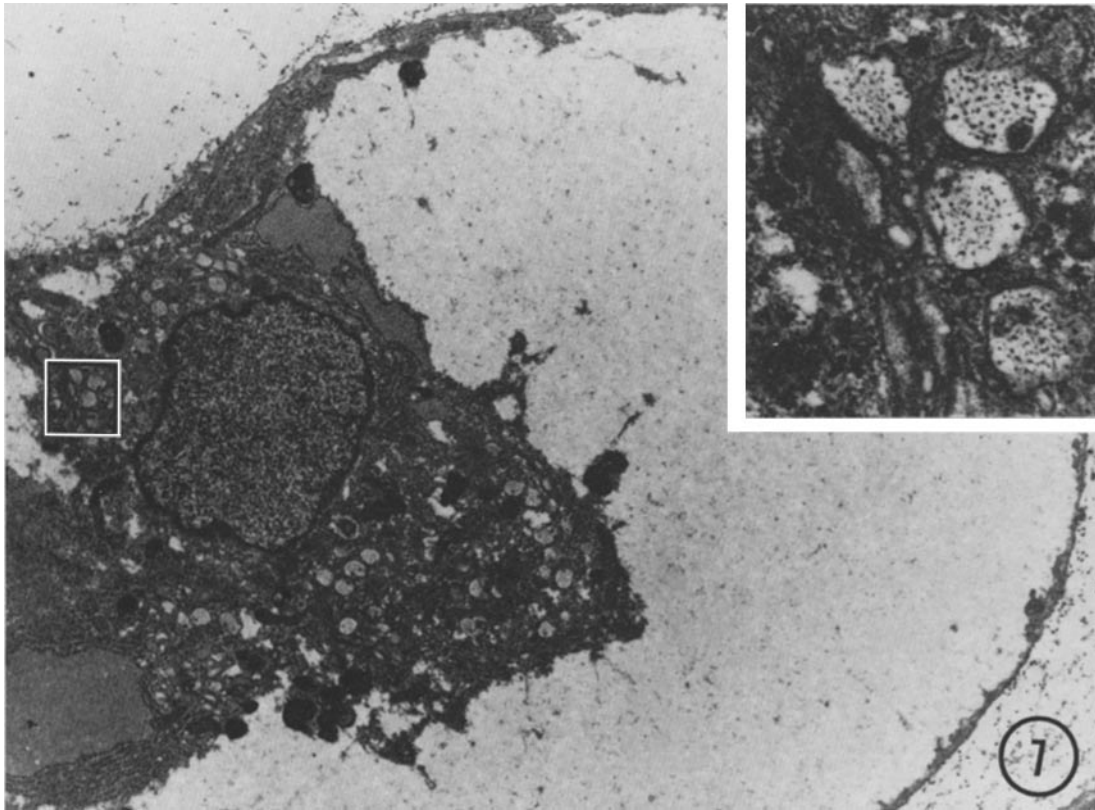
FIGURE 7   A typical hypertrophic cell from a homogeneous subcultured population of chondrocytes, 2 wk in culture. The cytoplasm has been pushed to the periphery of the cell by a large volume of included amorphous material. The *inset* shows a higher magnification of the secretory vesicles containing proteoglycanlike granules. Fixed with glutaraldehyde, osmium tetroxide, and ruthenium red. × 2,800. *Inset*, × 29,000.
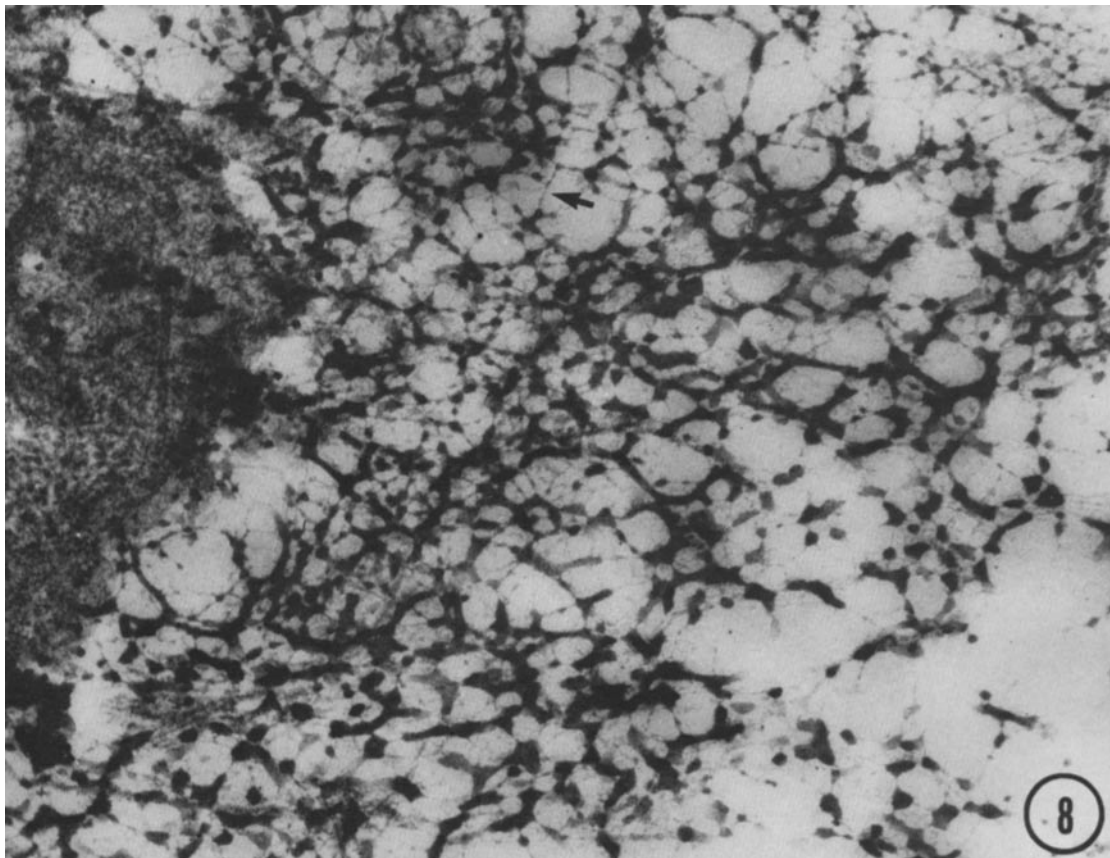

FIGURE 8   The extracellular matrix surrounding a subcultured chondrocyte, 2 wk in culture. Note the long branching, cylindrical granules forming a network. The granules are interconnected by thin unbanded fibrils (arrow). Fixed with glutaraldehyde, osmium tetroxide, and ruthenium red. × 45,400.

were shown to be precursors of type II collagen, according to several criteria. First, incubation of the medium proteins, before denaturation in SDS, with highly specific bacterial collagenase degraded these polypeptides into low molecular weight fragments. Secondly, incubation with pepsin without denaturation of the molecules converted these precursor polypeptides into collagen polypeptides which had an electrophoretic mobility of α_1(II) chains in SDS polyacrylamide slab gels (Fig. 11). No radioactivity in the α_2(I) position could be noted, suggesting that most of the procollagen synthesized was type II. Finally, the identification of the newly synthesized α chains as α_1 of type II collagen was obtained by CNBr peptide mapping. The α chains isolated by SDS PAGE were subjected to CNBr digestion, and the peptide pattern was examined by gel electrophoresis (Fig. 12). The pattern of ^{3}H-peptides was identical to that obtained with α_1(II) standard (see Fig. 10 C). Thus, the procollagen synthesized by the cells in subculture was almost exclusively in precursor form of type II collagen.

Proteoglycan Labeling in Primary and Secondary Cultures

The cells in primary and secondary culture actively incorporated $^{35}SO_4$ into proteoglycans (Table IV). When the guanidinium chloride extract (4 M, dissociative conditions) of the primary, secondary, and organ cultures was applied to a PD-10 column, the total ^{35}S-incorporated material eluted in the void volume. Organ-cultured calvaria (chondrocranial portions) were also studied in order to determine whether isolated cells retained the capacity to synthesize parent type proteoglycans. The total ^{35}S incorporated into proteoglycan by the subcultured chondrocytes (14-d culture) was 11-fold greater than that obtained for chondrocranial calvarium cultures (16 d in culture) when compared on a per cell basis. Further analysis of the proteoglycans was performed by Sepharose 2B-Cl chromatography. The elution profiles for the chondrocytic subculture and for posterior fragment organ cultures extracted under dissociative conditions (Fig. 13) show that the ^{35}S mon-

omer peaks are nearly identical (K_{avg} of subculture = 0.28 vs. K_{avg} of organ culture = 0.27) and elute in a position identical to that obtained for bovine articular chondrocyte culture (K_{avg} = 0.28). The elution profile of the subculture cell layer extracted under associative conditions (Fig. 14) shows that a large proportion of proteoglycans are present in the form of aggregates while a smaller proportion is present in the form of monomer; the percentage appears similar to the results obtained with bovine articular chondrocytes (70% aggregate of total ^{35}S-proteoglycan).

TABLE III

Differential NaCl Precipitation of [^{3}H]Hydroxyproline-containing Macromolecules Synthesized by Cartilage Cells *

| NaCl fraction | [^{3}H]Hydroxyproline/fraction in chondrocranium cultures | | | |
| | Medium | | Cell layer | |
	dpm‡	%§	dpm	%
2.6 M	84.1	47.6	50.1	30.3
3.2 M	62.1	35.2	63.9	38.6
4.0 M	20.5	11.6	32.5	19.6
Supernatant	9.8	5.6	19.0	11.5
Total	176.5	100.0	165.5	100.0

* Cartilage cells, 17 d after initiation of cultures, were labeled with [^{3}H]-proline, and the newly synthesized ^{3}H-labeled protein in medium and cell fractions was separately submitted to limited pepsin proteolysis. The pepsin digests were then dialyzed against 0.4 M NaCl/0.1 M Tris, pH 7.5. Subsequently, successive precipitations with 2.6, 3.2, and 4.0 M NaCl were performed, and the [^{3}H]hydroxyproline in the NaCl precipitates as well as in the final supernatant was assayed.
‡ The values are dpm × 10^{-3}/fraction; mean of two determinations.
§ The values are percent recovered in the corresponding fraction of the total [^{3}H]hydroxyproline in the digest.

TABLE I

Incorporation of [^{3}H]Proline into Protein and Synthesis of [^{3}H]Hydroxyproline by Cultured Cartilage Cells *

Source of cells	Total ^{3}H radioactivity	[^{3}H]Hydroxyproline	[^{3}H]Hydroxyproline × 100/ (Total ^{3}H radioactivity)
	cpm × 10^{-4}/ plate	cpm × 10^{-3}/ plate	%
Chondrocranium	189.8 ± 87.2	130.2 ± 54.2	6.8 ± 3.3

* Cartilage cells, 28 d after initiation of cultures from chondrocranium, were labeled with [^{3}H]proline, as described in Materials and Methods. The medium and cells from each plate were removed in the presence of protease inhibitors, and the samples were assayed for nondialyzable ^{3}H radioactivity and [^{3}H]hydroxyproline, as indicated in the text. The values are mean ± SD of five separate cultures.
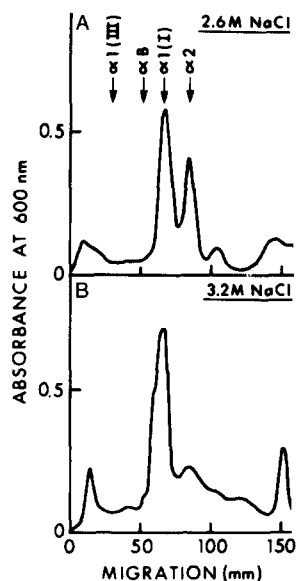

FIGURE 9 SDS PAGE of newly synthesized ^{3}H-collagenous protein isolated from the 2.6 M (A) and 3.2 M (B) NaCl fractions. Electrophoretic positions of α_1(I) and α_2 chains of type I collagen and α_1(III) and α chains of type V collagen are indicated in the figure.

TABLE II

Relative Distribution of Nondialyzable [^{3}H]Hydroxyproline between the Medium and the Cell Layer *

Source of cells	Total culture	[^{3}H]Hydroxyproline in medium	Cell layer
	dpm × 10^3/plate	dpm × 10^3/plate (%)	dpm × 10^3/plate (%)
Chondrocranium	66.5 ± 6.3	31.9 ± 3.0 (48.0)	34.6 ± 5.8 (52.0)

* Cartilage cells, 16 d after initiation of cultures, were labeled with [^{3}H]proline. The nondialyzable [^{3}H]hydroxyproline was then assayed separately in the medium and the cell layer. The values are mean ± SD of five separate cultures.
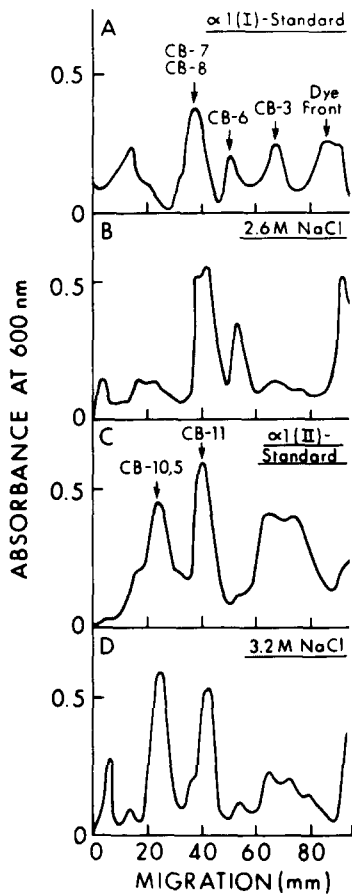
FIGURE 10 Comparisons of cyanogen bromide ^{3}H-peptide maps of standard α₁(I) chains from rat skin (A), α₁(I) chains from 2.6 M NaCl fraction of chondrocytes cultures (B), standard α₁(II) chains from rat sternum (C) and α₁(II) chains from 3.2 M NaCl fraction from chondrocyte cultures. Peaks corresponding to the CB-7, CB-8, CB-6, and CB-3 cyanogen bromide cleavage products of type I and the CB-10,5 and CB-11 cleavage products of type II collagens are indicated in the figure.

FIGURE 11 SDS PAGE of [^{3}H]proline-labeled medium proteins synthesized by sub-cultured chondrocytes. In the control sample (upper frame), the proteins were denatured in SDS and reduced with 2-mercaptoethanol without a proteolytic digestion. Part of the sample was subjected to limited pepsin proteolysis prior to denaturation in SDS (lower frame). The electrophoretic positions of pro-α, pc-α, and pn-α, and α chains of type II procollagen or collagen are indicated in the figure.

FIGURE 12 Cyanogen bromide peptide mapping of α chains isolated from the medium of subcultured chondrocytes after limited pepsin proteolysis. The electrophoretic positions of CB-10,5 and CB-11, and the cyanogen bromide cleavage products of type II collagen are indicated in the figure. Note the similarity of the cyanogen bromide peptide pattern to that derived from authentic type II collagen standard (Fig. 10).

DISCUSSION

Among the many cell types liberated enzymatically from the calvaria of rat fetuses, chondrocytes of the chondrocranium appear to be the elements that redifferentiate and perhaps proliferate when cultured in chemically defined incubation medium. Our observations provide strong testimony to the presence of differentiated chondrocytes in this system: first, the development of nodules enriched with alkaline phosphatase-positive cells and containing a newly elaborated, complex extracellular matrix tinctorially consistent with cartilage; second, the electron microscopic demonstration of the typical chondrocyte phenotype, and of extracellular collagen in close association with ruthenium red–positive, hyaluronidase-digestable granules; and, third, the de novo synthesis of type II collagen and cartilage-type proteoglycan monomer and aggregate (proteoglycans).

After a prolonged period in culture, most calvarium cells were seen to be chondrocytes associated with cartilage nodules. Ultrastructural examination of cultures maintained for 30 d in chemically defined medium revealed that the cells in the nodules were embedded in a dense extracellular matrix resembling the chondrocyte lacunae of native cartilage (15, 53). The extracellular matrix was composed of collagen fibrils which were faintly banded and averaged 19 nm in diameter. After ruthenium red staining, the banding of the collagen fibrils was more pronounced and proteoglycan aggregates were seen to be associated with them. This type of matrix has been described for many in vitro cartilage systems (2, 15, 16, 33, 38, 46, 53, 55). Additionally, thin fibrils were seen to connect the proteoglycan granules and may be composed of hyaluronic acid (19), since the fibrils disappeared after the hyaluronidase treatment.
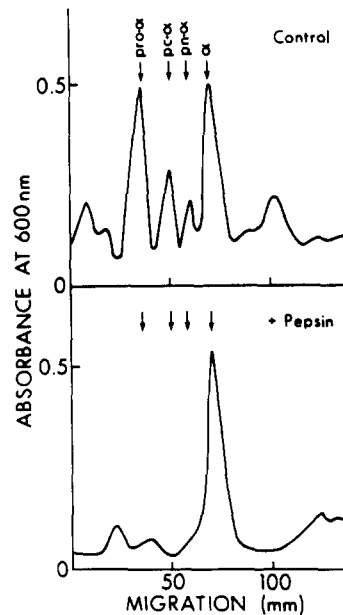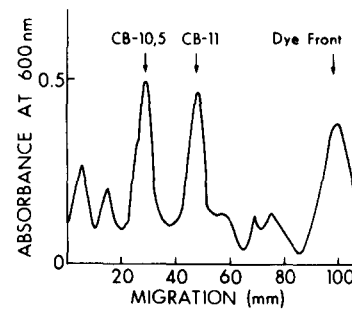
Similar findings have been reported for neural crest, sclerotome, and cornea in the embryonic chick (21). The extensive endoplasmic reticulum and well-developed Golgi apparatus, typical for chondrocytes in vivo (29, 32), suggested that the cells were actively synthesizing and secreting protein. The apparent perichondrium around the nodules and the clustering of cells into nodules may indicate that cultured cells faithfully express their in vivo destinies and may reflect specific cell-cell recognition and reaggregation.

Results of biochemical analyses substantiate our impression that chondrocytes emerge almost selectively when cells from fetal rat calvaria are cultured in a serum-free, chemically defined medium. Of particular interest was the presence of multiple types of newly synthesized collagens in the primary cultures, and the virtually exclusive presence of type II precursor molecules in the subcultures. In the mature primaries, clearly identifiable chondrocytes embedded in extracellular matrix were separated by flattened cells that were devoid of the chondrocyte phenotype. It is tempting to suggest that these are the cells that formed collagens other than type II. We cannot exclude the possibility that the flattened cells are morphologically and biochemically unspecialized chondrocyte pre-

TABLE IV

Total Cell Layer–associated $^{35}SO_4$ Incorporation

Source of cells	Incorporation
	cpm/10^5 cells
Primary culture of chondrocranium	1,080 ± 141
Subculture (passage 1)	12,528 ± 1,119
Chondrocranium organ culture	90,000*

Cell layers were extracted under dissociative conditions (4.0 M guanidinium chloride) after a 12-h pulse with $Na_2{}^{35}SO_4$. The extracts were chromatographed on PD-10 columns. The void volume was collected and counted in a scintillation counter. The results are means of four determinations ± SD.
* cpm/Chondrocranium.

FIGURE 13 Sepharose 2B-Cl elution profiles of ^{35}S-labeled monomer proteoglycans extracted from the chondrocytic subculture cell layer (upper frame) and the posterior (chondrocranium) fragment organ culture (lower frame). The extractions were performed under dissociative conditions (4M GuCl) and chromatographed under the same conditions.

FIGURE 14 Sepharose 2B-Cl column chromatographic patterns of ^{35}S-labeled aggregate and monomer proteoglycans extracted under associative conditions from chondrocytic subcultures. A large proportion of aggregate is seen in the cell-associated layer (———), whereas the medium (- - -) contains mostly monomers.

cursors. In fact, previous studies (11, 13, 30, 31) have shown that chondrocytes may assume a fibroblastic or chondrocytic appearance in culture and elaborate type I or type II collagen. For example, addition of serum and embryo extract to chondrocyte cultures can induce a more fibroblastic appearance and the elaboration of type I collagen. In contrast with the primary cultures, subcultured "floating" chondrocytes formed only type II collagen and its precursors, indicating that these cultures contained a virtually homogeneous chondrocyte population.

$^{35}SO_4$ incorporation studies tend to confirm the presence of chondrocytes in the primary and secondary cultures and suggest their preponderance in the latter. The $^{35}SO_4$ proteoglycan monomer elution profile of the purified chondrocyte culture was found to resemble that of intact chondrocranium. Furthermore, the subcultured cells incorporated ~11-fold more $^{35}SO_4$ into proteoglycan than did the primary culture, on a per cell basis. The presence of proteoglycan aggregate in the subcultured cells as the major fraction of proteoglycan was determined by associative extraction. This fraction represented ~70% of the total $^{35}SO_4$ proteoglycan synthesized, which is a finding similar to that for bovine articular chondrocyte cultures. Thus, the specific type II collagen synthesis of the secondary cultures as the only collagen species detectable and the cartilage type proteoglycan synthesized in the cultures provide convincing evidence that the secondary cultures were composed of a homogeneous population of chondrocytes.

Because cellular heterogeneity might complicate the use of the primary culture system for further biological studies, we explored the feasibility of subculturing known chondrocytes under chemically defined conditions. Indeed, it was possible to obtain a virtually pure chondrocyte population from the primary culture using floaters from cultures incubated in the absence of ascorbic acid. Whereas initiation in defined medium did not permit their attachment, the chondrocytes attached, proliferated, and redifferentiated when cultures were initiated in conditioned medium or in serum-containing medium and refed with chemically defined medium (serum-free) 4 h after seeding. Four explanations for this observation deserve mention. First, the cells in primary culture might have been under persistent stimulation by growth-promoting substances to which they were exposed in vivo, an influence that was dissipated during prolonged incubation in vitro. The fact that subcultured cells thrived after only a brief exposure to serum suggests that such influences, if operative, were relatively long lasting. Second, the capacity to elaborate growth-promoting stimuli, if any, may have evanesced during an initial period in culture. third, the chondrocytes subjected to subculture may not have been the source of growth-promoting activity; perhaps more primitive cells in the primary culture system were those that elaborated the necessary material. Fourth, the subcultured,
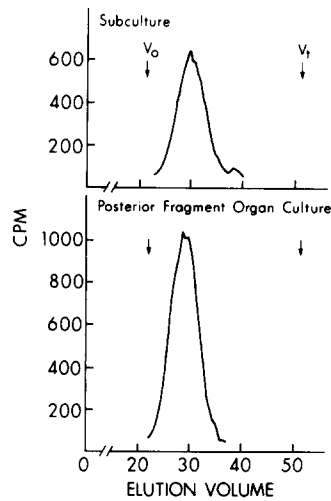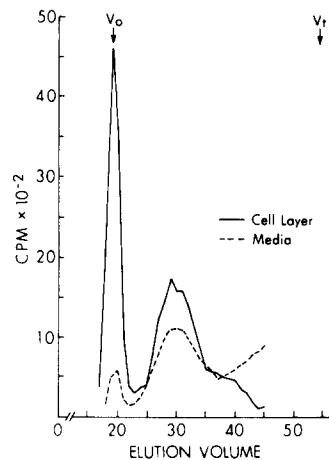
phenotypic chondrocytes might not have been able to produce an attachment factor, e.g., chondronectin (22).

Although neither skin nor periosteal fibroblasts survived under the culture conditions described herein, osteoblasts might nevertheless have been among the surviving elements. Preliminary results suggest that osteoblastlike cells (prepared from chondrocranium-free portions of the rat calvaria) attach to the culture dish substratum and remain viable when cultured separately in chemically defined medium but do not elaborate an extensive chondrocyte-type extracellular matrix. Osteoblasts included in the culture may have transformed into phenotypic chondrocytes, a transformation that has been noted in other culture systems (24).

Despite the low seeding efficiency of the cells used in the present experiments, the cell number more than doubled during the initial 14 d in culture. In light of the progressive emergence of chondrocytes in these cultures, chondrocyte precursors or chondrocytes themselves may have been the proliferating cells. Suitable testing of this hypothesis will require the characterization of the flattened cells residing between the cartilage nodules and the autoradiographic analysis of DNA synthesis among the cultured cells. The failure of the cells to exhibit any more than modest proliferation is not surprising in light of the absence of added serum, growth factors, or peptides. Although the replacement of serum with growth factors has been most successful in supporting the growth of transformed cells (3), some success has been achieved with hormone-supplemented, serum-free medium in supporting the growth of nontransformed cells (54). Kato et al. (25) reported enhancement of

chondrocyte proliferation and specialization in serum-free medium supplemented with MSA, although the presence of serum was required for culture initiation.

We have accumulated evidence that cells in this system, perhaps the fetal chondrocranial cells, elaborate a growth-promoting factor (or factors), and the evidence has been the subject of preliminary reports (8, 9). Perhaps cells of fetal cartilage origin thrive in the absence of added serum or growth factors by virtue of their capacity to elaborate an autostimulatory substance. Indeed, Solursh and Meier (50) and Solursh and Reiter (51) have shown that fetal chondrocytes produce a factor(s) that enhance(s) their own differentiation but not growth. We are now pursuing the purification and characterization of growth-promoting and differentiating factor(s) from defined medium conditioned by the fetal chondrocranial cells in primary culture.

Ascorbic acid has been shown to promote the synthesis of collagen in cartilage cultures (28). Hajek and Solursh (18) and Meier and Solursh (33) have reported that ascorbic acid, at a concentration of 50 μg/ml, not only stimulated proliferation of chondrocytes but was necessary for the normal secretion of collagen and the proper deposition of extracellular matrix in chick embryo sternal chondrocytes grown in the presence of fetal calf serum. In our serum-free system, the differentiated chondrocytes did not remain attached in primaries to the culture surface in the absence of the vitamin. When the cultures were routinely supplemented with the vitamin, the cells remained adherent and developed an extensive matrix. Thus, ascorbic acid may have stimulated the undifferentiated cells to synthesize and secrete factors necessary for the attachment of differentiated chondrocytes as well as being essential for the elaboration of the matrix.

Chondrocranial chondrocytes may be unique in their ability to thrive when cultured in serum-free medium. Certainly, chondrocytes from other tissue sites have not been reported to grow and differentiate without at least transient exposure to serum in vitro, and subsequent addition of growth factors in systematic studies employing conditions such as those described herein are lacking. Chondrocytes embedded as they are in vivo in an avascular tissue may be capable of adapting to nutritionally and humorally stringent environmental conditions. The in vitro system we describe should allow a detailed examination of the factors that govern chondrocyte behavior.

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