Gene Expression and Extracellular Matrix Ultrastructure of a Mineralizing Chondrocyte Cell Culture System

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Abstract. Conditions were defined for promoting cell growth, hypertrophy, and extracellular matrix mineralization of a culture system derived from embryonic chick vertebral chondrocytes. Ascorbic acid supplementation by itself led to the hypertrophic phenotype as assessed by respective 10- and 15-fold increases in alkaline phosphatase enzyme activity and type X synthesis. Maximal extracellular matrix mineralization was obtained, however, when cultures were grown in a nutrient-enriched medium supplemented with both ascorbic acid and 20 mM β-glycerophosphate. Temporal studies over a 3-wk period showed a 3–4-fold increase in DNA accompanied by a nearly constant DNA to protein ratio. In this period, total collagen increased from 3 to 20% of the cell layer protein; total calcium and phosphorus contents increased 15–20-fold. Proteoglycan synthesis was maximal until day 12 but thereafter showed a fourfold decrease. In contrast, total collagen synthesis showed a >10-fold increase until day 18, a result suggesting that collagen synthesis was replacing proteoglycan synthesis during cellular hypertrophy. Separate analysis of individual collagen types demonstrated a low level of type I collagen synthesis throughout the 21-d time course. Collagen types II and X synthesis increased during the first 2 wk of culture; thereafter, collagen type II synthesis decreased while collagen type X synthesis continued to rise. Type IX synthesis remained at undetectable levels throughout the time course. The levels of colla-

gen types I, II, IX, and X mRNA and the large proteoglycan core protein mRNA paralleled their levels of synthesis, data indicating pretranslational control of synthesis. Ultrastructural examination revealed cellular and extracellular morphology similar to that for a developing hypertrophic phenotype in vivo. Chondrocytes in lacunae were surrounded by a well-formed extracellular matrix of randomly distributed collagen type II fibrils (~20-nm diam) and extensive proteoglycan. Numerous vesicular structures could be detected. Cultures mineralized reproducibly and crystals were located in extracellular matrices, principally associated with collagen fibrils. There was no clear evidence of mineral association with extracellular vesicles. The mineral was composed of calcium and phosphorus on electron probe microanalysis and was identified as a very poorly crystalline hydroxyapatite on electron diffraction. In summary, these data suggest that this culture system consists of chondrocytes which undergo differentiation in vitro as assessed by their elevated levels of alkaline phosphatase and type X collagen and their ultrastructural appearance. During the development of a progressively mineralizing extracellular matrix, gene expression and synthesis shift from high levels of proteoglycans to high levels of collagen type II and subsequently to high levels of type X. The chondrocytes in vitro assemble and mineralize their matrix in an analogous fashion to that seen in vivo.

D URING embryogenesis of vertebrates, most bone formation occurs by a process of endochondral ossification in which undifferentiated mesenchyme condenses initially to form a core of differentiated chondrocytes. Bone formation is initiated peripheral to the cartilage core, and vascular invasion of the core follows, accompanied by cartilage resorption and replacement by marrow and trabecular bone (8, 26). In postembryonic development during both longitudinal bone growth at the epiphysis and unstable fracture healing, similar events take place. The cartilage tissue undergoes extensive ultrastructural changes characterized by cellular hypertrophy and extracellular matrix mineralization. These alterations are obtained through a highly coordinated temporal and spatial sequence of changes in the chondrocyte phenotype suggestive of cellular differentiation.

Collagen gene expression and synthesis have been intensively studied during endochondral development because of the major role of these proteins in the cartilage extracellular matrix. At least three unique collagen types (II, IX, and X) are known to be predominantly found in cartilage and have been examined both in vivo and in vitro during the endochondral sequence (43). Collagen type X has been localized immunologically and biochemically within only those areas of cartilage where the chondrocytes are hypertrophic

and has been associated with dense foci of mineralization (7, 35, 49, 55, 56). Such characteristics suggest that this collagen type may be important in either mineralization or matrix resorption (39). Collagen types II and IX are expressed throughout cartilage development, but unlike type X these collagen species are expressed in areas of chondrocyte proliferation in the growth plate and permanent cartilage (9, 35). Other phenotypic characteristics associated with hypertrophic chondrocytes are the expression of high levels of alkaline phosphatase and the accumulation of type II C-propeptide (chondrocalcin) in the extracellular matrix (25, 27).

Recent studies indicate that chondrocytes grown in vitro retain their ability to express different phenotypes characteristic of either the developmental stage at which they were isolated or their ultimate developmental fate. For example (27), bovine chondrocytes cultured from either growth plate, nasal or epiphyseal cartilage possess very different abilities to undergo calcification or to express collagen type X and chondrocalcin. Further, cultured embryonic chick chondrocytes obtained from one tissue that undergoes endochondral replacement during embryogenesis (ventral vertebra) and another that does not (caudal sterna) may be shown to have differences in total collagen synthesis, procollagen processing, and the distribution of collagen types (20).

Studies from several groups also indicate that environmental factors and culturing conditions alter chondrocyte phenotype and may promote cell hypertrophy. These included culture growth in a three-dimensional matrix (collagen gel or agarose) and the cell density at which cultures are plated (4, 9, 23, 24, 61, 62), the type of nutrient medium employed (62), and the presence of vitamin D_3 metabolites (22) and ascorbic acid (36). None of these investigations has concerned itself with a combination of these various influences on cell development and none has considered the possible corresponding effects on culture mineralization.

In the work reported here, different nutrient-enriched media and the addition of ascorbate and β-glycerophosphate were systematically examined with time to identify conditions in vitro which promote chondrocyte differentiation, cellular hypertrophy, and extracellular matrix calcification in an embryonic chick chondrocyte culture. Induction of high levels of alkaline phosphatase and type X collagen expression were used as phenotypic markers of cellular differentiation. Chondrocyte growth and the accumulation of protein, collagen, and mineral in the culture system were assessed in relation to the expression and synthesis of collagen and proteoglycan. Concurrently during the temporal progression of the culture, the elaboration of an extracellular matrix and the deposition of mineral were examined ultrastructurally to establish if such characteristics of the cells in vitro resembled those of cartilage tissue in vivo.

Materials and Methods

Vertebral Chondrocyte Cell Cultures

Chondrocytes were prepared from the ventral half of 12-d-old chicken embryo vertebra as described previously (15, 20). Cells were grown for a 7-d period in MEM and 10% FBS. After 7 d, only nonadherent cells were selected, treated with trypsin, and replated at 2.0 × 10⁶ cells per 100-mm dish in DME and 10% FBS. Cultures were grown for an additional 7 d during which 90% of the cells became attached to culture plates and grew in foci of polygonal cells. Experiments were initiated by changing the medium

to Fitton-Jackson modified (BGJ_b) supplemented with 50 μg/ml ascorbate, 10 mM β-glycerophosphate, and 10% FBS. Data points were numbered from the time cells were placed in BGJ_b medium. In some experiments cultures were maintained in DME supplemented with either or both 50 μg/ml ascorbate and 10 mM β-glycerophosphate or in BGJ_b medium supplemented with ascorbate alone.

Analysis of Collagen and Proteoglycan Synthesis

All radiolabeled compounds were from Amersham Corp. (Arlington Heights, IL). For protein and collagen analysis, cultures were labeled for 24 h using either 5 ml 50 μCi/ml ³H-proline (110 mCi/mmol) or ³H-leucine (120 mCi/mmol) as described previously (17). After the 24 h pulse, medium was removed, and cell layers were washed with PBS. The total cell-associated proteins were extracted using 6 M guanidine-HCl and secreted medium proteins were processed in the presence of proteolytic inhibitors (17). In some experiments collagenous proteins were extracted by pepsin treatment. From 500,000 cpm nondialyzable protein, both the cell layer and medium were combined, suspended in 5 ml of 5% (vol/vol) acetic acid and digested with 10 μg/ml pepsin at 4°C for 24 h. Samples were then dialyzed to neutrality against distilled water and subsequently lyophilized. Total collagen synthesis was determined by digested total proline labeled proteins (50,000 cpm) with collagenase and following the assay of Schwarz et al. (57). Radiolabeled proteins were analyzed by electrophoresis on 5-10% continuous gradient SDS–polyacrylamide gels (29) with 75,000 cpm applied per lane for total protein synthesis and 50,000 cpm per lane for pepsin extracted samples. Fluorography was carried out according to Bonner and Laskey (3) and Laskey and Mills (34). Quantitation of the fluorographs was performed on a densitometer (ultrascan II; LKB Instruments Inc., Bromma, Sweden).

For proteoglycan analysis, cell cultures were labeled with 50 μCi/ml Na$_2$³⁵SO$_4$ (21 mCi/ml) in 1.5 ml for 24 h. Proteoglycans were precipitated overnight with 1.3% potassium acetate (vol/vol) in 95% ethanol. Samples were collected by centrifugation at 16,000 g in an Eppendorf centrifuge (Brinkman Instruments Inc., Westbury, NY) for 30 min, resuspended, and reprecipitated. Incorporation of label was determined by glass filtration of the precipitate followed by scintillation counting (44).

Analysis of Culture Growth Conditions and Mineralization

For these experiments cells were replated into the specified medium at 3.75 × 10⁵ cells per 35-mm tissue culture well. All determinations were carried out in triplicate culture wells of at least duplicate cell preparations. Total DNA was quantitated by fluorometric analysis by reaction with 3,5-diaminobenzoic acid (67). Total protein, total collagen, calcium, and phosphate were determined by suspending whole cell layers in 0.5 ml 6 N HCl. Hydrolysis was carried out at 110°C for 24 h under vacuum after flushing with N₂. Hydrolysates were then dried under N₂, reconstituted in 0.5 ml of 0.01 N HCl and amino acid analysis was performed on an analyzer (model 121 M; Beckman Instruments, Inc., Palo Alto, CA) using a 200 μl aliquot of the hydrolysate. Collagen determination was based on hydroxyproline content. Mineral was measured on the remaining 300 μl hydrolysate with calcium assayed by atomic absorption spectrophotometry and phosphate by the method of Chen et al. (10). Alkaline phosphatase enzyme activity was determined as previously described (18).

RNA Extraction and Determination of mRNA Levels

Total RNA was extracted from embryonic chondrocytes using modification of the phenol-proteinase K method (17). RNA was electrophoresed on 25-cm agarose gels containing 2.2 M formaldehyde and blotting followed the method of Thomas (65). Random primer labeling and hybridization conditions (15, 17) were carried out using cDNA clones pCg45 (37), pCg54 (38), and pCs2 (68) for determination of pro α2(I), pro α1(I), and pro α1(II) collagen mRNA levels, respectively. Proteoglycan core protein was examined using a 1,200 bp cDNA (52). The α1(IX) mRNA levels were analyzed using an ~600 bp cDNA (20), and type X mRNA levels were determined by hybridization to a 33 base primer synthesized by Operon Technologies (San Pablo, CA). An antisense sequence from bp 290 to 323 was constructed from the reported sequence by Ninomiya et al. (46). Primer was labeled by polynucleotide kinase with γ³²P-ATP (41). Relative mRNA levels were determined by scanning densitometry (Ultrascan II; LKB Instruments, Inc.)

Table I. Culture Conditions and Their Effects on Culture Parameters

	DME	DME + Asc	DME + Asc + βGPO$_4$	BGJ$_b$ + Asc	BGJ$_b$ + Asc + βGPO$_4$
Mineralization*	−	+	+ +	+ + +	+ + + +
DNA content ($\mu g/33$-mm well)	5.8 ± 0.9	6.2 ± 0.5	7.4 ± 0.6	10.6 ± 0.6	13.7 ± 0.7
APase activity (nM p-nitrophenol/ μg DNA/30 min)	15.0 ± 4.0	140.0 ± 30.0	162.0 ± 26.5	148.0 ± 25.0	403.0 ± 46.0
Total protein synthesis[‡] (^3H-leucine/μg DNA)	1.07×10^5	ND[§]	ND	6.8×10^4	4.1×10^4
Percent collagen synthesis[‖]	34 ± 8	48 ± 12	53 ± 14	58 ± 14	62 ± 15
Type X/α1 Type (I + II)	0.24	0.66	0.35	ND	1.1

* Gross extracellular matrix calcification was assessed qualitatively by von Kossa staining.
‡ Total protein synthesis was assessed after 24 h of labeling of day 15 cultures and represents the addition of both medium and cell layer nondialyzable TCA precipitable counts per minute.
‖ Percent collagen synthesis was determined by collagenase treatement of day 15 cultures after 24 h ^3H-proline labeling and samples represent the addition of both medium and cell layer associated nondialyzable counts per minute.
§ Not determined.
¶ Ratio of collagen α1 type X to α1 type (I + II) was obtained from scanning densitometry of Fig. 1.
Data are mean values of triplicate samples of two separate experiments. Data variations are denoted by the total range of observed values.

of underexposed autoradiograms of Northern blots. These measurements were then compared to absolute levels found from slot blot analysis. Absolute quantities were derived using back hybridization, RNA to DNA ratio, and total hybridizable RNA content from slot blots (17, 20).

Histological and Ultrastructural Analysis of Chondrocyte Cultures

Alkaline phosphatase expression was initially examined by histochemical staining. Duplicate cultures were fixed with 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4 for 10 min, rinsed with fresh buffer, and stained for alkaline phosphatase activity using napthol AS-MX phosphate and Fast Red TR salts (Sigma Chemical Co., St. Louis, MO). Matrix calcification was determined by haematoxylin/eosin and von Kossa (phosphate) staining. For ultrastructural analysis, samples (cells and matrix) were fixed and embedded directly in culture plates using either aqueous (glutaraldehyde-OsO$_4$) or anhydrous (ethylene glycol) techniques (33). Specimens were sectioned ($\sim$80 nm) with diamond knives on an Ultrotome III (LKB Instruments, Inc., Gaithersburg, MD) and floated briefly on an aqueous (veronal acetate) or anhydrous (ethylene glycol) trough fluid. Some sections were stained with 8% uranyl acetate in absolute ethanol and with Sato's lead. Ultrastructural features of such sections were examined by conventional transmission microscopy at 60 or 80 kV in an electron microscope (model 300; Philips Electronic Instruments, Inc., Mahwah, NJ). Mineral in unstained sections was studied by selected area electron diffraction at 80 or 100 kV in the same instrument and by electron probe microanalysis at 25 kV in a STEM-modified scanning electron microscope (model JSM-50A; JEOL USA, Peabody, MA) (30, 31).

Results

Culture Conditions Promoting the Development of Chondrocyte Hypertrophy and Extracellular Matrix Mineralization

Comparisons made between two culture media, DME or BGJ$_b$, supplemented with ascorbic acid or ascorbic acid and β-glycerophosphate showed that addition of ascorbic acid was essential to promoting development of the hypertrophic

phenotype (Table I). Independent of the growth medium, ascorbic acid increased alkaline phosphatase activity $\sim$10-fold while stimulating overall collagen synthesis $\sim$20–30%. Assessment of type X collagen expression by analysis of
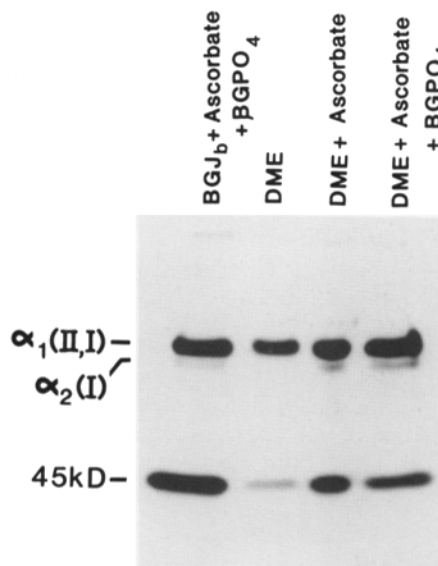

Figure 1. Effect of different culturing conditions on the synthesis of type X collagen (45 kD). Cultures were grown for 15 d under the conditions denoted in the figure. On day 15 cultures were labeled with ^3H-proline; total cell layer and medium proteins were combined and pepsin extracted as described in Materials and Methods. Total pepsin-extracted proteins were resolved in a 5–10% continuous gradient SDS-polyacrylamide gel. Fluorographic exposure was 48 h.
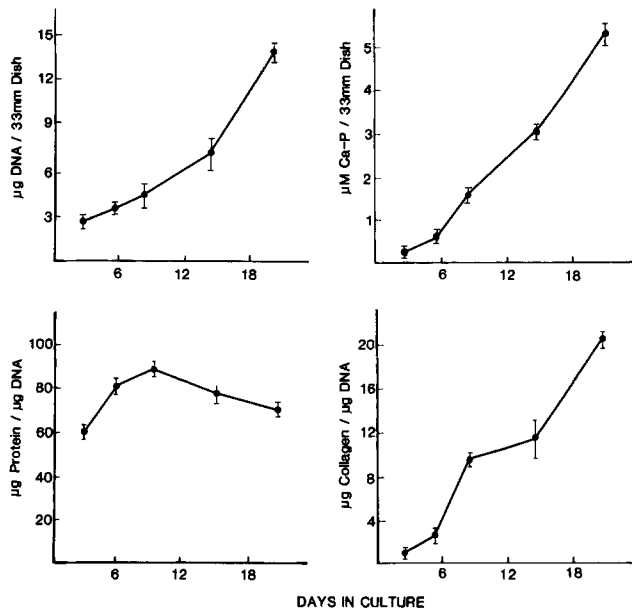
Figure 2. Culture growth, protein, and calcium and phosphorus accumulation with time in culture. Total DNA, protein, collagen, and calcium and phosphorus contents were determined in triplicate 33-mm culture dishes of duplicate experiments over a 21-d period. Calcium to phosphorus molar ratio was ~4:1 throughout the time course. Error bar denotes total range of the experimental variation.
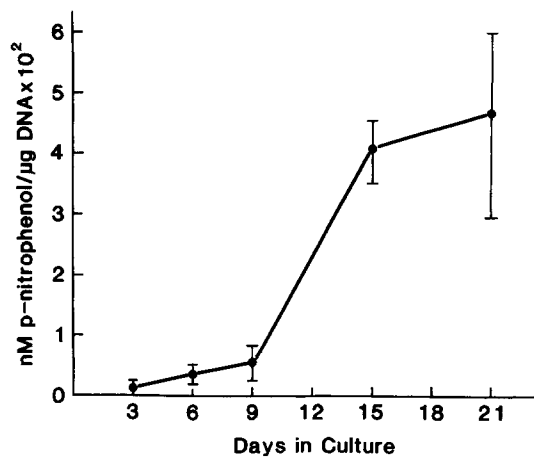

Figure 4. Alkaline phosphatase enzyme activity as a function of time in culture. Triplicate 33-mm culture dishes from three separate experiments were determined for every experimental point. Total nanomolar p-nitrophenol determined in a 30-min assay was used to determine values depicted in the figure. Error bar denotes the total range of the experimental variation.

SDS-polyacrylamide profiles of pepsin-extracted collagens (Fig. 1) and collagen $\alpha 1$ type $X/\alpha 1$ type (I + II) ratio (Table I) demonstrated that ascorbic acid alone increased collagen type X expression. In comparing DME and BGJ_b media, nutrient enriched BGJ_b also provided a 2-2.5-fold increase in cell number as determined from DNA content. Growth of cultures in BGJ_b with ascorbate and β-glycerophosphate had the greatest effect on promoting extracellular matrix calcification. Under these conditions, maximal levels of both alkaline phosphatase enzyme activity and collagen type X expression were also observed (Fig. 1 and Table I).

Chondrocyte Growth during Hypertrophy and Extracellular Matrix Mineralization

Cultures were initially examined for alkaline phosphatase activity and mineralization by histological staining to define

the time period required for establishment of the hypertrophic phenotype and extracellular matrix calcification. Both maximal alkaline phosphatase activity and calcification could be obtained by 21 d of culture (data not shown). Several growth parameters of these cultures over this period are summarized in Fig. 2. The cells remained replicative over the three weeks as demonstrated by their increasing DNA content. During this time, total protein contents of the cultures increased ~30% until day 12 and then showed a slight decrease, while total accumulated collagen increased from 5% of the total protein to ~33%. These results would suggest that collagen was increasing at a much greater rate than the noncollagenous proteins in the matrix. Over the 21-d period, total bulk calcium and phosphorus continuously increased in the cell layers with the first histologically observable calcification occurring by day 9 (data not shown).

The relationship of total protein and collagen accumulation of the chondrocytes to their extracellular matrix synthesis was determined by analyzing total proteoglycan [^{35}S incorporation], total collagen [^{3}H-proline release by collagenase], and total protein synthesis [^{3}H-proline] (Fig. 3).
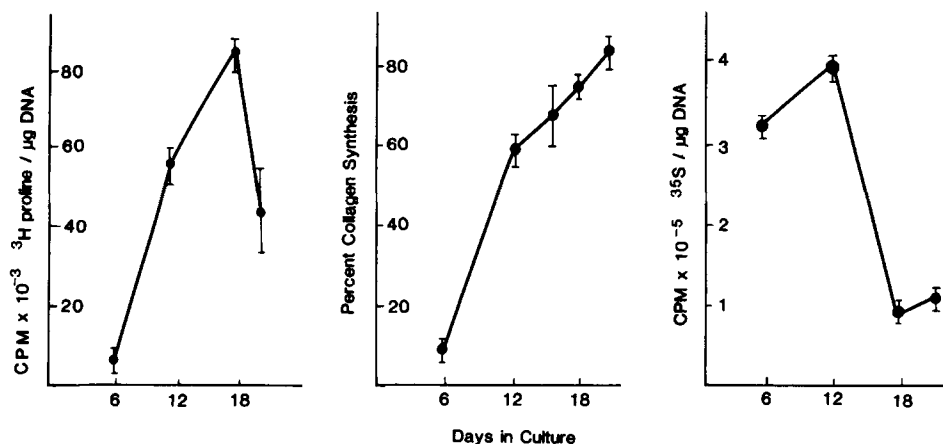

Figure 3. Total protein, collagen, and proteoglycan synthesis over 21-d growth period in culture. Total protein synthesis, percent collagen, and total proteoglycan synthesis were determined from triplicate 33-mm culture dishes of duplicate experiments. Cultures were labeled for 24 h on the days denoted in figure. Error bar denotes total range of the experimental variation.
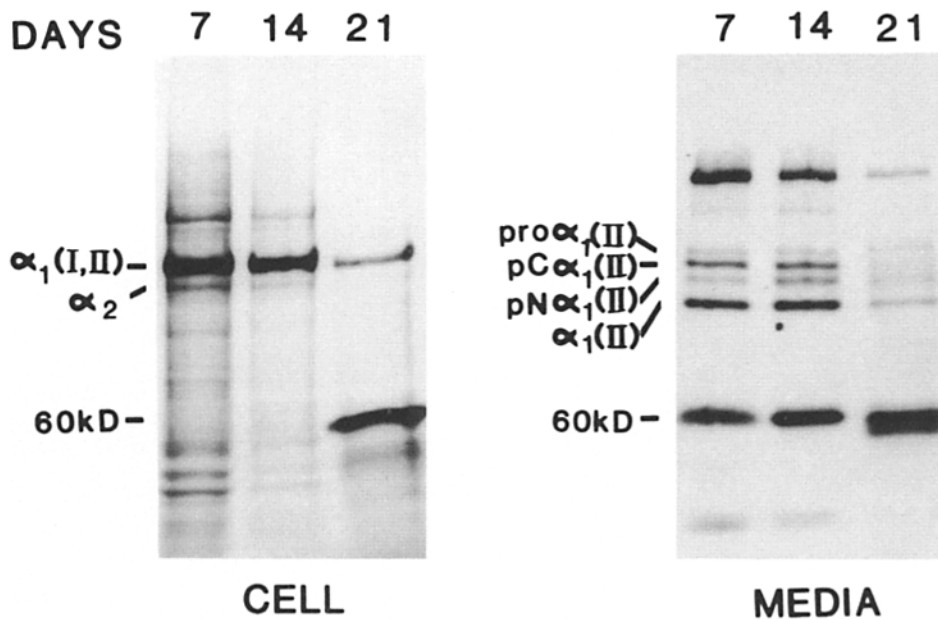
DAYS   7   14   21   7   14   21

α_1(I,II)—
α_2
60kD—

proα_1(II)
pCα_1(II)—
pNα_1(II)
α_1(II)
60kD—

CELL   MEDIA

Figure 5. Collagen synthesis as a function of time in culture. Intact procollagens and collagen α chains for types II and X (60 kD) of either the cell layer or the medium were separately extracted as described in Materials and Methods and separately analyzed on a 5–10% continuous gradient SDS–polyacrylamide gel. The position and intensity of collagen type X and the position and intensity of collagen type II containing the carboxy-terminal extension peptide (*pC*) or the amino-terminal extension peptide (*pN*) are denoted in the figure. Fluorographic exposure was for 3 d.

Total protein synthesis increased until day 15 after which it decreased 50%. During this same period, total collagen synthesis became a greater percentage of the total protein synthesized and increased from ∼10 to >75%. In contrast, total proteoglycan synthesis was maximal at early times (6 and 12 d) but thereafter decreased rapidly approximately three-fold and remained diminished to the day 21 end point. A comparison of these data to those of Fig. 2 indicates that the changes in the percentage distribution of accumulated colla-

gen were primarily caused by an increase in total collagen synthesis. Concurrent with analyses of the synthesis and accumulation of extracellular matrix markers in culture, alkaline phosphatase activity was measured as an indicator of the development of chondrocyte hypertrophy (Fig. 4). During the first 9 d of cellular growth in culture only a slight increase in this enzyme occurred. Thereafter an ∼10-fold increase in activity was observed to day 15 after which the levels were constant.

α_1(II) α_2(I)   PG

27S—

α_1(I) α_1(X) α_1(IX)

27S—
18S—

DAYS   6   12   18   21   6   12   18   21   6   12   18   21
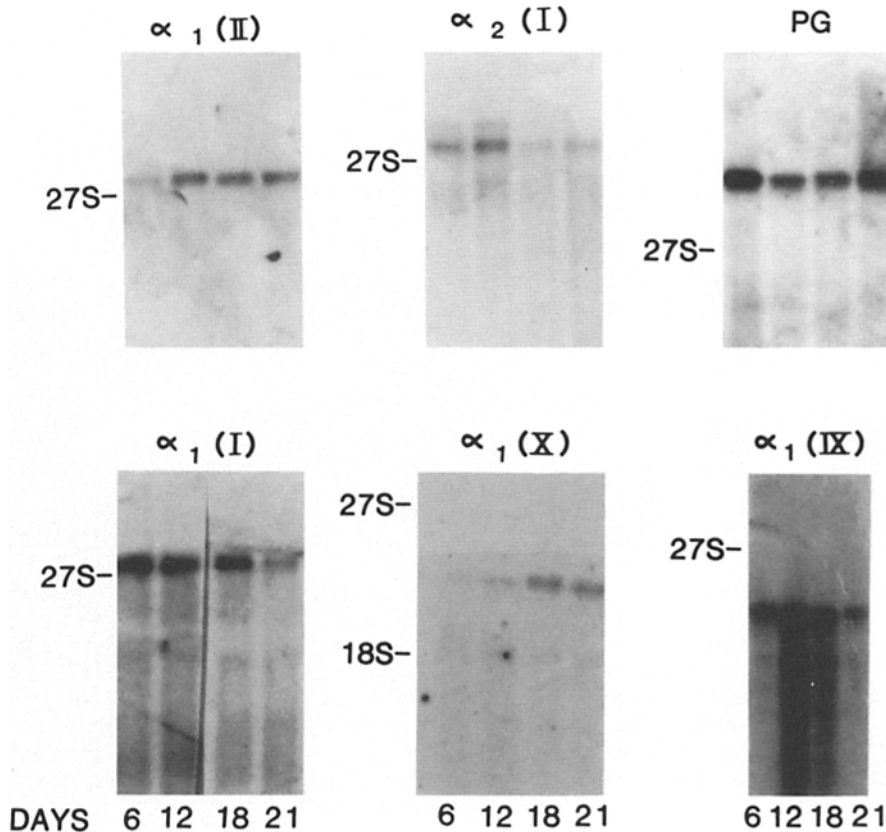
Figure 6. Northern blot analysis of extracellular matrix gene expression over 21 d of growth in culture. Four micrograms of total RNA were applied to each lane. The mRNA species analyzed and the day on which mRNA was harvested are denoted on the figure. Autoradiographic exposure with an intensifying screen varied from 24 h to 96 h depending on the probe used.
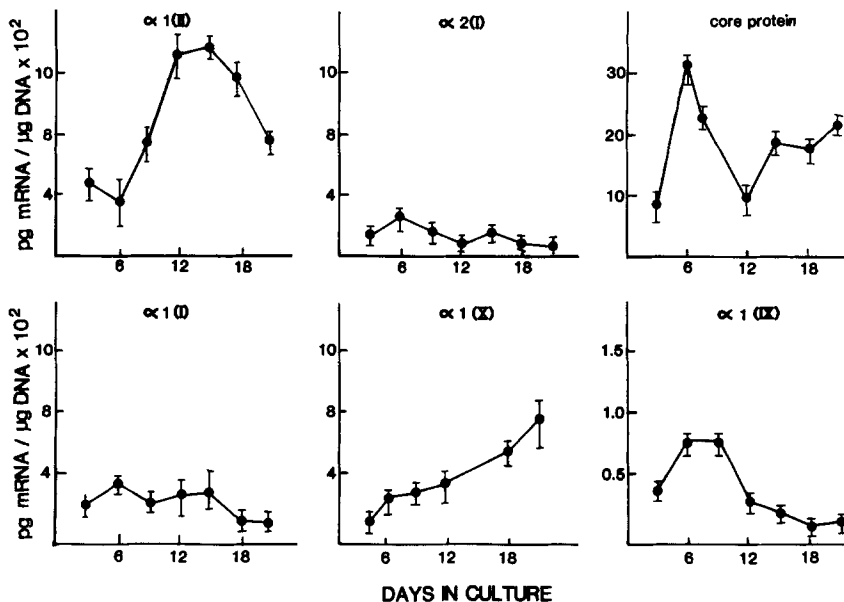
Figure 7. Quantitation of extracellular matrix gene expression as a function of culture time. Slot blot analysis and mRNA quantitation were carried out as described in Materials and Methods. Each measurement is an average of duplicate measurements of three concentrations from two separate experiments. The error bar denotes the total range of the experimental variation.

Gene Expression of Extracellular Matrix Components

During chondrocyte hypertrophy and the development of culture mineralization over the 21-d course of measurements, gene expression of certain extracellular matrix proteins was examined. Protein synthesis of the various collagen types appearing in culture was determined by SDS-PAGE of proline labeled samples (Fig. 5). The identification of procollagen and collagen species was based on their mobility and was consistent with that made by previous immunological and

biochemical determinations (20, 22, 55). Collagen type II synthesis was observed throughout the 21-d culture period. In the cell layer, type II was found predominantly as processed α chains although at early times unprocessed and partially processed procollagen type II could be detected. In contrast, the culture medium contained both pro, carboxy-terminal extension peptide, and amino-terminal extension peptide forms of type II molecules at all times. In both cell layer and medium, there was a progressive decrease in the relative ratio of type II to type X collagen (band at 60 kD).
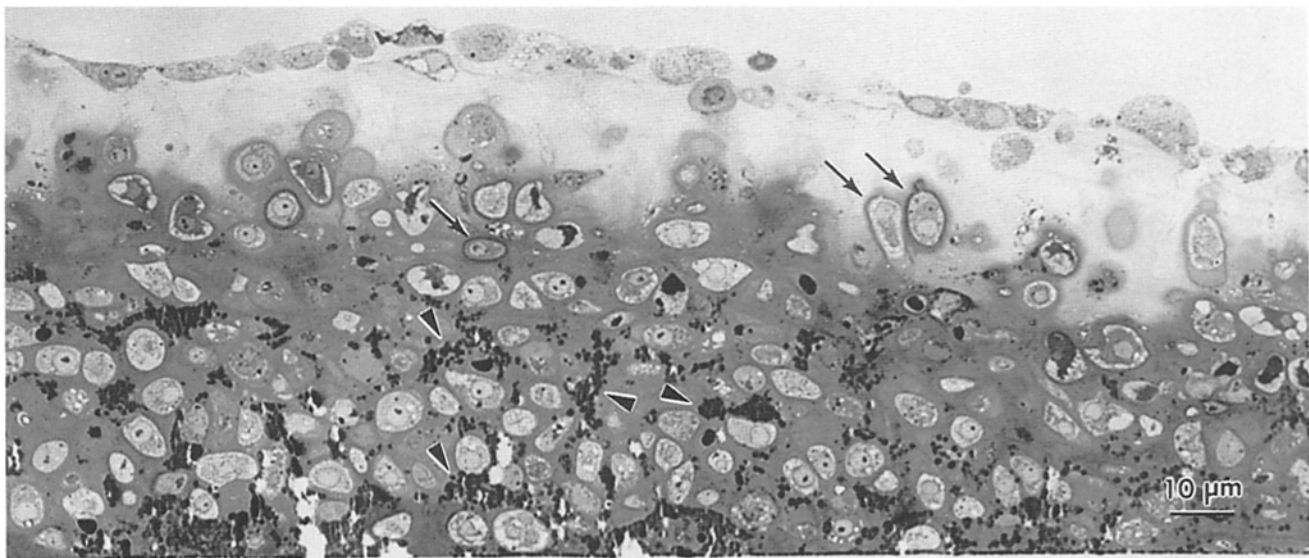

Figure 8. A light micrograph of an 18-d chondrocyte culture, sectioned through its full thickness (courtesy of Dr. M. D. McKee, presently at Department of Stomatology, Faculty of Dental Medicine, University of Montreal, Montreal, Canada). The sharp line along the bottom of the micrograph marks the interface between the chondrocyte culture and the Petri dish, now removed. The culture appears composed of many cells filling a wide portion of the field. There is an increased concentration of cells below the upper quarter of the culture; these cells possess a generally rounded shape within a lacuna and resemble a growth plate chondrocyte phenotype. The extracellular matrix stains heavily (toluidine blue) in the same zone of cells while it is relatively unstained in the upper quarter of the culture, capped by a palisade of somewhat flattened cells which are not immediately similar in appearance to those below them. There is a pericellular staining of some of the lower cells (*arrows*), and in intermediate and deeper portions of the culture there are numerous sites of dense mineral deposition (*arrowheads*). All samples (Figs. 8–12) have been fixed in glutaraldehyde and osmium tetroxide and stained with uranyl acetate and lead citrate.
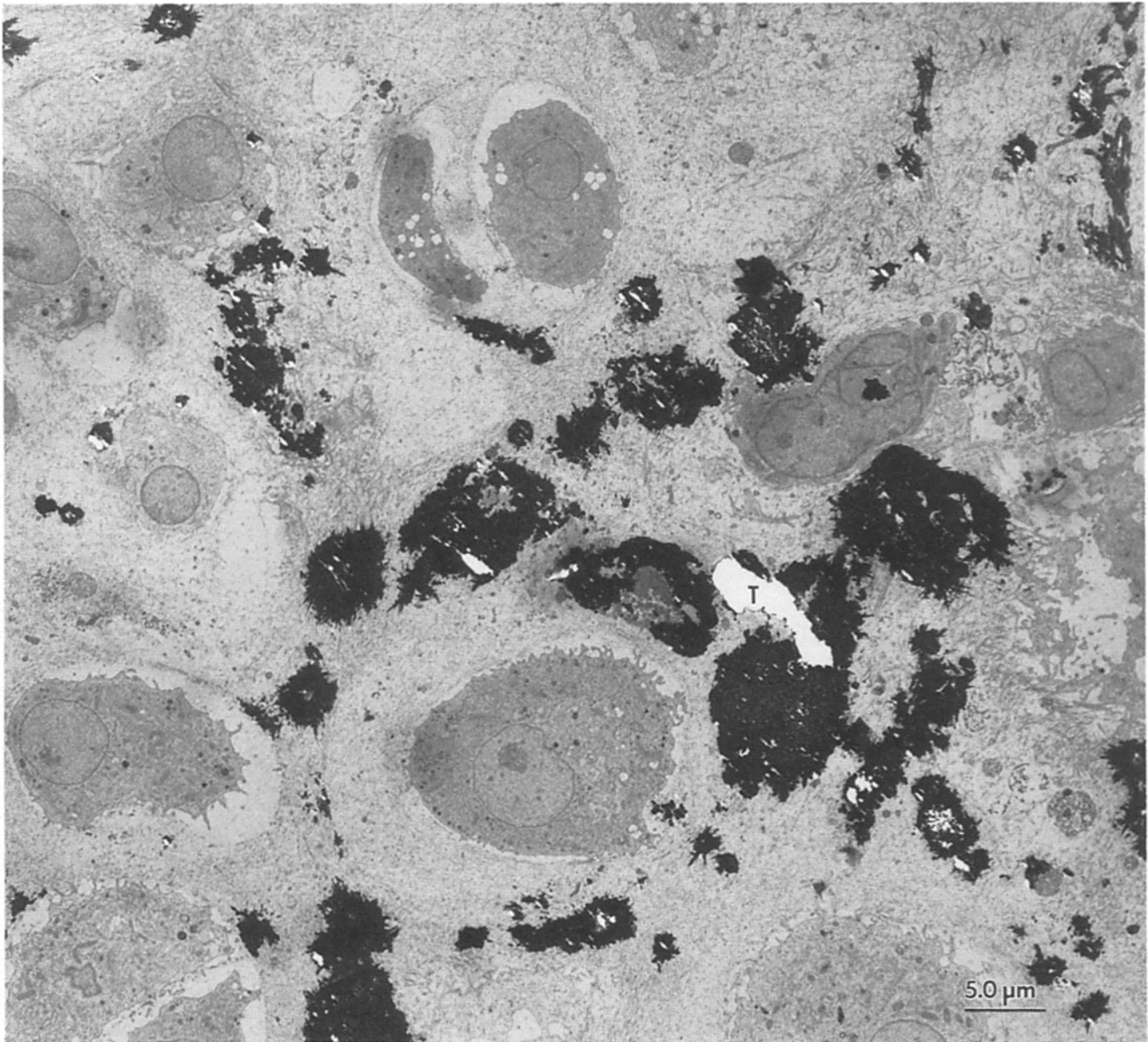
Figure 9. A relatively low magnification electron micrograph of a representative area from a 17-d calcified chondrocyte culture. The numerous cells have well developed nuclei with nucleoli and extensive cytoplasmic organelles. The cells are located in lacuni and surrounded by an unmineralized pericellular space. The extracellular matrix beyond such pericellular zones is distinguished by prominent collagen fibrils of narrow diameter and numerous extracellular vesicles. Mineralization is noted by highly electron dense deposits which are disposed about the pericellular lacuni and overlay the collagen fibrils. Cell sizes, shapes, and disposition as well as extracellular characteristics appear to resemble those observed in vertebrate growth plates in vivo. (*T*) A tear in the section.

An absence was noted in the accumulation of newly synthesized collagen type X in the culture cell layers during the first two time points examined, but at day 21 type X became the major protein synthesized by the chondrocytes, accumulated in the cell layer, and secreted into the medium. In accord with earlier work (20), showing adherent vertebral chondrocytes producing low levels of type I collagen, evidence of α2(I) chains was also detected in the present cultures (Fig. 5; cell layer, day 7), but the level of the type I synthesis appeared to diminish with time. Unlike sternal chondrocyte cultures in which collagen α1(IX) and α3(IX) chains were clearly detected (20), neither of these chains could be detected in the proline-labeled samples of these vertebral cultures, a result probably reflecting their very low levels of synthesis.

To determine the level of control for collagen and proteoglycan synthesis, steady state mRNA was measured for various collagen genes and the large proteoglycan core protein gene (Fig. 6 and 7). Message was examined by Northern blot analysis (Fig. 6) to assess the integrity of the isolated mRNAs, their size, and their relative quantity. Two molecular mass species observed for α1(IX) mRNA appeared throughout the time course. These hybridization profiles were consistent with those previously reported and are attributable to either different exon usage at the 5′ end of this gene or alternate polyadenylation (47, 63). Scanning densitometry of all of the Northern blots accounting for the multiple mRNA species for both α2(I) and α1(IX) demonstrated comparable relative differences of mRNA quantities over the 21-d time course as that determined from slot blot analysis.
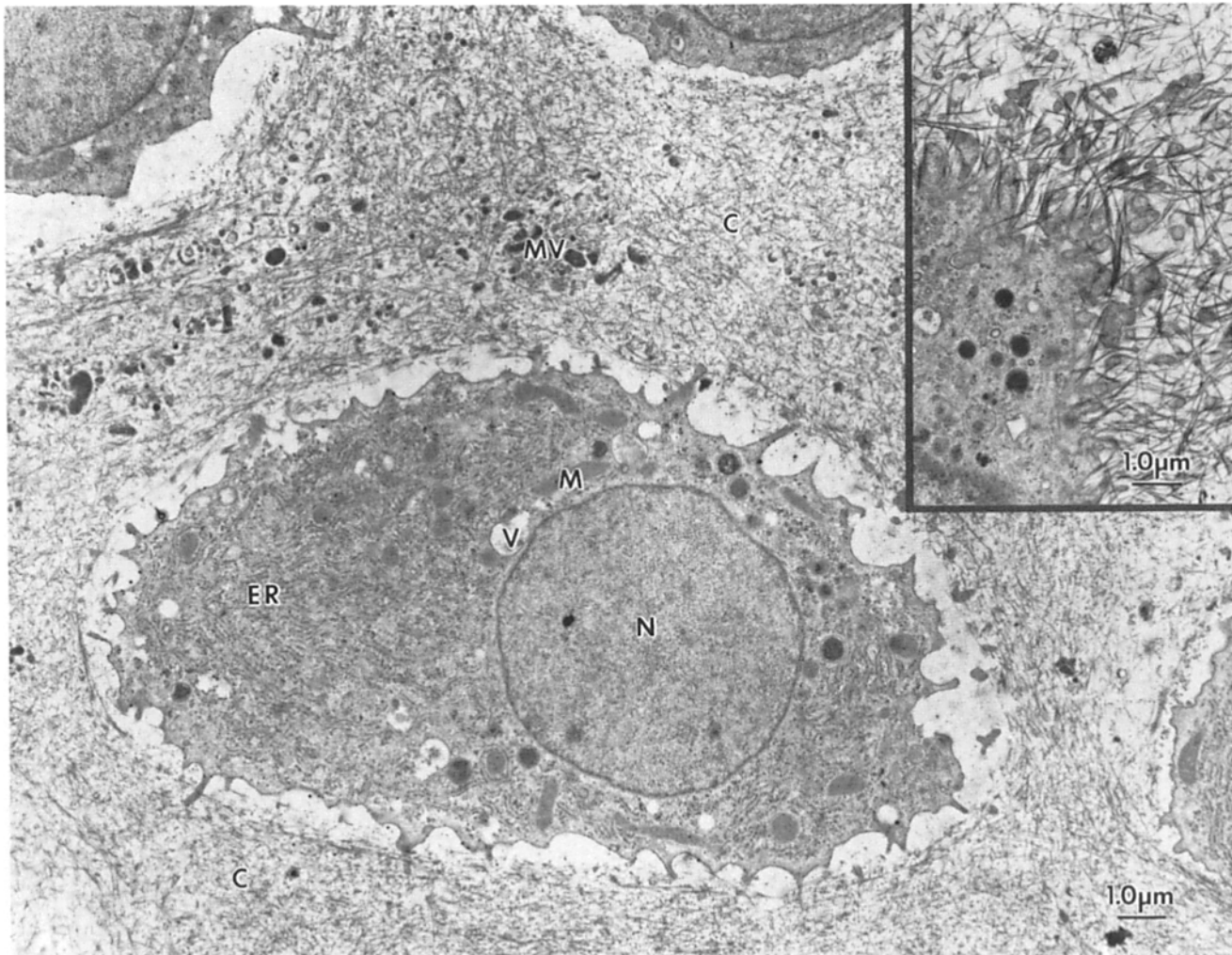
Figure 10. A cultured chondrocyte, typical of those found after 17 d. The cell resides in a lacuna similar to chondrocytes observed in vivo. A prominent nucleus (*N*) is present, as well as mitochondria (*M*), endoplasmic reticulum (*ER*), vacuoles (*V*), and other organelles indicative of a cell involved in active synthesis and secretion. Numerous cellular processes appear along the chondrocyte membrane. The extracellular matrix is marked by vesicles (*MV*) and by collagen (*C*) of a narrow microscopic diameter and inconspicuous periodicity, both features resembling that of type II fibrils found in vivo. (*Inset*) A portion of a cultured chondrocyte illustrating a region along the cell envelope in which extracellular vesicles appear to originate by infolding and budding of the chondrocyte membrane. Many collagen fibrils also lie adjacent to the chondrocyte membrane and in the same vicinity of the budding vesicles.

Absolute RNA amounts per cell, obtained by slot blot analysis (17, 20) and expressed as pg/μg DNA, are shown in Fig. 7. Measurements were made at 3-d intervals to obtain a more defined analysis of the mRNA levels with time. It was found that collagen type II and proteoglycan mRNAs were the most prominent species throughout the time course. The message for collagen α1(IX) was lowest relative to DNA. Collagen α1(I) and α2(I) messages were also present throughout the time course; but, relative to type II mRNA, they were greatest during the initial week of culture development and thereafter decreased. While type I mRNAs fell, collagen type X mRNA was increased throughout the culture time course, and by 21 d it approached a level equal to that of type II. Expression of the proteoglycan core protein and collagen α1(IX) mRNA appeared to parallel each other at maximal levels during the first 6–8 d of culture and then declined to levels below their initial values. These data show that expression of protein synthesis quantitatively follows the respective mRNA species.

Culture Ultrastructure: Chondrocyte Morphology, Matrix Formation, and Mineralization

The biochemical and molecular biological data described above suggest that the cultures have established an extensive extracellular matrix but do not address whether this matrix is ultrastructurally similar to that found in vivo. Correlative to the chemical studies, then, ultrastructural work also followed the time course development of these chondrocyte cultures to establish cell morphology, matrix production, and matrix–mineral interaction. By day 8–10, chondrocyte cultures have elaborated an extensive extracellular matrix as suggested by measurements of collagen synthesis, collagen accumulation, and alkaline phosphatase activity. Examination
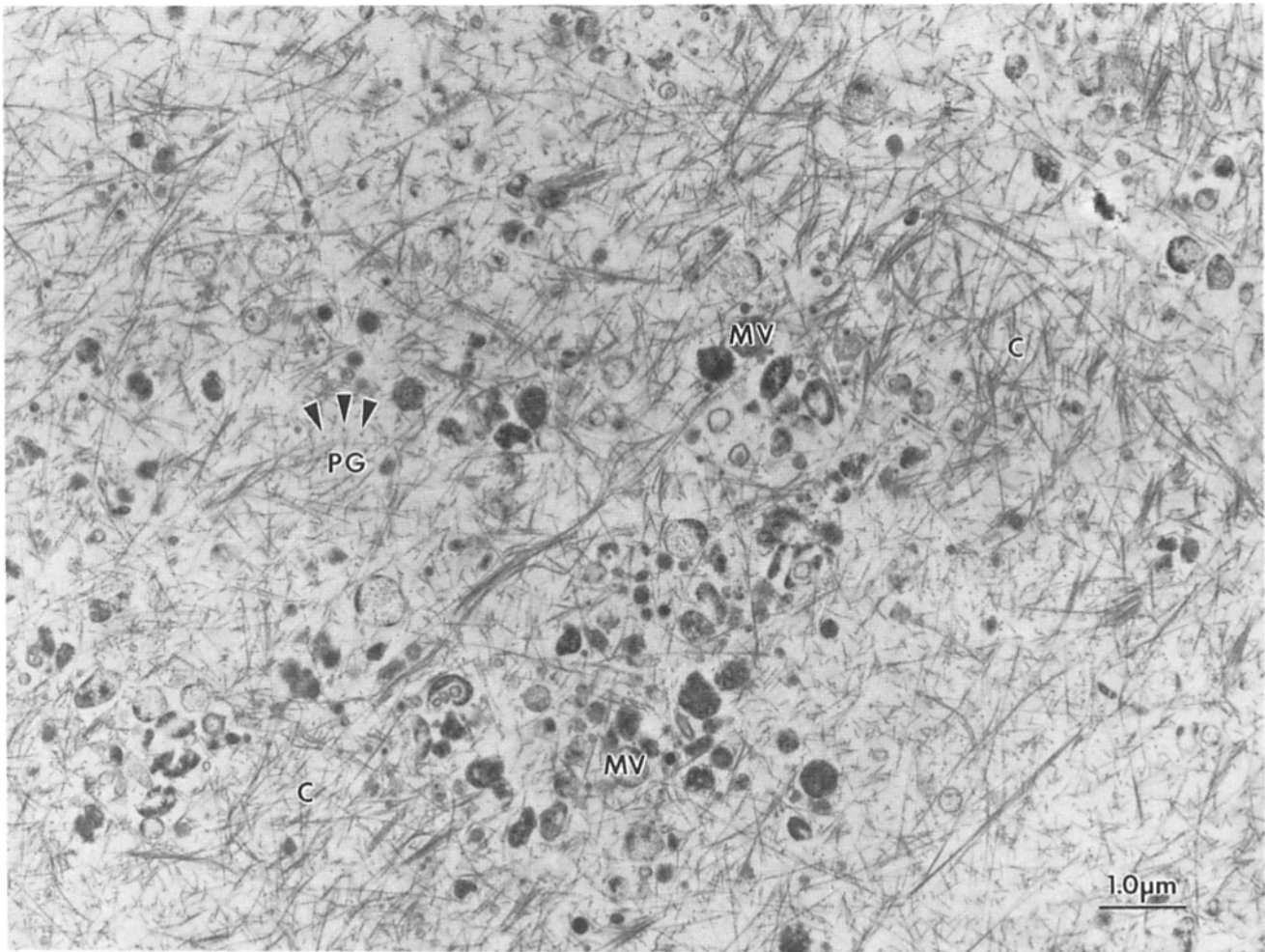
Figure 11. An enlargement of the extracellular matrix surrounding chondrocytes in culture. This region contains numerous vesicular organelles (*MV*), some having electron-dense material and others relatively electron lucent. The region exemplifies the wide diversity of structures resembling matrix vesicles normally observed in vivo. The extracellular milieu is also marked by thin type II collagen fibrils (*C*) and small electron dense condensations of putative proteoglycans (*PG*).

of cultures over this period by EM revealed the presence of numerous cells and a matrix containing collagen, extracellular vesicles, and other organic structures. With time, the matrix became progressively mineralized. As a representative example, the appearance of 17–18-d chondrocyte culture is shown in Figs. 8 and 9. The large numbers of cells are well formed, have prominent nuclei with nucleoli, and maintain a complement of various cytoplasmic organelles. These include mitochondria, cisternae and endoplasmic reticulum, vacuoles, and other structures indicative of cells active in protein synthesis and secretion. Cellular processes appear to give rise to extracellular vesicles (Fig. 10, *inset*) which ultimately contribute, together with many type II collagen fibrils and electron-dense condensations of putative proteoglycans, to the overall make-up of the extracellular matrices of these cultures (Fig. 11). Cells are located in lacunae (Figs. 8–10) about which a pericellular basket of collagen fibrils is observed. Peripheral to this territorial matrix, electron-dense mineral deposits are observed (Figs. 8, 9, and 12). The cell shapes and composition, evidence of lacunae, and extracellular characteristics of these cultures are similar to those of proliferating and maturing chondrocytes in a typical cartilage growth plate in vivo (54).

Generally the dense material in chondrocyte cultures physically obscures the extracellular matrix components with which it apparently associates (Figs. 9 and 12). In instances at higher magnifications, however, the deposits can be found extending closely along individual collagen fibrils (Fig. 12, *inset A*). There is no clear evidence for an association between the mineral and extracellular vesicles. As determined from cultures prepared by anhydrous means, the mineral contains calcium and phosphorus with Ca/P molar ratios ranging from ∼1.0–∼1.3 (Fig. 12, *inset B*). Selected area electron diffraction generates only a single broad halo of d ∼2.8 Å, the spacing corresponding to a group of principal reflections for hydroxyapatite (Fig. 12, *inset C*). In this case, the single halo and its indistinct resolution indicate the nature of the mineral in the culture is a very poorly crystalline hydroxyapatite.

Discussion

The initial focus of the experiments presented here was to define growth conditions in vitro which could promote cell hypertrophy and the development of a mineralized extracellular matrix in a culture system of embryonic chick vertebral
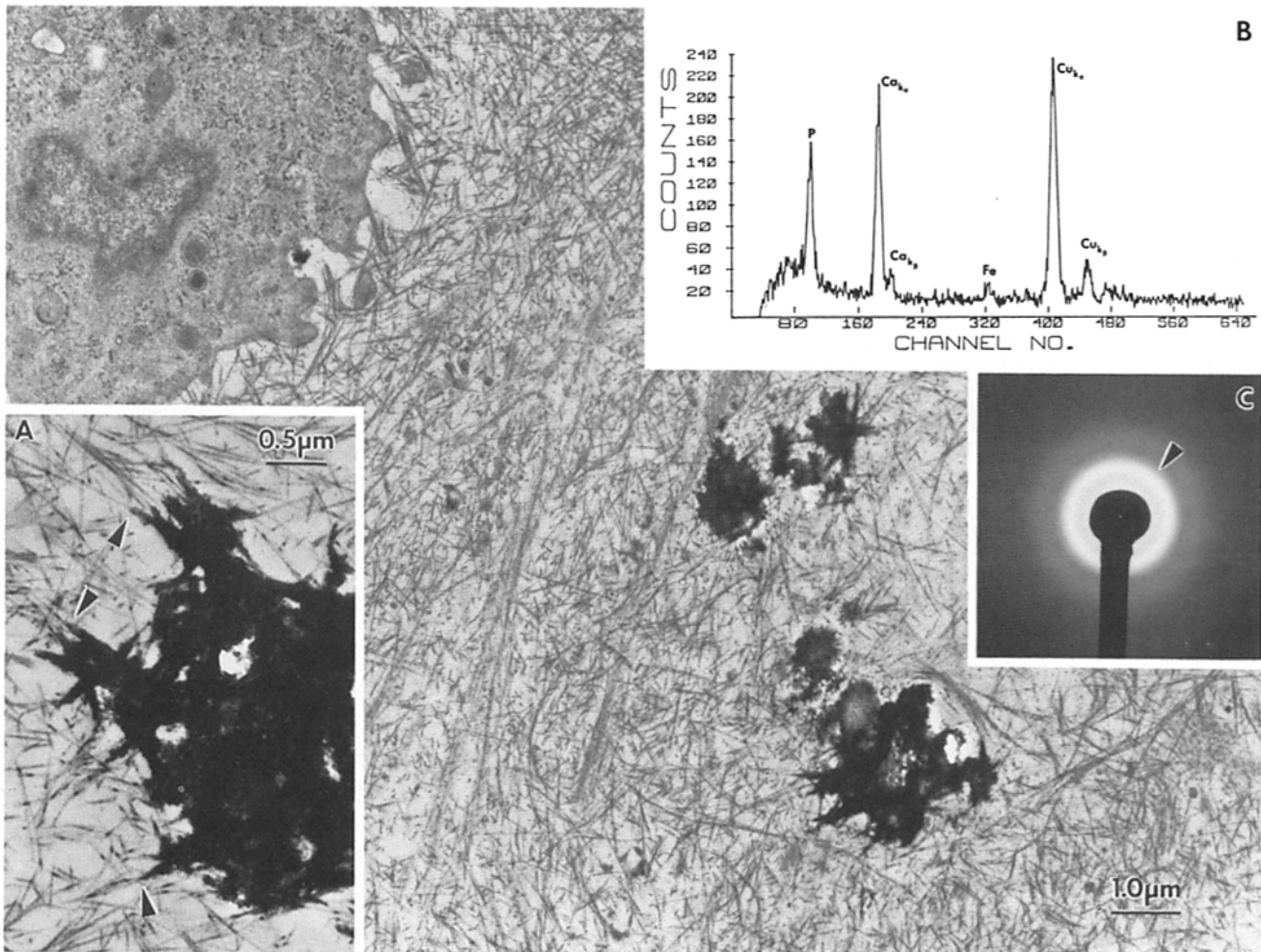
Figure 12. Mineral deposition within the extracellular culture matrix is characterized by relatively large punctuate dense clusters which are isolated from one another. The deposits obscure any organelles within or beneath them, making it difficult to identify specifically any possible association with matrix constituents. On the other hand, in some instances (*Inset A*) electron density at the periphery of the deposits appears to follow individual collagen fibrils located near or within the dense foci (*arrowheads*). An electron probe spectrum (*Inset B*) of a portion of a single density shows it consists of calcium and phosphorus (Ca/P ~1.1 here). Selected area electron diffraction (*Inset C*) yields a pattern consisting of a single, broad unresolved reflection (*arrowhead*) indicative of a poorly crystalline hydroxyapatite as the mineral phase of the cultures. Microanalysis and electron diffraction were performed using unstained sections of ethylene glycol–treated culture samples.

chondrocytes. From these investigations, the supplementation of the culture medium (either DME or BGJ$_b$) with ascorbic acid was the single critical factor leading to progressive culture growth and expression of the hypertrophic phenotype (assessed by elevated levels of alkaline phosphatase enzyme activity and collagen type X). Similar effects of ascorbate supplementation were observed with cephalic sternal chondrocytes (36) and tibial chondrocytes (25). While the mechanism by which ascorbic acid promotes chondrocyte differentiation is unclear, it is well documented to be essential in maintaining normal extracellular matrices and in promoting maximal collagen synthesis in connective tissues (11, 12, 40, 53, 57, 58, 59). The role of ascorbate in proline and lysine hydroxylation has been extensively demonstrated (48), and its absence during collagen synthesis in a wide variety of cultured cells has been shown to result in diminished rates of collagen synthesis and high rates of collagen turnover (45). The data presented here clearly show that ascorbic acid increases both total collagen synthesis

(from 30–100% depending on the nutrient medium) and total collagen deposition. Both ultrastructural and biochemical analyses further demonstrate that over the 21-d period of culture measurements, the chondrocytes elaborate an extensive extracellular matrix which retains features similar to those found in vivo. These results would suggest that effects mediated by ascorbate on the chondrocyte phenotype principally reflect its actions on the synthesis and deposition of a normal extracellular matrix.

It has been implied in other work that, once established, the extracellular matrix may exert effects on the chondrocytes. The influence may be twofold: (*a*) to maintain a normal rounded chondrocyte morphology, and (*b*) to establish normal cell–extracellular matrix interactions. Such suggestions are supported by work in which chondrocyte differentiation toward a hypertrophic phenotype can be demonstrated when cells are grown in association with collagen or agarose gels (4, 9, 23, 62). It was found that maintenance of a rounded cell configuration was necessary, but insufficient

alone, to promote hypertrophic chondrocyte differentiation since the growth of chick limb bud cells in cytochalasin D was permissive to chondrogenesis but not for further chondrocyte development to a hypertrophic state. In the present studies and those of others (4, 9, 36, 62), cellular differentiation was found to be time dependent, a result suggesting that the cells must provide or condition their growth environment to develop progressively. Such conditioning may represent the establishment of the extracellular matrix as demonstrated ultrastructurally here and in the studies of Bruckner et al. (4) and Tacchetti et al. (64). Provision, then, of specific cell–extracellular matrix interactions may provide the requisite signal(s) or be permissive by some means for further chondrocyte differentiation. It is interesting in this context to note that osteoblast differentiation occurs only when cells are grown in the presence of ascorbic acid (1, 18) and that certain proteins specific to the differentiated osteoblasts are expressed only after a collagenous matrix is established (19, 21).

While ascorbate is indicated to be of major significance in the development of the chondrocyte hypertrophic phenotype, maximal alkaline phosphatase and collagen type X expression and extracellular matrix mineralization were obtained only when cultures were grown in a nutrient-enriched medium supplemented with β-glycerophosphate. With respect to nutrition in general, previous results (61) showed that F_{12}, a more nutrient-rich medium which has higher amino acid and glucose contents than DME, increased cultured chondrocyte expression of collagen type X. The role of nutrition and oxidative metabolism as it relates to the oxygen tension of cartilage may be of considerable importance in vivo since the tissue undergoes progressive vascularization during endochondral development (16, 26, 42, 60). With regard to β-glycerophosphate supplementation, this additive has been shown by numerous researchers to stimulate extracellular matrix mineralization in cultured osteoblasts (2, 13, 18). It also appears to play a similar role in chondrocyte cultures, and its presence may suggest that the regulation of phosphate metabolism is important in mineralization processes in general.

Besides defining conditions of culture growth and promotion of cell hypertrophy, experiments were also concerned with extracellular matrix development, specifically by determining temporal relationships between collagen and proteoglycan gene expression and synthesis and matrix mineralization and by characterizing ultrastructural features of the chondrocytes, the matrix, and the mineral deposited there. Comparison of total protein/DNA ratios to the percentage of collagen accumulated in the cultures suggests that, as the extracellular matrix develops and the cells express the hypertrophic phenotype, proteoglycan is diminished in the matrix. Corroborative evidence for this conclusion is the inverse relationship found between total proteoglycan and collagen synthesis as a function of culture time. Decreased proteoglycan content and reduced proteoglycan synthesis in zones of cartilage hypertrophy and calcification have previously been reported (5, 6, 14, 50, 51). Analysis of the steady state mRNA levels for collagen types I, II, IX, and X and the large proteoglycan core protein also indicates that the synthetic levels of these matrix constituents are controlled by pre- rather than posttranslational events and may be part of the phenotypic changes of cellular hypertrophy. It is interesting to note the presence of low levels of type I collagen mRNA

and protein synthesis which decreased throughout the 21-d time course. These results are consistent with those obtained in vivo during limb bud development (66) and those seen in vitro with tibial chondrocytes undergoing differentiation and hypertrophy in which type I collagen expression decreased (9).

While such data suggest that matrix remodeling in the hypertrophic zone is mediated by proteolytic turnover of the proteoglycan (14), results presented here would indicate that matrix composition is also controlled and reflected by quantitative changes in the ratio of matrix components that are synthesized by the chondrocytes. Other data may imply this same possibility: morphometric analysis of growth cartilage cell and matrix volumes demonstrated an increased matrix volume in the hypertrophic zones (28). Moreover, biochemical studies have shown quantitative losses of proteoglycans in cartilage hypertrophic zones that are not associated with qualitative changes in either their size, aggregation with hyaluronic acid, or immunoreactivity (50, 51). The shift in matrix components noted here was manifested in changes in distribution and steady state mRNA levels from high collagen type II quantities to predominantly collagen type X, and in the tightly coupled appearance between high levels of collagen type X and alkaline phosphatase enzyme activity. Similar observations have been made (9, 25, 36), and they appear to correspond to the changes in collagen types II, X, and alkaline phosphatase observed in growth cartilage in situ in the spatial progression from nonmineralizing to mineralizing regions of this tissue. The present study also demonstrates that type X becomes the major collagen species in the cultures with greater than 50% of newly synthesized material remaining associated with the cell layer. In other experiments (Gerstenfeld, L.C., unpublished results) in which day 21 cultures are pepsin-extracted, stained gels showed an equal ratio of accumulated type II/type X collagen. These data provide evidence supporting the suggestion that type X collagen is cell-layer associated during mineralization of chondrocyte cultures (36).

Several interesting biochemical and ultrastructural correlations may be made by comparing the mineralization events observed in cultured osteoblasts (18, 19, 21) and chondrocytes. In both systems, high levels of alkaline phosphatase are induced at the onset of mineralization. With osteoblasts there is a transient elevation (18), whereas with chondrocytes the enzyme levels are ten times higher and remain elevated well after induction. As mentioned previously, ascorbate is an essential prerequisite for mineralization and β-glycerophosphate greatly enhances the rate of mineralization for both osteoblasts and chondrocytes. On the other hand, with both factors, mineralization of chondrocyte cultures occurs 4–6 d earlier than that seen in osteoblast cultures. The two culture systems also contain multilayers of cells which become incorporated into their respective accumulated matrices.

Ultrastructurally, chondrocyte and osteoblast cultures differ and retain many of their distinctive features present in vivo. For example, the thickness of chondrocyte cultures is generally severalfold that of their osteoblast counterparts, even though the numbers of cell layers and protein/DNA quantities are comparable. This result would suggest that there is extensive hydration of the chondrocyte matrix attributable to retention of water mediated by large quantities

of proteoglycan as found in normal cartilage tissue. In addition, while osteoblast cultures are comprised of a supramolecular assembly of large collagen type I fibrils (19), only uniformly thin (∼20 nm) type II fibrils with no definitive higher ordered structuring were seen. These observations are also consistent with those derived from normal specimens. Likewise, extracellular vesicles were commonly observed in the chondrocyte cultures; few vesicles were seen in the osteoblast system.

Finally, and to restate an important result, these cultured chondrocytes and osteoblasts (19) mineralize and do so reproducibly, unlike ostensibly similar systems in other laboratories. In the chondrocyte cultures here, mineral is not detected in association with extracellular vesicles and, unlike that in osteoblast cultures (19), small mineral foci were not dispersed throughout the matrix but were deposited at the periphery of the cells in a manner resembling the interterritorial matrix distribution of mineral described by Schenk et al. (54). While mineral distribution was dissimilar in this chondrocyte system and osteoblast cultures (19), the principal matrix–mineral interaction in each was between collagen and the inorganic species. The precise mechanism of mineralization related to collagen has yet to be detailed and may be different with respect to collagen types I and II/X and to osteogenic and chondrogenic systems (32). Nonetheless, the nature of the mineral, itself, in the two cultures considered here is a poorly crystalline hydroxyapatite whose Ca/P molar ratios vary typically in the range ∼1.1–1.5, considerably lower than that for stoichiometric hydroxyapatite (1.67) but consistent with measurements obtained in situ from normally mineralizing bone and growth cartilage (30, 31, 33).

In summary, the data presented here are interpreted to support the concept that elaboration of the extracellular matrix is a key factor in the promotion of chondrocyte differentiation toward a hypertrophic phenotype. Matrix compositional changes are associated with the progressive stages of the differentiation process in which type II and X collagens are initially expressed but the former is replaced by the latter with time. Accompanying increased type X synthesis is a decrease in proteoglycan gene expression and synthesis. Mineral accumulated by these cultures is an apatite whose distribution and interaction with matrix constituents resemble that determined for normally calcifying cartilage. This mineralizing chondrocyte culture system should be especially useful for describing further the basic chemical, physical, and biological events of cartilage growth and development.

References

1. Aronow, M. A., L. C. Gerstenfeld, T. A. Owen, M. S. Tassinari, G. S. Stein, and J. B. Lian. 1990. Factors that promote progressive development of the osteoblast phenotype in cultured fetal calvaria cells. *J. Cell. Physiol.* 142:213–221.
2. Bellows, C. G., J. E. Aubin, J. N. M. Heersche, and M. E. Antosz. 1986. Mineralized bone nodules formed *in vitro* from enzymatically isolated rat calvaria cell populations. *Calcif. Tissue Int.* 38:143–154.
3. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.
4. Bruckner, P., I. Horler, M. Mendler, Y. Houze, K. H. Winterhalter, S. G. Eich-Bender, and M. Spycher. 1989. Induction and prevention of chondrocyte hypertrophy in culture. *J. Cell Biol.* 109:2537–2545.
5. Buckwalter, J. A. 1983. Proteoglycan structure in calcifying cartilage. *Clin. Orthop. Relat. Res.* 172:207–232.
6. Campo, R. D., and J. E. Romano. 1986. Changes in cartilage proteoglycans associated with calcification. *Calcif. Tissue Int.* 39:175–184.
7. Capasso, O., G. Tajana, and R. Cancedda. 1984. Location of 64K collagen producer chondrocytes in developing chick embryo tibiae. *Mol. Cell. Biol.* 4:1163–1168.
8. Caplan, A., and D. G. Pechak. 1987. The cellular and molecular embryology of bone formation. *J. Bone Miner. Res.* 5:117–183.
9. Castagnola, P., B. Dozin, G. Moro, and R. Cancedda. 1988. Changes in the expression of cellular genes show two steps in chondrocyte differentiation in vitro. *J. Cell Biol.* 106:461–467.
10. Chen, P. S., T. J. Toribara, and H. W. Warner. 1956. Microdetermination of phosphorous. *Anal. Chem.* 28:1756–1758.
11. Daniel, J. C., B. U. Pauli, and K. Kuettner. 1984. Synthesis of cartilage matrix by mammalian chondrocytes in vitro. III. Effects of ascorbate. *J. Cell Biol.* 99:1960–1969.
12. Dunn, D., 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.
13. Ecarot-Charrier, B., F. A. Glorieux, M. van der Rest, and G. Pereira. 1983. Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. *J. Cell Biol.* 96:639–643.
14. Ehrlich, M. G., A. L. Armstrong, R. G. Neuman, M. W. Davis, and H. J. Mankin. 1982. Patterns of proteoglycan degradation of a neutral protease from human growth plate epiphyseal cartilage. *J. Bone. Jt. Surg.* 64A:1350–1354.
15. Finer, M. H., L. C. Gerstenfeld, D. Young, P. Doty, and H. Boedtker. 1985. Collagen expression in embryonic chicken chondrocytes treated with phorbol myristate acetate. *Mol. Cell Biol.* 5:1415–1424.
16. Floyd, E. W., D. J. Zaleske, A. L. Schiller, C. Trahan, and H. J. Mankin. 1987. Vascular events associated with the appearance of the secondary center of ossification in the murine distal femoral epiphysis. *J. Bone Jt. Surg. Am. Vol.* 69A:185–190.
17. Gerstenfeld, L. C., D. R. Crawford, H. Boedtker, and P. Doty. 1984. Expression of type I and type III collagen gene during differentiation of embryonic chicken myoblasts in culture. *Mol. Cell. Biol.* 4:1483–1492.
18. Gerstenfeld, L. C., S. D. Chipman, J. Glowacki, and J. Lian. 1987. Expression of differentiated function by mineralizing cultures of chicken osteoblasts. *Dev. Biol.* 122:49–60.
19. Gerstenfeld, L. C., S. D. Chipman, C. Kelly, K. J. Hodgens, D. D. Lee, and W. J. Landis. 1988. Collagen expression, ultrastructural assembly and mineralization in cultures of chicken embryo osteoblasts. *J. Cell Biol.* 106:979–989.
20. Gerstenfeld, L. C., M. Finer, and H. Boedtker. 1989. Quantitative analysis of collagen expression in embryonic chick chondrocytes having different development rates. *J. Biol. Chem.* 264:5112–5120.
21. Gerstenfeld, L. C., Y. Gotoh, M. D. McKee, A. Nanci, W. J. Landis, and M. J. Glimcher. 1990. Developmental appearance and ultrastructural immunolocalization of the major 66 kDa phosphoprotein by chicken osteoblasts during *in vitro* mineralization. *Anat. Rec.* 228:93–103.
22. Gerstenfeld, L. C., C. M. Kelley, M. von Deck, and J. B. Lian. 1990. Effects of 1,25(OH)₂D₃ on induction of chondrocyte maturation in culture: extracellular matrix gene expression and morphology. *Endocrinology.* 126:1599–1609.
23. Gibson, G. J., S. L. Schor, and M. E. Grant. 1982. Effects of matrix macromolecules on chondrocyte gene expression: synthesis of a low molecular weight collagen species of cell cultures within collagen gels. *J. Cell Biol.* 93:767–774.
24. Gibson, G. J., B. W. Beaumont, and M. H. Flint. 1984. Synthesis of a low molecular weight collagen of chondrocytes from the presumptive calcification region of the embryonic chick sterna. The influence of culture with collagen gels. *J. Cell Biol.* 99:208–216.
25. Habuchi, H., E. Conrad, and J. H. Glaser. 1985. Coordinate regulation of collagen and alkaline phosphatase levels in chick embryo chondrocytes. *J. Biol. Chem.* 260:13029–13034.
26. Hall, B. K. 1987. Earliest evidence of cartilage and bone development in embryonic life. *Clin. Orthop. Relat. Res.* 225:255–272.
27. Hinek, A., A. Reiner, and A. R. Poole. 1987. The calcification of cartilage matrix in chondrocyte cultures: studies of the C-propeptide of type II collagen (chondrocalcin). *J. Cell Biol.* 104:1435–1441.
28. Hunziker, E. B., R. Schenk, and L. M. Cruz-Orive. 1987. Quantitation of chondrocyte performance in growth-plate cartilage during longitudinal

bone growth. *J. Bone Jt. Surg.* 69A:162–173.

29. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 277:680–685.

30. Landis, W. J., and M. J. Glimcher. 1978. Electron diffraction and electron probe microanalysis of the mineral phase of bone tissue prepared by anhydrous techniques. *J. Ultrastruct. Res.* 63:188–223.

31. Landis, W. J., and M. J. Glimcher. 1982. Electron optical and analytical observations of rat growth plate cartilage prepared by ultracryomicrotomy. *J. Ultrastruct. Res.* 78:227–268.

32. Landis, W. J., and K. J. Hodgens. 1990. Visualization of sulfur-containing components associated with proliferating chondrocytes from rat epiphyseal growth plate cartilage. Possible proteoglycan and collagen comigration. *Anat. Rec.* 226:153–167.

33. Landis, W. J., M. C. Paine, and M. J. Glimcher. 1977. Electron microscopic observations of bone tissue prepared anhydrously in organic solvents. *J. Ultrastruct. Res.* 59:1–30.

34. Laskey, R. A., and D. A. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335–341.

35. Leboy, S. P., I. M. Shapiro, B. D. Uschmann, O. Oshima, and D. Lin. 1988. Gene expression in mineralizing chick epiphyseal cartilage. *J. Biol. Chem.* 263:8515–8520.

36. Leboy, S. P., L. Vaias, B. Uschmann, E. Golub, S. L. Adams, and P. Pacifici. 1989. Ascorbic acid induces alkaline phosphatase, type X collagen and calcium deposition in cultured chick chondrocytes. *J. Biol. Chem.* 264:17281–17286.

37. Lehrach, H., A. M. Frischauf, D. Hanahan, J. Wozney, F. Fuller, R. Crkvenjakov, H. Boedtker, and P. Doty. 1978. Construction and characterization of a 2.5-kilobase collagen clone. *Proc. Natl. Acad. Sci. USA.* 75:5417–5421.

38. Lehrach, H., A. M. Frischauf, D. Hanahan, J. Wozney, F. Fuller, and H. Boedtker. 1979. Construction and characterization of pro α1 collagen complementary deoxyribonucleic acid clones. *Biochemistry.* 18:3145–3152.

39. Linsenmayer, T. F., E. Gibney, and T. M. Schmid. 1986. Segmental appearance of type X collagen in developing avian notochord. *Dev. Biol.* 113:467–473.

40. Lyons, B. L., and R. I. Schwarz. 1984. Ascorbate stimulation of PAT cells causes an increase in transcription rates and a decrease in degradation rates of procollagen mRNA. *Nucleic Acids Res.* 12:2569–2579.

41. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pg. 123.

42. Matsumoto, H., K. DeBolt, and I. M. Shapiro. 1988. Adenine, guanine and inosine nucleotides of chick growth cartilage: relationship between energy status and the mineralization process. *J. Bone Miner. Res.* 3:347–352.

43. Mayne, R., and I. H. Irwin. 1986. Collagen types in cartilage. *In* Articular Cartilage Biochemistry. K. Kuettner, editor. Raven Press, New York. 23–35.

44. Mikuni-Takagaki, Y., and B. P. Toole. 1979. Shedding of hyaluronate from the cell surface of Rous sarcoma virus transformed chondrocytes. *J. Biol. Chem.* 254:8409–8415.

45. Nimni, M. E. 1973. Metabolic pathways and control mechanisms involved in biosynthesis and turnover of collagen in normal and pathological connective tissues. *J. Oral Pathol.* 2:175–202.

46. Ninomiya, Y. M., M. Gordon, M. van der Rest, T. Schmid, T. Linsenmayer, and B. Olsen. 1986. The developmentally regulated type X collagen gene contains a long open reading frame without introns. *J. Biol. Chem.* 261:5041–5050.

47. Nishimura, I., Y. Muragaki, and B. Olsen. 1989. Tissue specific forms of type IX collagen-proteoglycan arise from the use of two widely separated promoters. *J. Biol. Chem.* 264:20033–20041.

48. Peterkovsky, B. 1972. The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during growth of cultured fibro-

blasts. *Arch. Biochem. Biophys.* 2:318–326.

49. Poole, A. R., and I. Pidoux. 1989. Immunoelectron microscopic studies of type X collagen in endochondral ossification. *J. Cell Biol.* 109:2547–2554.

50. Poole, A. R., I. Pidoux, and L. Rosenberg. 1982. Role of proteoglycans in endochondral ossification: immunofluorescent localization of link protein and proteoglycan monomer in bovine fetal epiphyseal growth plate. *J. Cell Biol.* 92:249–260.

51. Poole, A. R., Y. Matsui, A. Hinek, and E. R. Lee. 1989. Cartilage macromolecules and calcification of cartilage matrix. *Anat. Rec.* 224:167–179.

52. Sai, S., T. Tanaka, R. Kosher, and M. Tanzer. 1986. Cloning and sequence analysis of a partial cDNA for chicken cartilage proteoglycan core protein. *Proc. Natl. Acad. Sci. USA.* 83:5081–5085.

53. Sandell, L. J., and J. C. Daniel. 1988. Effects of ascorbic acid on collagen mRNA levels in short term chondrocyte cultures. *Connect. Tissue Res.* 17:11–22.

54. Schenk, R., P. S. Eggli, and E. B. Hunziker. 1986. Articular cartilage morphology. *In* Articular Cartilage Biochemistry. K. Kuettner, editor. Raven Press, New York. 3–22.

55. Schmid, T. M., and E. Conrad. 1982. A unique low molecular weight collagen secreted by cultured chick embryo chondrocytes. *J. Biol. Chem.* 257:12444–12450.

56. Schmid, T. M., and T. F. Linsenmayer. 1985. Developmental acquisition of type X collagen in the embryonic chick tibiotarsus. *Dev. Biol.* 107:373–381.

57. Schwartz, E., R. S. Bienkowski, B. Coltoff-Schiller, S. Goldfischer, and O. O. Blumenfeld. 1982. Changes in the components of extracellular matrix and in growth properties of cultured aortic smooth muscle cells upon ascorbate feeding. *J. Cell Biol.* 72:462–470.

58. Schwarz, R. I., L. Colarusso, and P. Doty. 1976. Maintenance of differentiation in primary cell cultures of avian tendon cells. *Exp. Cell Res.* 102:63–71.

59. Schwarz, R. I., P. Kleinman, and N. Owens. 1983. Ascorbate can act as an inducer of collagen pathway because most steps are tightly coupled. *Ann. NY Acad. Sci.* 498:172–185.

60. Shapiro, F. 1987. Epiphyseal disorders. *N. Eng. J. Med.* 317:1702–1710.

61. Solursh, M., K. L. Jensen, N. C. Zanetti, T. F. Linsenmayer, and R. S. Reiter. 1984. Extracellular matrix mediates epithelial effects on chondrogenesis in vitro. *Dev. Biol.* 105:451–457.

62. Solursh, M., K. L. Jensen, R. S. Reiter, T. M. Schmid, and T. F. Linsenmayer. 1986. Environmental regulation of type X collagen production by cultures of limb mesenchyme, mesectoderm and sternal chondrocytes. *Dev. Biol.* 117:90–101.

63. Svoboda, K. K., I. Nishimura, S. P. Sugrue, Y. Ninomiya, and B. R. Olsen. 1988. Embryonic chicken cornea and cartilage synthesize type IX collagen molecules with different amino-terminal domains. *Proc. Natl. Acad. Sci. USA.* 85:7496–7500.

64. Tacchetti, C., R. Quarto, L. Nitsch, D. J. Hartmann, and R. Cancedda. 1987. In vitro morphogenesis of chick embryo hypertrophic cartilage. *J. Cell Biol.* 105:999–1006.

65. Thomas, P. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201–5205.

66. von der Mark, K. 1986. Differentiation, modulation and dedifferentiation of chondrocytes. *Rheumatology.* 10:272–315.

67. Vytasek, R. 1982. A sensitive fluorometric assay for the determination of DNA. *Anal. Biochem.* 120:243–248.

68. Young, M. F., G. Vogeli, A. M. Nunez, M. P. Fernandez, M. Sullivan, and M. E. Sobel. 1984. Isolation of cDNA and genomic DNA clones encoding type II collagen. *Nucleic Acids Res.* 12:4207–4228.

69. Zanetti, V. C., and M. Solursh. 1984. Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton. *J. Cell Biol.* 99:115–123.