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Induction of Bone-Related Proteins, Osteocalcin and Osteopontin, and Their Matrix Ultrastructural Localization With Development of Chondrocyte Hypertrophy In Vitro

J.B. Lian, M.D. McKee, A.M. Todd, and L.C. Gerstenfeld

Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655 (J.B.L.); Department of Stomatology, Université de Montréal, Montréal, Québec, Canada H3C 3J7 (M.D.M.); Harvard University School of Dental Medicine, Department of Orthodontics, Boston, Massachusetts 02115 (A.M.T.); Laboratory for the Study of Skeletal Disorders and Rehabilitation, Department of Orthopedic Surgery, Harvard Medical School, Boston, Massachusetts 02115 (L.C.G.)

Abstract Endochondral bone formation occurs by a series of developmentally regulated cellular events from initial formation of cartilage tissue to stages of calcified cartilage, resorption, and replacement by bone tissue. Several studies have raised the question of the possibility that the hypertrophic chondrocytes associated with the calcifying cartilage matrix can acquire properties similar to osteoblasts. We have addressed this possibility by measuring synthesis within hypertrophic chondrocytes in vitro of two bone-related proteins, osteopontin and osteocalcin. Chondrocytes derived from chick embryo ventral vertebral tissue were cultured under conditions that promoted extracellular matrix mineralization and differentiation towards the hypertrophic phenotype as indicated by the induction of Type X collagen, alkaline phosphatase, and diminished expression of Type II collagen and the core protein of large proteoglycan. In these cultures, osteopontin synthesis was detected in early cultures in the absence of a calcified matrix; in contrast, an absence of the bone-specific protein osteocalcin was observed. However, with onset of development of the hypertrophic phenotype an induction of protein expression for osteocalcin was observed with a significant (twofold) increase in osteopontin. Maximal levels of osteocalcin synthesis occurred with the peak of alkaline phosphatase activity and Type X collagen mRNA levels. The levels of osteocalcin synthesis were induced fiftyfold from the earliest level of detection but this level was only one one-hundredth of that observed for mature chick osteoblast cultures. Osteocalcin and osteopontin were characterized by several criteria (electrophoresis, immunoblotting, chromatographic characteristics, and response to $1,25(OH)_2D_3$) which confirmed their molecular properties as being identical to osteoblast synthesized proteins. The coordinate change in the cellular phenotype to the hypertrophic chondrocyte was shown to be concurrent with ultrastructural maturation of the cells and the accumulation of osteocalcin and osteopontin in the extracellular matrix associated with hydroxyapatite at sites of mineralization. Since the ultrastructural features of the cells in vitro and the extracellular matrix surrounding the lacunae have features of the hypertrophic chondrocyte and associated matrix in vivo, the induction of the bone-specific protein osteocalcin suggests that at least a population of these cells may develop osteoblastic phenotypic markers in association with mineralizing matrix. The detection of osteocalcin and the high level of synthesis of osteopontin may represent an advanced stage of chondrocyte hypertrophy or the possibility of a trans-differentiation of the chondrocytes to an osteoblastic-like cell.

Key words: bone formation, hypertrophic chondrocytes, osteocalcin, osteopontin, Type X collagen

Endochondral bone formation involves a complex series of precisely programmed events in which chondrocytes undergo maturation from resting zone cells to hypertrophic cells in the zone of calcification. The hypertrophic chondrocytes express both quantitatively different and unique extracellular matrix components from either hyaline cartilage at an articular surface or the resting zone chondrocytes within a growth plate. Numerous studies have documented both morphological and biochemical differences among the cells at distinct stages of chondrocyte

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Address reprint requests to Louis C. Gerstenfeld, Ph.D., Children's Hospital Medical Center, 300 Longwood Ave., Boston, MA 02115.

differentiation within the growth cartilage which include changes in synthesized collagen types, proteoglycan levels, alkaline phosphatase enzyme activity, response to hormones, and mineralization of the extracellular matrix [Schmid and Linsenmayer, 1985; Habuchi et al., 1985; Leboy et al., 1988, 1989; Boyan et al., 1988; Schwartz et al., 1989; Takigawa et al., 1988; Gerstenfeld et al., 1989a,b, 1990a; Franzen et al., 1987; Hinek et al., 1987].

Several in vitro systems have clearly demonstrated that chondrocytes isolated from different cartilages which are at specific developmental stages or which have different developmental fates retain their phenotypes in vitro, and show distinct biochemical parameters and unique phenotypic gene expression [Gerstenfeld et al., 1989a; Hinek et al., 1987; Boyan et al., 1988; Schmid and Linsenmayer, 1985]. In studies from this laboratory and from several others, culture conditions have been established which promote chondrocyte differentiation towards a hypertrophic phenotype [Daniel et al., 1984; Morris and Balian, 1985; Castagnola et al., 1988; Leboy et al., 1989; Tacchetti et al., 1987; Gerstenfeld and Landis, 1991]. In these studies, time dependent induction of Type X collagen, alkaline phosphatase, and diminished expression of Type II collagen and the core protein of the large cartilage proteoglycan were observed. This coordinate change in the cellular phenotype was further shown to be concurrent with the ultrastructural maturation of the cells and the extracellular matrix in which hydroxyapatite deposition and the presence of matrix vesicles were observed [Gerstenfeld and Landis, 1991]. These observations raise the possibility that other specialized proteins associated with mineral deposition might become expressed with a change to the distinct hypertrophic chondrocyte phenotype.

Both in vivo [Yoon et al., 1987; McKee et al., 1990, 1992] and in vitro studies utilizing both chick embryonic or rat calvaria-derived osteoblasts have shown up to several hundredfold induction of several non-collagenous extracellular matrix proteins with the onset of extracellular matrix mineralization of the bone-like matrix [Aronow et al., 1990; Owen et al., 1990; Gerstenfeld et al., 1987, 1990c]. These include the bone specific and vitamin K–dependent protein, osteocalcin (or bone Gla protein) and the phosphoprotein, osteopontin (or SppI, 44kD). The induction of these proteins has been shown to be transcriptionally regulated during the phase in which the extracellular matrix begins to mineralize [Owen et al., 1990, 1992; Moore et al., 1991].

In the present study, the synthesis of osteocalcin and osteopontin was examined in chicken ventral vertebral chondrocyte cultures grown either under conditions that promote extracellular matrix mineralization or control conditions in which the matrix remains uncalcified in order to determine whether mineralization of the cartilage matrix may also provide signals for induction of these proteins. We observed an induction and fiftyfold increase of protein expression for osteocalcin and a significant (twofold) increase in osteopontin with the onset of mineralization during in vitro chondrocyte differentiation to the hypertrophic phenotype. The ultrastructural localization of these proteins in the extracellular matrices accumulated by these cultures was determined and found associated with calcified cartilage matrix.

MATERIALS AND METHODS

Vertebral Chondrocyte Cell Cultures

Chondrocytes were prepared from the ventral half of 12-day-old chicken embryo vertebra as described previously [Gerstenfeld et al., 1989a,b]. Cells were grown for a 7-day period in minimum essential medium (MEM) and 10% fetal bovine serum (primary cultures). After 7 days, only non-adherent cells were selected, treated with trypsin, and replated at 2.0×10^6 cells per 100 mm dish in Dulbecco's modified essential medium (DME) and 10% fetal calf serum (secondary cultures). Cultures were grown for an additional 7 days, during which time 90% of the cells became attached to culture plates and grew in foci of polygonal cells. Experiments were initiated by a changing the medium to BGJ_b (Fitton-Jackson modified) and 10% fetal calf serum supplemented with 50 μg/ml ascorbate, 10 mM β-glycerophosphate (β-GPO_4), designated complete medium. Data points were numbered from the time cells were placed in BGJ_b complete medium. In some experiments, cultures were maintained in DME and 10% fetal calf serum supplemented with either 50 μg/ml ascorbate or 10 mM βGPO_4 supplemented with 50 μg/ml ascorbate. Some cultures were treated with $1,25(OH)_2D_3$ (kindly provided by M. Uskokovic, Hoffman LaRoche, Inc., Nutley, NJ). Hormone was added by dilution in media of 10^{-3} M stock

in absolute ethanol. No hormone addition exceeded 0.10% of the final volume of the media with ethanol, and control cultures contained vehicle only.

Biochemical Analyses of the Cultures

All determinations were carried out on triplicate culture wells of at least duplicate cell preparations. Growth conditions were monitored by total DNA, quantitated by fluorometric analysis after reaction with 3,5-diaminobenzoic acid [Vysatek, 1982]. The total calcium content in the cell layer assayed by atomic absorption spectrophotometry after hydrolysis of the whole cell layers in 0.5 ml 6N HCl while alkaline phosphatase enzyme activity was determined in cell layer homogenates using reagents from Sigma Chemical Co. (St. Louis, MO) as previously described assay. Identification of osteocalcin and osteopontin were carried out by polyacrylamide gel electrophoresis [Laemmli, 1970] followed by Western blot analysis [Towbin and Gordon, 1984]. Buffer conditions and current used for transfer were as described in Gerstenfeld et al. [1990b]. Transfer time for osteocalcin was 15 min and osteopontin 30 min. The IgG fraction of anti-chicken osteocalcin rabbit serum [Gundberg et al., 1987] and whole anti-chicken osteopontin rabbit serum [Gotoh et al., 1990] were utilized for these studies.

Osteocalcin was quantitated in the media by radioimmunoassay as previously described [Gundberg et al., 1987]. Osteocalcin was present in unincubated media containing 10% FCS at low detectable levels of 1–2 ng/ml. Characterization of the osteocalcin which had been synthesized and secreted into media was carried out using pooled media from cells cultured 24 h in the absence of 10% fetal bovine serum from 20 (100 mm) cultures. Media were dialyzed against water in SpectroporII (Spectrum Industries, Los Angeles, CA), then was fractionated on a C18 reverse phase column (Rainin Instruments) using a 40 min gradient of 10–70% acetonitrile in 0.1% trifluoroacetic acid or an anion exchange column (Brownlee AX300, Rainin Instruments) using a 60 min gradient from 0.1–1 M sodium phosphate buffer, pH 8. Each 1 ml fraction was quantitated for osteocalcin by radioimmunoassay. Position of elution was determined with osteocalcin standard that was purified from 12–14-week chick cortical long bone as previously described [Hauschka et al., 1989] and was the same protein used for standards and iodinated tracer in the radioimmunoassay procedure as detailed elsewhere [Gundberg et al., 1987]. The immunoreactive fraction was pooled and rechromatographed either alone for further biochemical analyses or in the presence of 10,000 CPM of ^{125}(I) iodinated chick osteocalcin to confirm coelution of the chondrocyte synthesized product with purified chick osteocalcin standard.

Osteopontin was quantitated in the media using a fluorometric slot blot assay (Schaffer J. and Gerstenfeld L.C., manuscript in preparation). In brief, this assay is as follows. For each measurement, duplicate media samples of 5 and 10 μl were applied to nitro-cellulose using a Millipore Slot Blot device (Millipore, Bedford, MA). A standard curve was developed for each blot using 6 ng, 3 ng, 1.5 ng, 0.75 ng, and 0.275 ng which were concurrently blotted with each set of experimental samples. Fluorographs were then developed using the ECL chemiluminescence detection kit as described by the manufacturer (Amersham Corp., Arlington Heights, FL). Fluorograms were scanned on an LKB Ultrascan II densitometer (LKB, Broma, Sweden), and for each sample the relative densitometric areas were extrapolated from the slopes of the line generation from the standard.

Ultrastructural Examination and Immunocytochemistry

Chondrocyte cultures were initiated as described above, but the vertebral derived chondrocytes were plated on Lux plastic coverslips. At designated times, media was removed and the coverslips were immediately flooded at room temperature with fixative containing 1% glutaraldehyde and 0.5% calcium chloride in 0.08 M sodium cacodylate buffer, pH 7.3. Following fixation for 20 min, the coverslips with their adherent cell layers were subsequently washed overnight at 4°C with 0.1 M sodium cacodylate buffer, pH 7.3. For morphological examination, the cultures were then post-fixed with potassium ferrocyanide–reduced osmium tetroxide for 1 h at 4°C, followed by further washing and dehydration through a graded ethanol series. The cultures were then infiltrated and embedded in Tabb Epon 812 epoxy resin (Mecalab, Inc., Montreal, Canada) and the resin was polymerized at 60°C for 2 days.

Cell cultures for immunocytochemistry were prepared as described above, but were left unosmicated and were embedded in Lowicryl K4M (Chemische Werke Lowi GmbH, Germany) fol-

lowing dehydration in a graded ethanol series. The Lowicryl was polymerized with ultraviolet light (360 nm wavelength) at −30°C. Semi-thin survey sections for light microscopy were cut perpendicular to the culture surface with glass knives and stained with toluidine blue to select regions containing mineralized matrix for electron microscopy. From these, thin sections (80 nm) were cut with diamond knives, mounted on formvar- and carbon-coated nickel grids, processed for immunocytochemistry as described below, and conventionally stained with uranyl acetate and lead citrate. Sections were examined by transmission electron microscopy at 80 kV with a JEOL JEM 2000FX-II. Some sections were not processed for immunocytochemistry and were left unstained to assess the presence of mineral by electron diffraction and X-ray microanalysis.

The high resolution protein A–gold immunocytochemical technique [reviewed in Bendayan, 1989] was applied to thin sections of the cultures as described previously [McKee et al., 1990]. Briefly, grid-mounted tissue sections were floated on a drop of sodium metaperiodate (osmicated tissues only) for 1 h at room temperature. They were washed with distilled water, floated for 5 min on a drop of 0.01 M phosphate-buffered saline (PBS) containing 1% ovalbumin (Sigma Chemical Co., St. Louis, MO), and then transferred and incubated for 1 h at room temperature on a drop of diluted rabbit anti-chicken osteocalcin [Gundberg et al., 1987] or rabbit anti-chicken osteopontin [Gotoh et al., 1990] antiserum. After incubation, sections were rinsed with PBS and placed again on PBS–1% ovalbumin for 5 min, followed by incubation for 30 min at room temperature with protein A–gold complex (gold particles of approximately 14 nm). Tissue sections were then washed thoroughly with PBS, rinsed with distilled water, and air dried. For control experiments, grid-mounted sections were incubated with 1) protein A–gold alone, 2) non-immune serum, and in some cases 3) antigen adsorbed with excess antibody.

RESULTS

Expression of Bone-Related Proteins in Mineralizing Chondrocyte Cultures

Baseline levels of both osteocalcin and osteopontin were assayed in the chick chondrocyte cultures. In secondary cultures of the chick vertebral chondrocytes at early times up to day 3, only background levels of osteocalcin ranging from 1.9–2.3 ng/ml or 0.2–0.3 ng/μg DNA media were detected and this value is probably derived in part from the partial cross-reactivity of the chick and bovine osteocalcins [Gundberg et al., 1987]. In contrast, the levels of osteopontin were slightly higher: 24 ng/ml, or 4.8 ng/μg DNA; however, from previous studies no cross-reaction of the antisera was observed with the mammalian forms of this protein. During this initial period, barely detectable levels of alkaline phosphatase activity per cell were also seen in the cell layer prior to the onset of mineralization.

In initial experiments, synthesis of these proteins in relation to development of the hypertrophic phenotype was established by several criteria. In previous studies, the development of chondrocyte hypertrophy and matrix mineralization was shown to be dependent on the nutrient content of the growth media (DME, a minimal media, vs. BGJ_b, a nutrient rich media) and the supplementation of the media with ascorbic acid and β-GPO_4 [Gerstenfeld and Landis, 1991]. Figure 1 compares the levels of synthesis for osteocalcin and osteopontin as assessed in the media and normalized to DNA quantities for cultures grown under three different culturing conditions. Cultures were assayed on day 15 after mineralization was initiated and Type X collagen was expressed, a marker of the hypertrophic phenotype [Fig. 1B from Gerstenfeld and Landis, 1991]. It can be seen that in the BGJ_b media, supplemented with both ascorbic acid and β-GPO_4, that a fifty- to one hundred-fold greater level of secreted osteocalcin per cell was detected in media, while osteopontin showed a two- to threefold induction under these same growth conditions. In contrast, intermediate levels of these proteins were observed in the less nutrient rich DME media supplemented with ascorbic acid and β-GPO_4, while very low levels of these proteins were observed in the unsupplemented DME media. The extent of development of the hypertrophic phenotype was indicated by the induction of the DNA normalized values for alkaline phosphatase enzyme activity and collagen type α,1(X)/α,1(II and I) ratio. As can be seen by comparing these results with those for the osteopontin and osteocalcin, the development of the hypertrophic phenotype is clearly associated with the induction of both osteocalcin and osteopontin. Maximal levels of both osteopontin and osteocalcin were seen in BGJ_b media supplemented with ascorbate acid and β-GPO_4

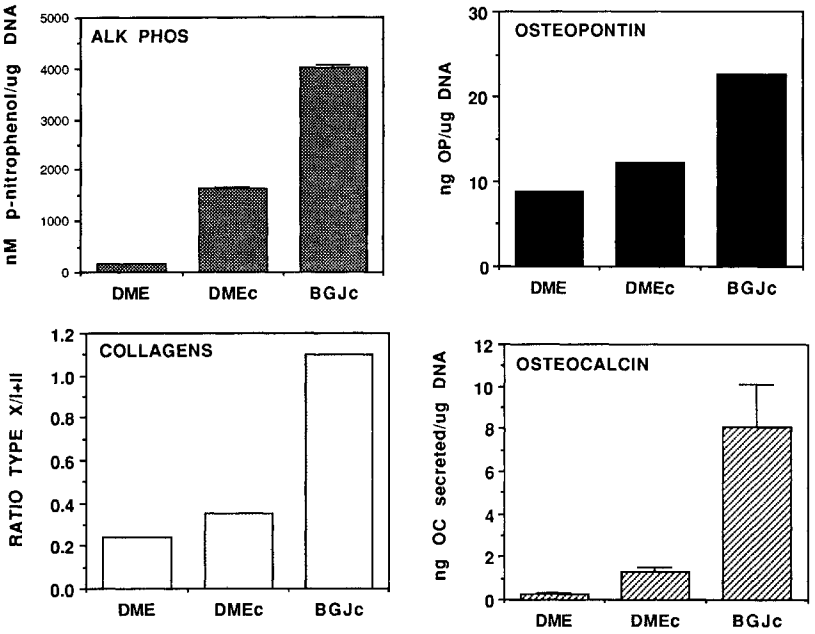

Fig. 1. Markers of the hypertrophic chondrocyte phenotype and expression of osteocalcin and osteopontin. The levels of alkaline phosphatase enzyme activity (ALK PHOS) and the ratio of Type X collagen, a marker of chondrocyte hypertrophy, to Types I and II are indicated (COLLAGENS) in chick chondrocytes cultured in three different media. Cells in secondary culture where harvested 15 days after initiation of the experiment as described in Materials and Methods when cells were maintained either in control medium (DME) with 10% fetal calf serum or complete media containing serum, ascorbic acid, and β-glycerol phosphate (DMEc or BGJc = media supplemented with 50 ug/ml ascorbate). By 15 days, calcification had initiated in DME + ascorbate and to a greater extent in BGJ_b + ascorbate. In BGJ_b + ascorbate, the induction of osteocalcin is sixfold greater and for osteopontin twofold greater than observed in DMEc.

both of which maximally promoted extracellular matrix synthesis and calcification.

While the presence of osteopontin and osteocalcin in the media provided evidence of their synthesis, the molecular nature of these proteins and their accumulation in the culture's extracellular matrix was confirmed by Western blot analysis of these proteins which were deposited in the cell layer (Fig. 2). These analyses clearly demonstrated that proteins of appropriate molecular weights consistent with previous analyses for chicken osteocalcin [Hauschka et al., 1989] and chicken osteopontin [Gotoh et al., 1990] were accumulated within the extracellular matrix. Osteocalcin, which has an actual molecular mass of 6 kDa, for its processed form, electrophoresed with molecular weight markers at 10 kDa while two osteopontin species of 66 kDa and 45 kDa were detected using chicken specific antisera.

The molecular characteristics of the osteocalcin which was synthesized by the chondrocytes was further analyzed by comparing its HPLC elution characteristics to that of chicken osteo-

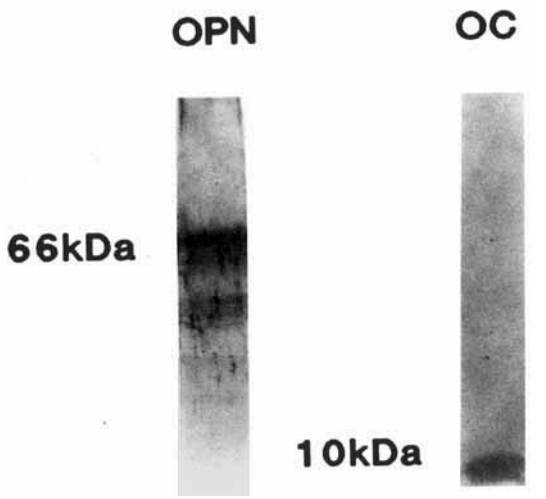

Fig. 2. Western blot analysis of osteocalcin (OC) and osteopontin (OPN). Cells where cultured in complete BGJ_b media and harvested 21 days later for analysis of the cell layer accumulated proteins which where solubilized for Laemmli gel analysis followed by transfer to nitrocellulose and incubation with protein specific antisera as described in Materials and Methods.

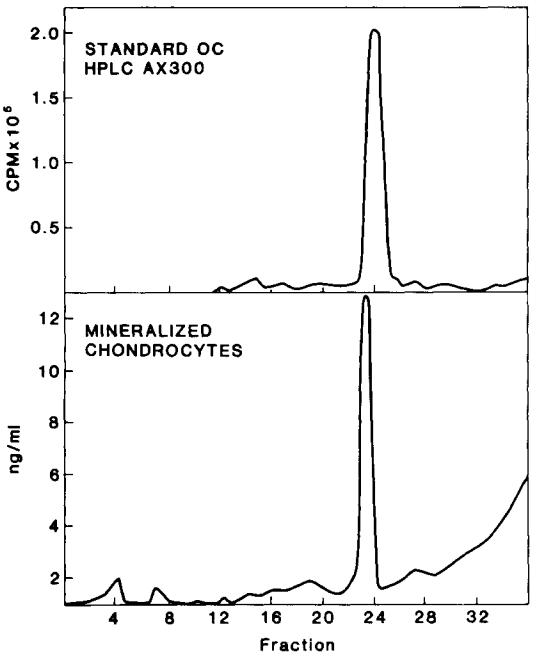

Fig. 3. Proof of identity of chondrocyte synthesized osteocalcin. Secreted osteocalcin was dialyzed in the presence of inhibitors and initially fractionated by reverse-phase chromatography. The immunoreactive fractions were pooled and rechromatographed over an AX300 column (**lower panel;** mineralized chondrocyte cultures) to demonstrate elution at the identical position of iodinated purified chick bone osteocalcin standard (**upper panel**). Osteocalcin standard was detected by assay of each fraction in a Perkin Elmer gamma counter. Chondrocyte derived osteocalcin was quantitated by radioimmunoassay of each fraction (ng/ml).

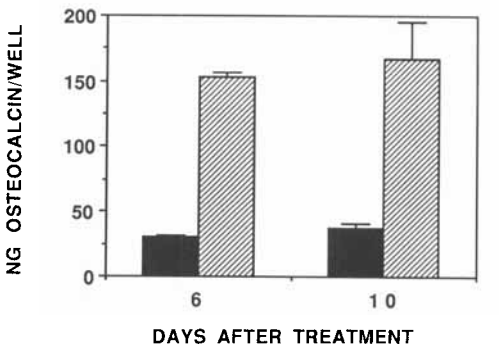

Fig. 4. Hormone responsiveness of chondrocyte synthesized osteocalcin. Chondrocytes were treated with 10^{-8} M $1,25(OH)_2D_3$ as of day 4 after initiation of chondrocyte differentiation in complete BGJ_b media. Assay of conditioned media (48 h) from control (solid bars) and vitamin D–treated cells (hatched bars) from n = 3 wells, harvested on day 6 and again on day 10, demonstrates the increase in synthesized secreted osteocalcin in response to hormone.

calcin purified from bone. The osteocalcin that was synthesized by both chondrocytes and bone had the same position of elution in a reverse-phase (C18) chromatography system. Rechromatography of the immunoreactive fraction from the media co-eluted with radiolabeled osteocalcin (data not shown). Furthermore, rechromatography using an HPLC anion exchange column chromatography also showed identical chromatography patterns between chondrocyte-synthesized and bone isolated osteocalcins (Fig. 3). These data suggest therefore, that the chondrocytes both proteolytically process and post-translationally modify this protein in an identical fashion as in bone, since changes in either the protein gla content or pro-osteocalcin processing would have altered its elution characteristics on HPLC [Nishimoto and Price, 1985; Hauschka et al., 1989].

The effect of $1,25(OH)_2D_3$ on osteocalcin expression in chondrocytes was also examined in order to determine if the gene was regulated in those cells in a similar fashion as observed in osteoblasts (Fig. 4). When the cultures under mineralizing growth conditions were continuously cultured in the presence of 10^{-8} M $1,25(OH)_2D_3$ a threefold greater level of synthesis of this protein was observed by measurement of secreted osteocalcin in the culture media. Those results together with the results depicted in the previous figures provide evidence by independent methodologies for the presence of both osteocalcin and osteopontin in cultures of chick chondrocytes.

Temporal Expression of Osteocalcin and Osteopontin During Development of Chondrocyte Hypertrophy and Matrix Mineralization

The level of synthesis of osteocalcin and osteopontin expression was examined in relation to maturation of the vertebral derived chondrocytes towards the hypertrophic phenotype in vitro, and mineralization of the extracellular matrix (Fig. 5). For these experiments, cultures were maintained in the complete BGJ_b media for 21 days. Osteocalcin and osteopontin synthesis showed a typical correlation to increased phenotypic features of the hypertrophic chondrocyte as reflected by alkaline phosphatase activity (Fig. 5) and an increase in collagen Type X expression. The onset of osteocalcin synthesis occurred concomitantly with initiation of Type X expression and mineral deposition (day 10) and increased in parallel with the rise in alkaline

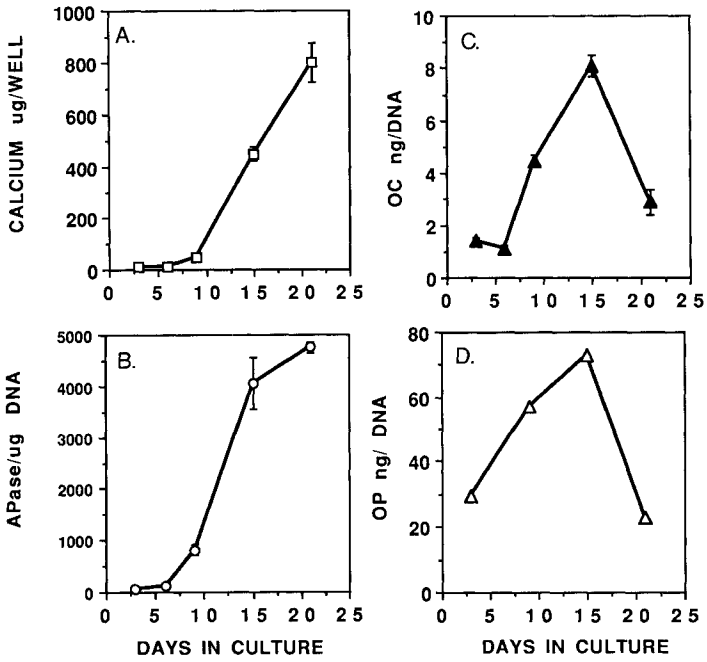

Fig. 5. Temporal expression of osteocalcin and osteopontin in relation to development of the hypertrophic phenotype in chick chondrocyte cultures. With the onset of initiation of calcium deposition (A) on day 10 the initial rise in alkaline phosphatase (B) is indicated At this time on day 10, the first induction of osteocalcin (C) and the rise in osteopontin (D) is observed with levels of these proteins peaking on day 15 and then declining in heavily mineralized cultures on day 20.

phosphatase activity and calcium accumulation. In contrast, higher baseline levels of osteopontin were observed in the early cultures and a smaller induction (twofold) was seen during mineralization of the extracellular matrix. By day 21, total DNA contents of the cultures were twofold greater than on day 15 (data not shown). Thus, when normalized to cell number as in Figure 5, a decline in synthesis of osteocalcin and osteopontin was observed between days 15 and 21. The levels peaked on day 15 and were ~50% lower on day 21.

Ultrastructural Analysis of the Association of Bone-Related Proteins With Mineralized Cartilage Matrix

The histological development and ultrastructural characteristics of the vertebral chondrocyte culture system used in the present study have been described previously [Gerstenfeld and Landis, 1991] and these cultures were characterized as having cellular and extracellular matrix ultrastructure comparable to that seen in hypertrophic cartilage in vivo. In the present study in vitro, immunocytochemical analysis of the chondrocyte cultures were carried out to determine how these proteins were localized within the extracellular matrix and if they were specifically associated with the mineralizing region of the matrix.

By light microscopy, 30-day vertebral chondrocyte cultures sectioned perpendicular to the culture dish appeared as multiple layers of cells encapsulated by cartilage matrix (Fig. 6). The perilacunar matrix often appeared more darkly stained with toluidine blue than the interterritorial matrix, and the matrix in the uppermost regions of the culture was only very lightly stained. Within each culture dish, mineralization was more advanced in some regions than in others, and where present, appeared as discrete irregular loci throughout the matrix or as small punctuate loci of mineralization at the interface with the plastic coverslip. The presence of mineral at these sites was confirmed at the light microscopic level by von Kossa staining (data not shown), and at the electron microscopic level by electron diffraction and X-ray microanalysis as shown previously [Gerstenfeld and Landis, 1991]. Matrix mineralization was most extensive in the near vicinity of the chondrocytes, and frequently completely surrounded these cells

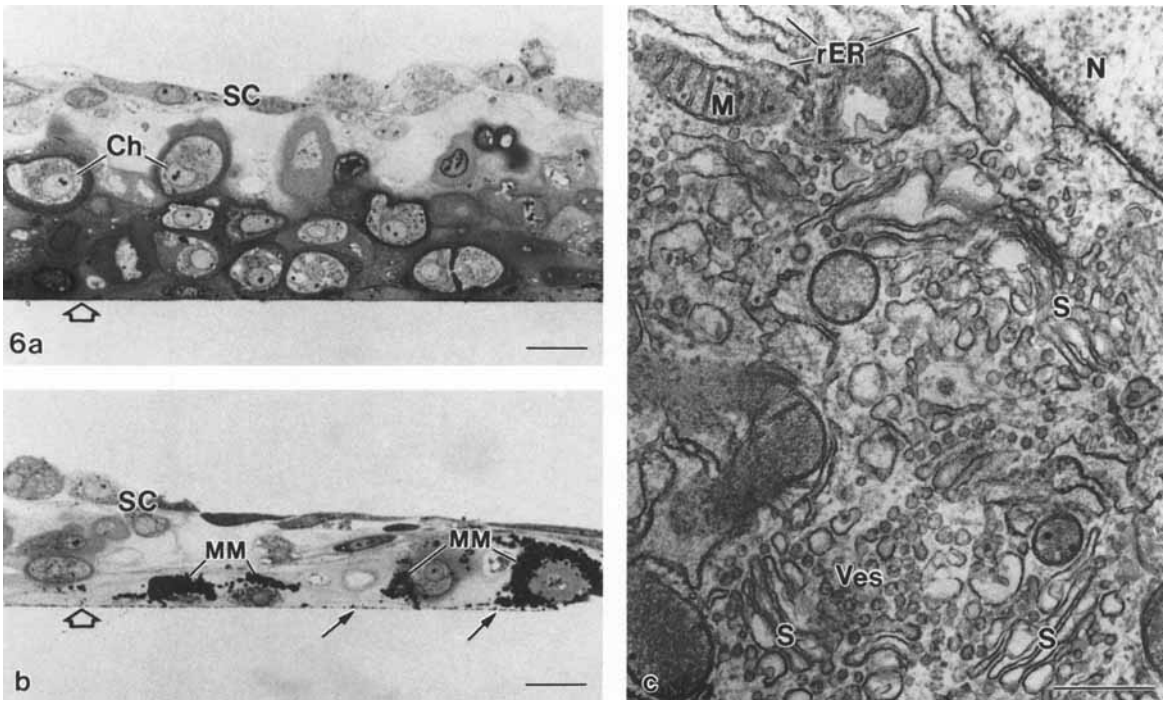

Fig. 6. Light micrographs (**a,b**) of a 30-day chondrocyte culture showing numerous large round chondrocytes (Ch) in lacunae defined by dark staining perilacunar cartilage matrix. Mineralized matrix (MM) is frequently associated with the cells or is found throughout the cartilaginous matrix and at the interface of the matrix with the plastic substrate (arrows). Cells at the surface of the cultures (SC) were frequently flattened and more darkly stained. The open arrow indicates the uppermost surface of the plastic coverslip. By electron microscopy (**c**), cells frequently showed morphological evidence of protein secretion as indicated by the presence of rough endoplasmic reticulum (rER) and an extensive Golgi apparatus consisting of numerous stacked Golgi saccules (S) and vesicles (Ves). M, mitochondria, N, nucleus. Epon sections stained with toluidine blue (a,b) or uranyl acetate and lead citrate (c). Bars equal 10 μm (a), 0.5 μm.

(Fig. 6b). Cells at the surface of the cultures were generally of two varieties, being either pale and somewhat rounded, or more darkly stained and flattened. By electron microscopy (Fig. 6c), chondrocytes were observed throughout the cultures that showed morphological features of cells typically engaged in the production and release of proteins. These features included a wide range of intracellular organelles including extensive rough endoplasmic reticulum, a well-developed Golgi apparatus, and numerous vesicles, mitochondria, and microtubules.

Following the aqueous incubation steps required during the immunocytochemical processing of the sections, and staining with uranyl and lead, the mineral is lost from the sections [discussed in McKee et al., 1990]. Thus, stained material remaining in the sections, while originally corresponding to sites of mineral deposition among the matrix components, represents only organic matrix. After incubation with the osteocalcin and osteopontin antibodies, these sites of apparent mineralization were intensely labeled with gold particles. Immunolabeling patterns using both these antibodies (Figs. 7–9) were primarily found over granular, electron-dense organic matrix either peripheral to the cells (Figs. 7, 8) or somewhat distant from them (Fig. 9). Throughout the cultures, labeling was predominantly associated with small loci of mineralization among the collagen fibrils and at the surface of the plastic substrate, and with larger masses of calcified matrix often surrounded by a labeled lamina limitans–like structure (Fig. 8). Frequently also associated with these structures were accumulations of unlabeled, amorphous organic material of unknown origin sometimes interposed between the cell membrane and the sites of mineralization (Figs. 7, 8), or further out within the matrix (Fig. 9). All control incubations showed only background labelling (data not shown).

DISCUSSION

In previous studies of cultured chicken embryonic vertebral chondrocytes, conditions had been

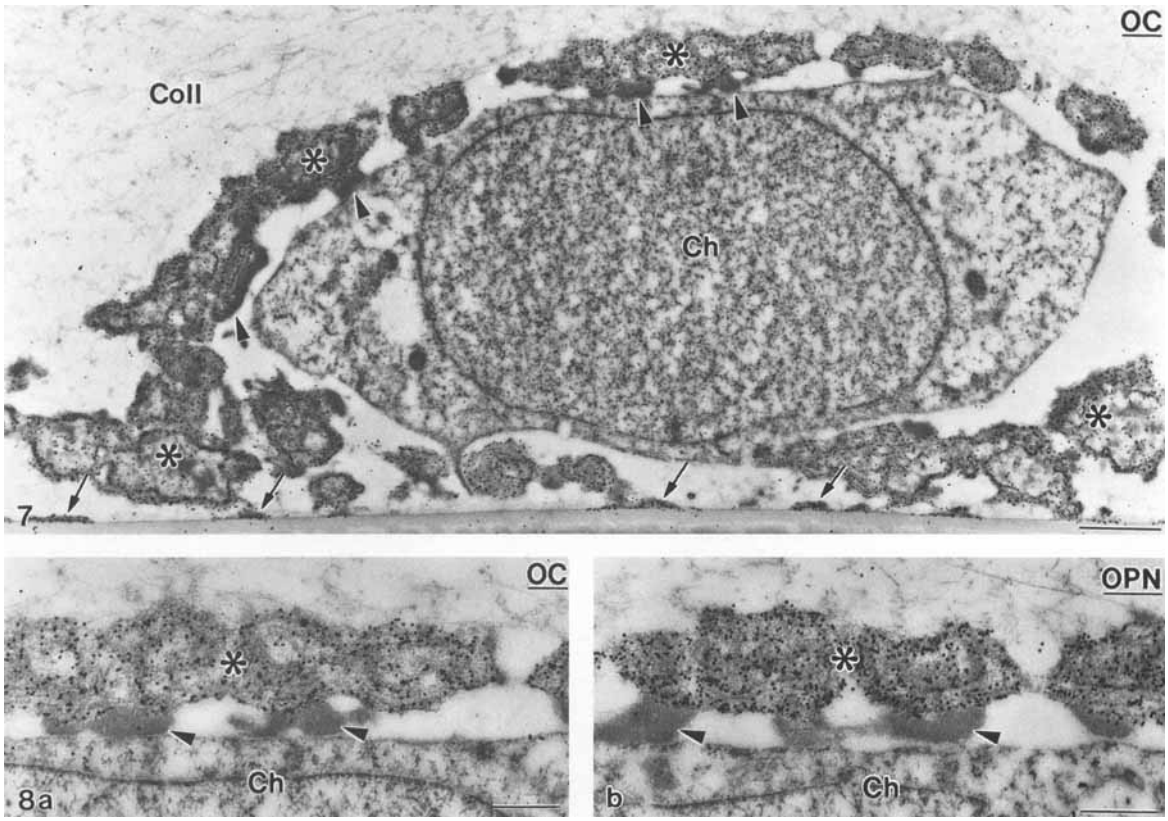

Fig. 7. Relatively low magnification of a chondrocyte (Ch) adjacent to the plastic coverslip and surrounded by a collagenous (Coll) matrix containing an extensive area of originally mineralized matrix (asterisks) in the immediate vicinity of the cell. This originally mineralized matrix, subsequently demineralized during immunocytochemical procedures and staining, is intensely labeled with gold particles following incubation of tissue sections with anti-chicken osteocalcin antibody and the protein A–gold complex. Labeled organic matrix is also present at the surface of the plastic substrate (arrows). Arrowheads indicate unlabeled amorphous, electron-dense organic material of unknown origin interposed between the cell and the labeled matrix. Lowicryl section stained with uranyl acetate and lead citrate. Bar equals 1 μm.

Fig. 8. Higher magnification of labeled matrix (asterisks) adjacent to cells after incubation with anti-osteocalcin (**a**) or anti-osteopontin (**b**) antibody. In both these examples, the majority of gold particles are randomly distributed over the mineralized matrix which is sometimes separated from the cell by unlabeled, amorphous organic material (arrowheads). Lowicryl sections stained with uranyl acetate and lead citrate. Bars equal 0.5 μm.

established which promoted development of the hypertrophic phenotype and mineralization of the extracellular cartilage matrix. In these cultures, development of the hypertrophic phenotypes was shown to occur over a 21-day period, and was characterized by increased collagen Type X and alkaline phosphatase enzyme activity with concurrent decreases in collagen Type II and proteoglycan expression [Gerstenfeld and Landis, 1991]. In the present studies, protein synthesis and matrix deposition of two proteins, osteocalcin and osteopontin, previously shown to be synthesized by differentiated osteoblasts and deposited within the mineralized matrix of bone [Owen et al., 1990; Gerstenfeld et al., 1987, 1990c], were demonstrated during the development of chondrocyte hypertrophy and mineralization of the extracellular matrix (ECM) within these cultures. Similar to the osteoblast cultures, these proteins were deposited in the chondrocyte produced ECM in association with mineral aggregates among the collagen fibrils.

In immunocytochemical studies, both osteocalcin and osteopontin have been localized within the mineralized extracellular matrix of hypertrophic zone cartilage of the chicken [McKee et al., 1992] and the rat [Boivin et al., 1987; Mark et al., 1988]. In contrast, in non-mineralizing zones

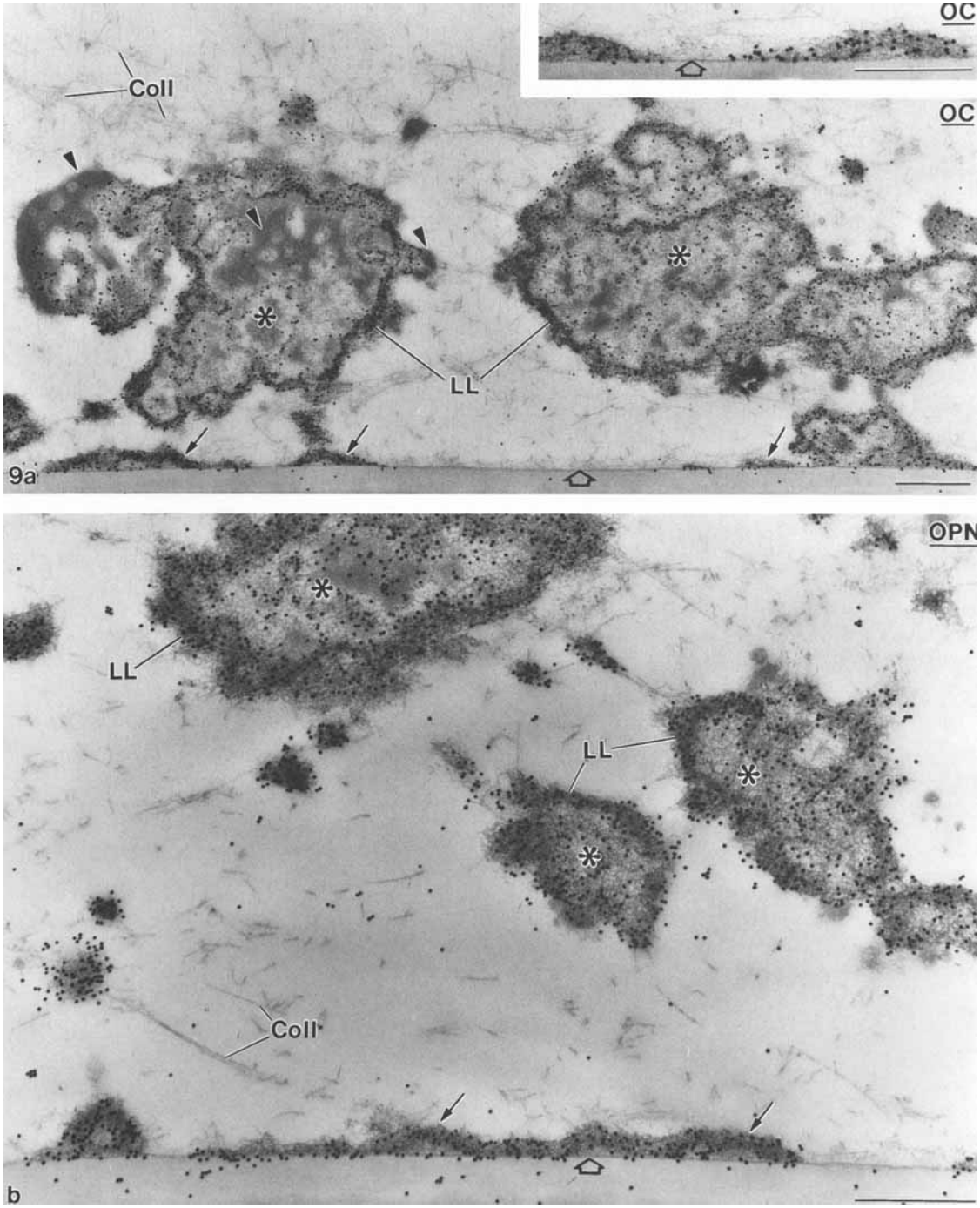

Fig 9 Areas of mineralized matrix are dispersed throughout the cultures and are not always found in close association with cells as shown in Figs 7 and 8 Here both small loci of mineralization throughout the matrix and at the surface of the culture dish (arrows and inset) and larger masses of calcified cartilage (asterisks) are intensely labeled with the anti osteocalcin (**a**) and anti osteopontin (**b**) antibodies Frequently such larger masses of calcified cartilage are surrounded by a lamina limitans–like structure (LL) showing a relatively high concentration of gold particles Occasionally accumulations of unlabeled amorphous organic material (arrowheads) are associated with the sites of calcified cartilage Coll collagen fibrils open arrows upper surface of plastic substrate Lowicryl sections stained with uranyl acetate and lead citrate Bars equal 0 5 μm

of the growth plate neither osteopontin nor osteocalcin can be detected within the matrix. However, chondrocytes within their lacunae are immunoreactive for osteopontin but not osteocalcin [Takashi et al., 1992]. In one study of developing long bones in rats, no expression of these genes was observed by in situ hybridization in the hypertrophic chondrocytes within the growth plate, but this was discussed as related to the technical difficulties of the in situ hybridization within the cartilage growth plate [Weinreb et al., 1990]. While such studies are suggestive that chondrocytes synthesize these proteins, their exact synthetic origin can only be inferred since protein diffusion into the extracellular matrix from either the adjacent bone or the vascular supply may be possible.

In the present study both osteopontin and osteocalcin were definitively shown to be synthesized by cultures of hypertrophic chondrocytes in vitro. The quantitatively higher baseline levels of osteopontin expression in the non-mineralizing cultures and its associated increase in expression during progressive cellular hypertrophy and matrix mineralization is consistent with both the previous in vivo immunocytochemical studies of whole bone as described above, as well as in vitro biochemical analysis of its expression by fetal articular cartilage derived rat chondrocytes [Barone et al., 1991] and cultured embryonic chicken tibial chondrocytes treated with trypsin to prevent adherence [Castognola et al., 1991]. In contrast, osteocalcin expression was induced and increased over a tenfold range in mineralizing cultures. Its expression was observed at very late time points over the 21-day culture period, suggesting that its expression was either restricted to a very late stage of the hypertrophic differentiation of the chondrocyte or its expression was induced by signals from the extracellular mineralized matrix. The prerequisite development of a collagenous matrix for the induction of these proteins within cultured embryonic osteoblasts has been previously demonstrated [Aronow et al., 1990; Gerstenfeld et al., 1987]. In the present experiments, the intermediate levels of expression of osteocalcin and osteopontin in complete DME media supplemented with ascorbate and β-GPO_4 is suggestive that the development of the extracellular mineralized matrix is also necessary in part for their expression in these cells. A concern of these observations is that osteocalcin has been well documented as a bone-specific protein with undetectable mRNA levels in various non-calcified cartilages [Lian et al., 1989; Hale et al., 1987].

Other evidence that hypertrophic chondrocytes express osteocalcin and bone-related proteins comes from studies of murine mandibular condyles grown in vitro [Strauss et al., 1990]. Hypertrophic chondrocytes were shown to express osteopontin mRNA by in situ hybridization, and osteocalcin was detected after several days in culture in cells that produced osteoid. While the studies of Strauss et al. [1990] demonstrated osteocalcin positive cells in the transition region between the hypertrophic zone and osteoid in mandibular condyle cultures, to date this is the first in vitro study of cultured chondrocytes with hypertrophic features that has shown either mRNA or protein expression of osteocalcin. In the studies of Barone et al. [1991], osteocalcin was not produced at any time over a 4-week culture period of articular cartilage-derived cells which never developed a mineralized matrix. Since osteocalcin is only induced with chondrocyte hypertrophy associated with mineralized matrix, and at a level of 1/100 synthesized in mature osteoblasts, the protein can still be considered restricted largely to osteoblastic or odontoblastic cells [Lian et al., 1989].

Because the chondrocyte cultures are derived from a tissue which normally undergoes bone replacement, there is the possibility that osteoprogenitor cells may be present in the cell population grown in these cultures. However, a number of aspects concerning how these cultures were prepared do not favor this possibility. The chondrocytes used for these studies were derived from 11–12-day embryonic vertebrae which are still composed entirely of cartilage, since bone formation does not begin until day 13 [Shapiro, 1992]. In the preparation of these cells the vertebral tissue was initially pre-treated for 20 min in trypsin collagenase to remove the surface progenitor cells that give rise to the future osteoblasts. Finally, only floating non-adherent cells were selected after 1 week of growth in MEM media to initiate the hypertrophic cultures. Both light and EM analysis of these cultures demonstrated that the morphological appearance of these cultures was similar to that of hypertrophic cartilage in vivo. The majority of the cells had a large rounded appearance in well-defined lacunae throughout the cell layer as shown in Figure 5, and only randomly distributed collagen Type II fibrils ($\sim$ 20 nm diam) were seen in the extracellular matrix. Both osteocalcin and osteopontin showed specific immuno-

localization to electron-dense regions of mineralization peripheral to the cells and throughout the matrix, in an identical fashion as seen in vivo. While both proteins are associated with sites of mineralization, their ultrastructural appearance and localization of these proteins is unique to cartilage and very different from bone [McKee et al., 1992]. These results, therefore, suggest that the chondrocytes throughout the culture cell layer were synthesizing these proteins. It is interesting to note, however, the unique morphological appearance of the surface cells most proximal to the media.

The fate of hypertrophic chondrocytes within tissues undergoing endochondral replacement has been of considerable speculation. Morphological data has long suggested that these cells undergo programmed death; however, it has also been proposed that at least some of these cells may persist and are incorporated into the endochondral bone [Farnum and Wilsman, 1987; Hunziker et al., 1984]. Strauss et al. [1990] and Silberman et al. [1983] reported in their studies that at least some of the cells of the mandibular condyle have the potential to express the bone phenotype. This may result from artifactual conditions produced by culturing the mandible in vitro or alternatively may reflect differences in the developmental pattern of the mandible compared to that of a long bone; however, the cell populations in both long bones and the mandible are mesodermal in origin [Hall, 1987], suggesting that chondrocytes might have the potential to express bone-related proteins. Several reports have demonstrated that at least some hypertrophic cells do indeed survive [e.g., Kahn and Simmons, 1977; Yoshioka and Yagi, 1988] and undergo additional phenotypic changes into a unique cell type showing some of the phenotypic properties of osteoblasts [Strauss et al., 1990]. Most interesting are observations in the culture system described by Descalzi-Cancedda et al. [1992] in which clonal cells derived from chick embryo limb cartilage differentiate beyond the stage of collagen Type X expression and express a marker, Ch21, unique to both hypertrophic chondrocytes and osteoblasts as well as a 63 kDa protein found only in the late stage of hypertrophic chondrocytes in culture. The finding of a clonal cell line with such properties supports the concept that our results of osteocalcin expression may be derived from further differentiation of the hypertrophic chondrocyte in their lacunae to cells that produced bone matrix which immunostained for collagen Type I and osteocalcin. In the experiments of Thesingh et al. [1991], periosteal free embryonic metatarsal at the cartilage stage was co-cultured with cerebral tissue, and the matrix produced by the cells in their lacunae was shown to be immunoreactive for Type I collagen and osteocalcin. These authors hypothesized that a transdifferentiation (i.e., via a dedifferentiation stage) occurred between the chondrocyte and osteoblastic lineages. Thus, the possibility exists that in our cultures, a subset of cells may first dedifferentiate and then be activated to differentiate osteoblasts by factors accumulated in the mineralized matrix or within the culture media.

It is clear from the several previous studies discussed above and those presented herein that both cartilage tissue and chondrocytes in vitro appear capable of expressing bone-like phenotypic properties. To resolve the question as to whether hypertrophic chondrocytes further progress into a unique cell type or into osteoblasts which produce low levels of osteocalcin, or whether only a subpopulation of cells have changed their phenotype to an osteoblastic cell, in situ hybridization for osteocalcin and Type X collagen mRNA need to be carried out. If both markers appear within the same cell, definitive proof that osteocalcin expression is associated with the hypertrophic chondrocytes would be provided. Notably, osteocalcin expression (mRNA levels) was not found associated with pathologically calcified tissue in the rat subcutaneous model of calcergy [Boivin, Lian, and Stein, unpublished observations].

These studies have provided evidence for the induction of osteocalcin and increased levels of osteopontin in hypertrophic chondrocytes within a calcifying cartilage matrix. These results raise the question as to the biological function of these proteins which are expressed in the hypertrophic chondrocyte. Osteocalcin appears in fetal bone tissue at the onset of mineralization. Both osteocalcin and osteopontin have been demonstrated by immunocytochemical techniques in bone tissue to be synthesized by osteoblasts and to accumulate at sites of mineralization within bone. Similarly, these same proteins are found in calcified cartilage in the hypertrophic zone of the growth plate. Taken together, these observations suggest a potential function in contributing to mineral deposition [Glimcher, 1989; Boskey, 1989] in these tissues. However, both proteins have also been implicated in mediating osteoclastic resorption of hydroxyapatite. Previous studies have documented the role of osteocal-

cin in recruitment and differentiation of osteoclast progenitors [Glowacki et al., 1991] and the role of osteopontin in osteoclast interaction via its R-G-D sequence [Oldberg et al., 1986; Helfrich et al., 1992; Reinholt et al., 1990; Gotoh et al., 1990; Miyachi et al., 1991]. Thus, the induction of osteocalcin synthesis and increased osteopontin synthesis in hypertrophic-like chondrocytes in the mineralizing matrix may potentially relate to their ability to serve as signals for osteoclast resorption of the calcified cartilage matrix.

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NOTE ADDED IN PROOF

M.I. Roach provided evidence using immunostaining for osteocalcin, osteopontin, and type I collagen that chick chondrocytes in their lacunae produce a bone matrix in organ culture. Bone and Mineral (1992) 19:1–20.

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