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Morphological and Biochemical Characterization of Mineralizing Primary Cultures of Avian Growth Plate Chondrocytes: Evidence for Cellular Processing of Ca^{2+} and Pi Prior to Matrix Mineralization

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Abstract Advances in the culture of mineralizing growth plate chondrocytes provided an opportunity to study endochondral calcification under controlled conditions. Here we report that these cultures synthesize large amounts of proteins characteristically associated with mineralization: type II and X collagens, sulfated proteoglycans, alkaline phosphatase, and the bone-related proteins, osteonectin and osteopontin. Certain chondrocytes appeared to accumulate large amounts of Ca^{2+} and Pi during the mineralization process: laser confocal imaging revealed high levels of intracellular Ca^{2+} in their periphery and X-ray microanalytical mapping revealed the presence of many Ca^{2+}- and Pi-rich cell surface structures ranging from filamentous processes 0.14 ± 0.02 μm by 0.5–2.0 μm, to spherical globules 0.70 ± 0.27 μm in diameter. Removal of organic matter with alkaline sodium hypochlorite revealed numerous deposits of globular (0.77 ± 0.19 μm) mineral (calcospherites) in the lacunae around these cells. The size and spatial distribution of these mineral deposits closely corresponded to the Ca^{2+}-rich cell surface blebs. The globular mineral progressively transformed into clusters of crystallites. Taken with earlier studies, these findings indicate that cellular uptake of Ca^{2+} and Pi leads to formation of complexes of amorphous calcium phosphate, membrane lipids, and proteins that are released as cell surface blebs analogous to matrix vesicles. These structures initiate development of crystalline mineral. Thus, the current findings support the concept that the peripheral intracellular accumulation of Ca^{2+} and Pi is directly involved in endochondral calcification.

Key words: chondrocytes, cell culture, mineralization, calcospherites, Ca and P mapping, matrix vesicles

With recent advances in the ability to grow normal growth plate chondrocytes, primary cultures now provide an opportunity to study cell-mediated mineralization in vitro under controlled conditions. However, a prerequisite for such systems is that the cultured cells must undergo progressive development and differentiation similar to events known to occur in vivo. Furthermore, the deposition of mineral in the extracellular matrix should closely match the normal pattern seen in vivo.

Several chondrocyte culture systems have been described recently that support formation of mineral in vitro. These include chick embryo sternal suspension cultures in agarose [Bruckner et al., 1989], micromass cultures of embryonic chick limb buds in serum-containing media [Boskey et al., 1992], secondary cultures of either *nonadherent* chick embryo vertebrae [Lian et al., 1993] or of *adherent* chick embryo tibiae, followed by suspension culture [Cancedda et al.,

Abbreviations used: ACP, amorphous calcium phosphate; AP, alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PG, proteoglycan; Pi, inorganic P; PMSF, phenylmethylsulfonylfluoride; PS, penicillin/streptomycin solution; SCL, synthetic cartilage lymph; SEM, scanning electron microscopy; TCA, trichloroacetic acid; TMS, Tris-Mg^{2+}-sucrose buffer; TMT, Tris-Mg^{2+}-Triton X-100 buffer.

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1992]. In addition, primary cultures of chick embryo cephalic and caudal sternal chondrocytes [Coe et al., 1992], and organ cultures of chick embryo mesenchymal limb buds [Zimmerman et al., 1992] have been reported. Previous work from our laboratory has shown that primary cultures of growth plate chondrocytes from 6–8 week old broiler-strain chicken undergo apparently normal mineralization only when supplemented with ascorbate [Wu et al., 1989, 1992] and *physiological* levels of inorganic P (Pi) in the medium [Ishikawa and Wuthier, 1992]. These, and the above studies, point to the importance of phosphate supplementation, either by addition of β-glycerophosphate or Pi to the culture medium, for proper matrix calcification.

However, the factors that trigger the sequential development of the chondrocytes to terminal differentiation and calcification are still unknown. It is also unclear when the set of distinctive proteins associated with mineral deposition become expressed during the different stages of cellular differentiation.

In the present study, we explored the synthesis of proteins characteristic of cartilage phenotype, as well as those associated with the process of mineralization. We found that the cultures synthesized large amounts of type II and X matrix collagens, and sulfated proteoglycans, during the course of the culture period. Immunoblot analyses revealed that, with increasing age of the cultures, the mineralizing chondrocytes also synthesized significant amounts of bone-related proteins such as osteonectin and osteopontin. Laser confocal Ca^{2+} imaging, scanning electron microscopy (SEM), and X-ray microanalytical mapping showed enrichment of Ca and P in specific cells, prior to buildup of mineral in the matrix. Globular mineral (calcospherite) and progressively transformed clusters of crystallites were found to occupy in the walls of cell lacunae. The findings from these mineralizing cultures, together with earlier studies [Wuthier and Gore, 1977; Boskey et al., 1980; Wuthier, 1993], indicate that intracellular accumulation of Ca^{2+} and Pi leads to formation of complexes of amorphous calcium phosphate (ACP) complexed with cell membrane lipids and proteins. These structures are released as cell surface blebs analogous to matrix vesicles that initiate the development of crystalline mineral in the extracellular matrix.

MATERIALS AND METHODS

Chondrocyte Cultures

Chondrocyte isolation. Chondrocytes were isolated from the epiphyseal growth plate cartilage of the tibiae of 6–8-week-old hybrid broiler-strain chickens by a modification of previously described methods [Wu et al., 1989]. The cartilage (4–6 g, from 3 tibiae) was cut into 2–3 mm cubes and digested with 0.25% trypsin in 5 ml of synthetic cartilage lymph (SCL) [Majeska and Wuthier, 1975] at 37°C for 20 min. The majority of the trypsin solution was removed with a sterile Pasteur pipette, then 10 ml of crude collagenase (Worthington CLS II, 126 units/mg, 0.03% final concentration) solution in SCL was added and digestion continued for 30 min at 37°C. The digestion solution was then removed and 15 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin (DMEM/FBS/PS), and additional collagenase (0.025%, final concentration) were added. The digestion was continued at 34°C overnight. The cells were isolated the next day as follows: after replacing the digesting medium with 15 ml of fresh collagenase-free DMEM/FBS/PS, the tissue suspension was vortexed three times, 1 min each, followed by dilution with medium to 25 ml, and centrifugation for 5 sec at 3,000 rpm in a clinical centrifuge to sediment the undigested tissue. The supernatant was then centrifuged for 15 min at 3,000 rpm to sediment the released cells. This pellet was demineralized with isotonic pH 6.0 buffer (80.5 mM trisodium citrate, 8.0 mM citric acid) for 10 min at 34°C. After centrifugation at 3,000 rpm for 15 min, the demineralized cell pellet was then washed with DMEM/FBS/PS and resuspended in the same medium.

Chondrocyte cultures. Chondrocytes were plated at a density of 4.5×10^5 cells per 35-mm dish and cultured in 2 ml of DMEM/FBS/PS at 37°C under an atmosphere of 95% air/5% CO_2. The culture medium was changed every 3–4 days. For serum-free media, on day 6 or 7, cells were switched to a medium composed of a 1:1 mixture of DMEM/FBS/PS and serum-free HL-1 medium; from day 10 onward, cells were given only HL-1. For DATP5 medium, cells were switched to DMEM supplemented with 5% FBS, 8 amino acids (in mM: Ala 7, Asn 0.4, Asp 4, Glu 10, Gly 7, Pro 3, Ser 2, taurine 2), insulin (5 μg/ml), transferrin (5 μg/ml), selenite (5 ng/ml), and 1 mM Na_2HPO_4 on day 6 or 9 [Ishikawa

and Wuthier, 1992]. Ascorbate was provided from day 3 onward at a concentration of 50 μg/ml. For SEM work, cells were plated in Falcon cell culture dishes with Cyclopore membrane inserts; for observation of calcospherites after hypochlorite treatment, Corning 35 mm dishes were used. Serum-free and serum-containing media were used to determine which is more suitable for future studies of the effect of growth factors on chondrocyte development.

Cell harvest. Chondrocytes were harvested from the 35-mm culture dishes after removal of the medium. The cell layer was rinsed twice with 1 ml of TMS (50 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, and 10% (w/v) sucrose) and was scraped from the culture dishes after addition of 2 ml of TMS. The procedure was repeated twice with 1 ml TMS to ensure removal of all cell and matrix material. This suspension was sedimented at 3,000 rpm for 30 min using the clinical centrifuge, and 1 ml of TMT (10 mM Tris, pH 7.5, 0.5 mM $MgCl_2$, 0.1% Triton X-100) was added. The suspension was then frozen at −20°C, and later after thawing, the harvested material was dispersed by sonication (~ 10 s), and alkaline phosphatase (AP) activity and total cellular protein were determined. Typically, cultures were studied up to day 30 after heavy mineral fronts had occurred. In some cases, cultures were grown to day 48 to investigate the expression of proteins characteristically associated with long-term cultures and to determine if the cultured chondrocytes would revert to an osteoblastic phenotype.

Chemical analyses. Protein content was analyzed using bovine serum albumin as a standard [Lowry et al., 1951]. AP activity was determined using 2 ml of 1 mM *p*-nitrophenylphosphate as substrate containing 750 mM 2-amino-2-methyl-1-propanol and 0.25 mM $MgCl_2$, pH 10.3, measuring from the absorbance at 402 nm, 37°C, the release of *p*-nitrophenol (ϵ_{402} = 19,050 M^{-1} cm^{-1}) after addition of 50–100 μl of sonicated TMT cell suspension [Cyboron and Wuthier, 1981].

For analysis of the calcium and phosphate content of the cell cultures, the TMT cell suspension was centrifuged at 3,000 rpm for 40 min and the TMT supernatant removed. The resulting mineral-containing cell/matrix pellet was treated with 1 ml of 0.1 N HCl to dissolve the mineral. In some advanced cultures with heavy mineralization, after scraping the cell/matrix layer (see Cell Harvest), 1 ml of 0.1 N HCl was added to the scraped dishes, and after 1 h at room temperature, transferred to the cell/matrix pellet and kept at 4°C overnight. The 0.1 N HCl extract was centrifuged as above, and aliquots were taken for Ca^{2+} and Pi analysis. Ca^{2+} was measured using *o*-cresolphthalein complexone [Baginsky et al., 1973], and Pi using the ammonium molybdate method [Ames, 1966]. For spectrophotometric analysis of proteoglycan (PG) content of the cultures, to the 0.1 N HCl pellet (in test tubes) was added 0.5 ml of a mixture of 4 M guanidine hydrochloride, 75 mM sodium acetate buffer (pH 5.8), 5 mM benzamidine, and 15 mM EDTA, and incubated overnight at 4°C. An aliquot of this extract was then mixed with dimethylmethylene blue, and the blue color monitored at 595 and 520 nm [Chandrasekhar et al., 1987].

Analyses of Cellular Synthesis

General protein and collagen in the culture medium. After labeling the cultures with 2 μCi/dish of [^{3}H]proline (35 Ci/mmol, NEN, Boston, MA) for 48 h, the culture media (2 ml) was collected. The total protein present in 0.5 ml of media was precipitated using trichloroacetic acid (TCA, 15%, w/v), and after washing with acetone, the TCA precipitates were dissolved in 100 μl of sample buffer [Laemmli, 1970] by heating in boiling water. A collagen-enriched fraction was precipitated from 0.5 ml of media using 30% ammonium sulfate saturation. The collagen-containing fractions were dissolved in 125 μl of 0.5 N acetic acid. All samples were then added to 5 ml of Beckman Ready Protein scintillant and counted.

Proteoglycan. Synthesis of medium and cell/matrix layer proteoglycan (PG) was analyzed by incorporation of radiolabeled sulfate. Chondrocyte cultures were labeled on specified days by addition of 10 μCi of [^{35}S]sulfate (43 Ci/mg S, ICN Biomedicals, Costa Mesa, CA) to each 35 mm dish containing 2 ml of media. After 24 h labeling, the culture medium was collected, protease inhibitors (5 mM EDTA, 1 mM benzamidine, 0.2 mM PMSF) were added, the medium was clarified by centrifugation for 10 min at 3,000 rpm, and a 100 μl sample of the supernatant was added to 1 ml of ethanol and precipitated overnight at 4°C. The precipitate was collected by centrifugation at 10,000 rpm for 10 min; the pellet was washed once with ethanol, and then dissolved in Laemmli sample buffer and counted. The cell/matrix layer was also analyzed for PG content after rinsing twice with

TMS buffer. To solubilize the cell/matrix PG, 1 ml of 75 mM sodium acetate buffer (pH 5.8) containing 4 M guanidine hydrochloride, 5 mM benzamidine, and 15 mM EDTA was added and incubated at 4°C overnight with gentle agitation [Heinegard and Sommarin, 1987]. The extract was transferred to test tubes and centrifuged for 10 min at 3,000 rpm. A 100 μl sample of the supernatant was added to 1 ml of ethanol, precipitated, washed, redissolved, and counted.

SEM specimen preparation. Cultures grown on Falcon membrane inserts were examined by SEM on various days after reaching confluency for the morphology and progression of calcium phosphate mineral development. The cell layer was fixed by emersion of the membrane inserts in 2% glutaraldehyde, 0.1 M sodium cacodylate buffer (containing 1 mM $CaCl_2$), pH 7.4, overnight. After rinsing 3 times with the buffer, the preparations were postfixed with 2% OsO_4 for 60 min and then rinsed with buffer. After stepwise dehydration in graded ethanol (25%, 50%, 75%, 95%, 100%, 100%, 10 min each), the preparations were exchanged twice with isoamyl acetate (10 min each) and then critical-point dried. The dried culture preparations were cut from the inserts, mounted on SEM specimen stubs, and coated with either gold or carbon. SEM observation was performed using a Hitachi scanning electron microscope (S-2500 Delta) at an accelerating voltage of 20 kV. Some carbon-coated samples were further analyzed for Ca and P mapping using an EDS-SEM (JEOL T200) scanning electron microscope.

To examine the underlying layers of the cultures, laboratory compressed air was blown over the surface of the samples to remove the upper layers of the culture and expose the underlying cells and mineral deposits. These air-blown samples were then coated with either carbon or gold before SEM examination.

In some cultures, beyond day 24, the dense mineral deposits were revealed by removal of organic matter with sodium hypochlorite (NaOCl) treatment [Boyde and Sela, 1978]. The culture dishes were soaked in alkaline (pH 11) 5.25% NaOCl solution for 30 min with gentle agitation, rinsed with distilled water, and dehydrated.

Confocal image analysis of intracellular Ca^{2+}. For the measurement of intracellular free Ca^{2+}-ion concentrations, live cells cultured in Nunc glass coverslips chambers, after reaching confluency, were loaded with a fluorescent Ca^{2+} indicator, Indo 1-AM (0.5–2 μM) at 25°C for 30–60 min [Grynkiewicz et al., 1985]. The cells were then rinsed with PBS, and fluorescent images were obtained using a Meridian ACAS 570 laser confocal scanning system. The excitation wavelength was set at 355 nm and the emission wavelength at 405 and 485 nm. Unbound Indo 1 within the cell emits at 485 nm, whereas Ca^{2+}-bound Indo 1 emits at 405 nm. The ratio of Ca^{2+}-chelated to free Indo-1 (405/485) in the cells was used to generate a relative Ca^{2+} response curve.

SDS-PAGE analysis. Secreted media and cell/matrix proteins and collagens, equivalent to 0.10–0.15 ml of original media (or as indicated) were analyzed by 7.5–15% gradient SDS-PAGE [Laemmli, 1970]. Media proteins were precipitated with TCA (15%, w/v), acetone washed, and then boiled in Laemmli sample buffer. Collagens were isolated from the media by 30% saturated ammonium sulfate (v/v), followed by digestion in 0.5 M acetic acid in the presence of pepsin (Sigma, P-7012, 150 μg/ml) at 4°C overnight. Samples of the cell/matrix layer, after harvesting and sonicating in 1 ml of TMT, were also analyzed by SDS-PAGE. Collagens in the cell/matrix layer were isolated by incubating the harvested TMS cell layer pellet with 0.5 M acetic acid (0.5 ml/dish; n = 3) containing the previously mentioned protease inhibitors at 4°C overnight. The acetic acid-cell layer mixture was sonicated to yield a uniform suspension, and then was digested with pepsin. Collagen present in the supernatant after centrifugation of the pepsin digest was precipitated with TCA, washed with acetone, or precipitated with 2.75 M NaCl, and then boiled in Laemmli sample buffer before SDS-PAGE.

Immunoblot analysis. Media and cell/matrix layer proteins after SDS-PAGE, were transferred to nitrocellulose sheets and probed with anti-osteonectin, and anti-osteopontin antibodies by the method of Towbin and Gordon [1984].

Materials. Alkaline phosphatase substrate (*p*-nitrophenylphosphate), TES (N-tris [hydroxymethyl] methyl 2-aminoethane sulfonic acid), Trizma base, Triton X-100, trypsin (type III from bovine pancreas), Dulbecco's modified Eagle's medium (DMEM), ascorbic acid, and pepsin (porcine, 2,900 units/mg protein), various amino acids, chondroitin sulfate A, bovine serum albumin, and *o*-cresolphthalein complexon (phthalein purple) were all from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum

TABLE I. Changes in the Levels of Total Protein and Alkaline Phosphatase Activity During Culture of Growth Plate Chondrocytes

Medium	Time in culture (days)	Alkaline phosphatase activity (nmol pNPP/min/dish)	Total protein (μg/dish)	AP specific activity $\left(\frac{\text{nmol pNPP/min}}{\text{mg protein}}\right)$
HL-1[a]	13	45 ± 11	168 ± 15	264 ± 64
	17	27 ± 5	187 ± 12	138 ± 22
	20	104 ± 38	399 ± 36	243 ± 72
	24	105 ± 46	388 ± 66	241 ± 67
	28	131 ± 50	608 ± 44	205 ± 69
	31	41 ± 21	560 ± 41	67 ± 29
	35	49 ± 19	971 ± 74	52 ± 18
	38	103 ± 15	1,132 ± 42	91 ± 13
	41	29 ± 10	992 ± 102	28 ± 10
	48	14 ± 5	1,090 ± 19	13 ± 5
DATP5[b]	3	73 ± 10	134 ± 12	543 ± 25
	6	129 ± 5	190 ± 5	667 ± 21
	9	119 ± 4	264 ± 6	452 ± 9
	12	148 ± 16	375 ± 12	390 ± 40
	15	206 ± 31	480 ± 20	428 ± 51
	18	247 ± 3	576 ± 21	430 ± 11
	21	725 ± 55	738 ± 16	983 ± 71
	24	1,094 ± 226	805 ± 82	1,348 ± 147
	27	885 ± 43	859 ± 48	1,036 ± 112

[a]HL-1 is a serum-free medium that contains 10 mM β-glycerophosphate.
[b]DATP5 medium contains 5% fetal bovine serum and 1 mM Na_2HPO_4, but does not contain any added organic phosphate.

(FBS) was from Hyclone (Logan, UT), and antibiotic (100×) (penicillin G, sodium 10,000 units, and streptomycin sulfate, 25 μg/ml normal saline) were purchased from GIBCO (Grand Island, NY). HL-1 serum-free medium was from Ventrex (Portland, ME); collagenase (CLS II) was from Worthington Biochemicals (Freehold, NJ). [^{3}H]Proline (35Ci/mmol) was from NEN (Boston, MA) and [^{35}S]Na_2SO_4 (carrier-free, ~43 Ci/mg S) were from ICN Biomedicals (Costa Mesa, CA). Sterile culture dishes were obtained from Corning Glass Works (Corning, NY). Indo-1AM was from Molecular Probes, Inc. (Eugene, OR), and 1,9-dimethyl-methylene blue was from Aldrich Chemical Co. (Milwaukee, WI).

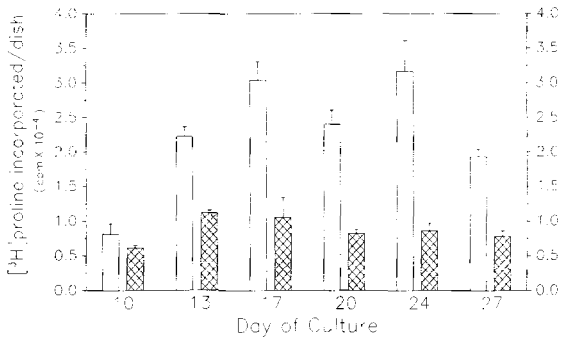

Fig. 1. Synthesis of general protein and collagen in the HL-1 culture medium. After labeling the cultures for 48 h with [^{3}H]proline (2 μCi/dish), two 0.5 ml portions of 2.0 ml of media were collected on each specified day. The total protein was precipitated with TCA and the collagen fraction was enriched by precipitation with 30% saturated ammonium sulfate, harvested, boiled in sample buffer, and counted. Open bars = general protein; cross-hatched bars = collagen.

RESULTS

Growth and Development of Cultured Growth Plate Chondrocytes

This report characterizes chicken growth plate chondrocyte primary cultures in terms of the development and differentiation of the cell/matrix layer, and the formation and morphology of the mineral deposits. In *HL-1 media,* there were progressive increases in total protein throughout the culture; AP became prominent by day 28, and after that underwent cyclic change (Table I). Secreted media proteins were investigated by following [^{3}H]proline incorporation into both the TCA (general protein) and 30% saturated ammonium sulfate-precipitable (collagen-enriched) fractions (Fig. 1). General protein synthesis increased progressively up to day 17, with

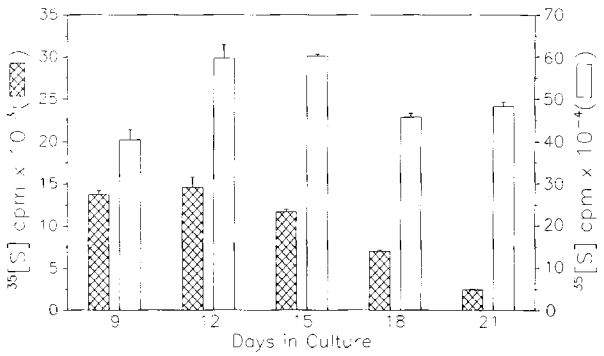

Fig. 2. Synthesis of proteoglycan in the HL-1 culture medium and the cell/matrix layer. Chondrocyte cultures were labeled with 10 μCi of [^{35}S]sulfate for 24 h prior to harvest. On the specified day, both the medium and the cell/matrix layer were separately fractionated to precipitate the proteoglycan as described in Materials and Methods. Values from 50 μl samples of 2 ml of the medium, and 50 μl samples of 1 ml cell/matrix layer homogenate, are shown. Open bars = culture medium; cross-hatched bars = cell/matrix layer.

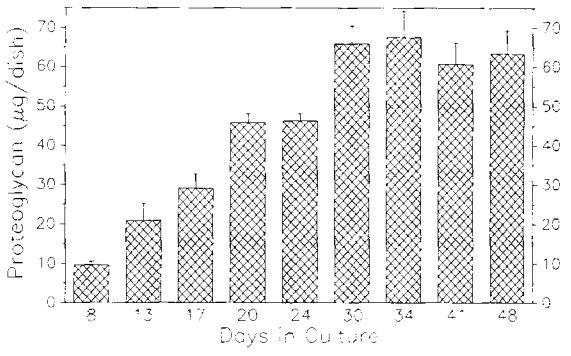

Fig. 3. Changes in proteoglycan content of the cell/matrix layer during the course of chondrocyte culture in HL-1 medium. Culture dishes were harvested in TMT buffer on specified days of the culture period as described in Materials and Methods. After centrifugation of the TMT suspension, the resulting mineral-containing cell/matrix pellet was treated with 0.1 N HCl to dissolve the mineral for Ca^{2+} and Pi analyses. Following centrifugation of the 0.1 N HCl suspension, to the pellet was added 0.5 ml of a solution containing 4 M guanidine · HCl, 75 mM sodium acetate (pH 5.8), 5 mM benzamidine, and 15 mM EDTA, and the mixture incubated overnight at 4°C. A 25 μl sample of this extract was then mixed with 0.4 ml of 50 mM sodium acetate (pH 6.0) and 1.6 ml of dimethylmethylene blue, and the blue color monitored at 595 and 520 nm. Proteoglycan levels were calculated from the difference in absorbancy at these wavelengths, using known amounts of chondroitin sulfate as standards.

variable levels thereafter; labeled collagen levels in the culture medium reached a maximum after 13–17 days and then remained relatively constant. In serum-containing *DATP5 media,* chondrocytes grew faster and expressed higher AP activities than in HL-1 medium (Table I).

Figure 2 shows the production of proteoglycan in HL-1 medium, monitored by using [$^{35}SO_4$]-labeling. Proteoglycan production in the cell layer peaked between days 9 and 12, and declined progressively thereafter to day 21. Levels of proteoglycan in the media reached peak values around days 12 to 15, but remained relatively constant thereafter. Using the dimethylene blue dye binding assay (Fig. 3), the total proteoglycan content of the cell/matrix layer was shown to increase steadily from day 8 to day 30, thereafter remaining unchanged.

Progression and Morphology of Calcium Phosphate Mineral Formation

Figure 4 shows the accumulation of Ca^{2+} and Pi mineral in the cell/matrix layer in HL-1 and DATP5 media. Significant Ca^{2+} and Pi mineral deposition was first noted at day 17–18, and increased progressively thereafter. In HL-1 medium (Fig. 4, top), mineral deposition reached its highest level by day 48, with Ca^{2+}/Pi molar ratios averaging 1.73 ± 0.13 from day 17 onward. With 5% serum-containing DATP5 medium (Fig. 4, bottom), similar mineral deposition was seen, with Ca^{2+}/Pi molar ratios averaging 1.56 ± 0.04.

Examination of the developing cell cultures on days 15, 18, and 21 at low magnification by SEM revealed multilayers of cells (Fig. 5). On the surface layer, the cells were more rounded and appeared smaller than the more flattened underlying cells. The surfaces of the rounded cells was variable: some were covered with many short hair-like processes (Fig. 5A,D), some were smoother with a wrinkled and ruffled outer membrane surface (Fig. 5A–C). Many of the underlying cells had numerous filamentous microvillar processes and ruffled membranes. Often, a web of matrix protein appeared to be associated with the cell surface (Fig. 5C). In "undisturbed" preparations, the mineral deposits were hidden beneath the superficial layer. However, when the surface layer was blown away by a jet of air, the underlying cells often revealed the presence of numerous cell surface structures ranging from filamentous processes 0.14 ± 0.02 μm diameter by 0.5–2.0 μm long, to spherical globules 0.25 to 2.0 μm (mean ± SD = 0.70 ± 0.27 μm) in diameter (Fig. 5E,F). These surface features may reflect different stages of chondrocyte maturation. Using EDAX analysis, the cell surface globules appeared to be rich in Ca and P. Significant amounts of S were also detected at these sites,

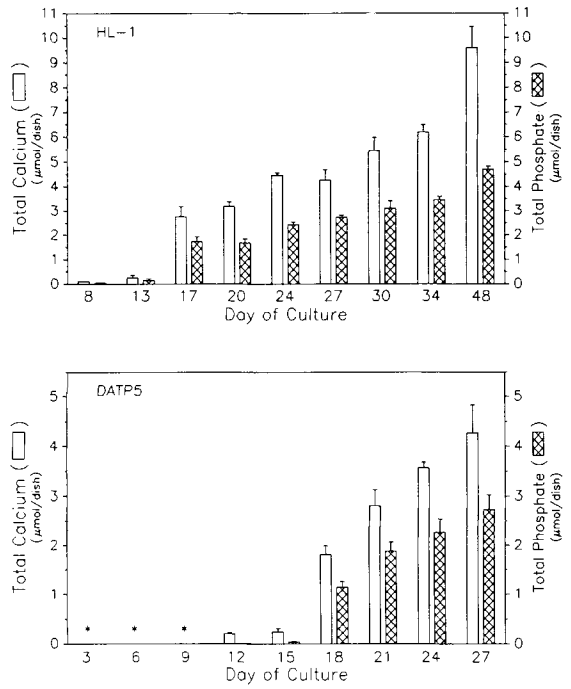

Fig. 4. Changes in total Ca^{2+} and Pi content of the cell/matrix layer during the course of chondrocyte culture. Samples of the 0.1 N HCl extract (See legend to Fig. 3) were analyzed for Ca^{2+} and Pi content as described in Materials and Methods. **Top:** HL-1 medium. **Bottom:** DATP5 medium. *Not analyzed.

indicative of sulfated proteoglycans (data not shown).

Four independent approaches were used to ascertain whether the apparent presence of Ca^{2+}-rich cell surface features was valid: 1) analysis of live cultures by phase-contrast microscopy; 2) examination of living chondrocytes for intracellular Ca^{2+} using the fluorescent Ca^{2+} probe, Indo-1, and laser confocal imaging; 3) examination of the mineralizing cultures after removal of the organic material with alkaline NaOCl; and 4) elemental mapping under SEM of Ca and P distribution in mineralizing cultures.

In early stages of mineral deposition, under phase-contrast light microscopy, we frequently noted that the cells developed a pericellular ring of opaque (dark) material, apparently rich in Ca^{2+} (Fig. 6A). These structures were closely correlated with subsequent mineral formation. The opaque rings of mineral were detected by phase contrast microscopy around the cells on day 21 in both DATP5 and HL-1 cultures. Essentially the same features were present between day 17 and 30, but the number of opaque rings increased with time and the surrounding area became extensively dark and mineralized. Confocal laser imaging of the live, mineralizing cell cultures also revealed the presence of tiny, intensely Ca^{2+}-rich structures associated with the perimeter of the cell surface membrane (Fig. 6B). Also note that $[Ca^{2+}]_{ic}$ varied widely from cell to cell.

Similarly, after alkaline NaOCl treatment on advanced cultures (Fig. 7), a mineralized wall appeared to surround each cell lacunae (15.1 ± 2.6 μm diameter) in the mineralizing cultures (Fig. 7A). At higher magnification, small globular (0.77 ± 0.19 μm diameter) mineral deposits were seen lining the walls of the lacunae (Fig. 7B); these appear to correspond to the cell surface globules seen in the standard SEM preparations (Fig. 5E,F). More mature globules (seen in upper left corner of Fig. 7B) appeared to transform into clusters of needle-like crystallites ~0.1 μm thick and ~1.0 μm long (Fig. 7C,D). Note that different stages of mineral deposition are evident in most culture dishes. In the field shown, both globular and needle-like structures are present.

Finally, SEM preparations of mineralizing cultures similar to those shown in Figure 5 were mapped for Ca and P using secondary electron imaging techniques (Fig. 8). Figure 8A shows the SEM image of the cultured cells, Figure 8B shows the Ca map, and Figure 8C the P map of the cultures. It is evident that at this stage of mineral formation the Ca and P maps coincide over highly selected cells.

Synthesis of Characteristic Proteins During Chondrocyte Maturation

SDS-PAGE was used to analyze the proteins and collagens secreted into the HL-1 media and deposited in the cell/matrix layer at different stages of the culture. Figure 9A shows the pattern of proteins present in the TCA precipitates of the media. Since serum was present in the media on day 8, the huge band at ~66 kDa appears to represent bovine serum albumin. The most notable proteins in the medium between days 20 to 34 of the culture were bands of MW ~80, 70, 42, 38, 30, and 21 kDa; on days 41 and 48, there was an increase in a doublet of 52–56 kDa, and a decrease in the bands at 38 and 30 kDa.

Figure 9B shows the pattern of collagens secreted into the *culture medium,* obtained by ammonium sulfate precipitation and pepsin digestion. Small amounts of collagen were detected in the medium from day 8 to 17. From

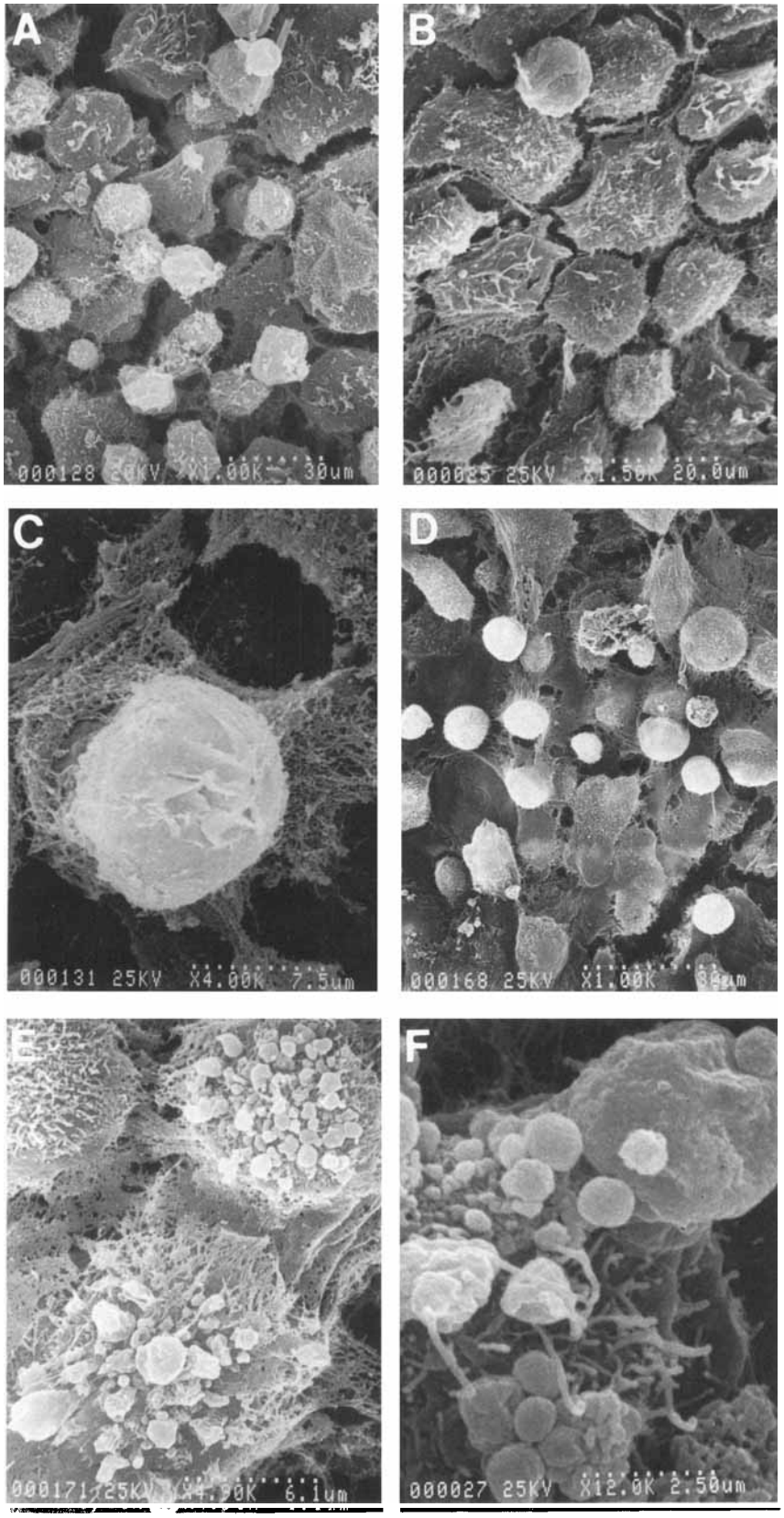

Fig. 5. Scanning electron microscopy of the morphology of the developing chondrocyte cell cultures. The culture dishes were fixed, dehydrated in graded ethanol, and then critical point dried as described in Materials and Methods. **A:** Day 15, undisturbed sample, overview, ×1.0 K. **B:** Day 18, undisturbed sample, overview, ×1.5 K. **C:** Day 15, undisturbed sample, monolayer cell and matrix, ×4.0 K. **D:** Day 21, air-blown sample, overview, ×1.0 K. **E:** Day 21, air-blown sample, cell surface features, ×4.9 K. **F:** Day 18, air-blown sample, cell surface features, ×12.0 K.

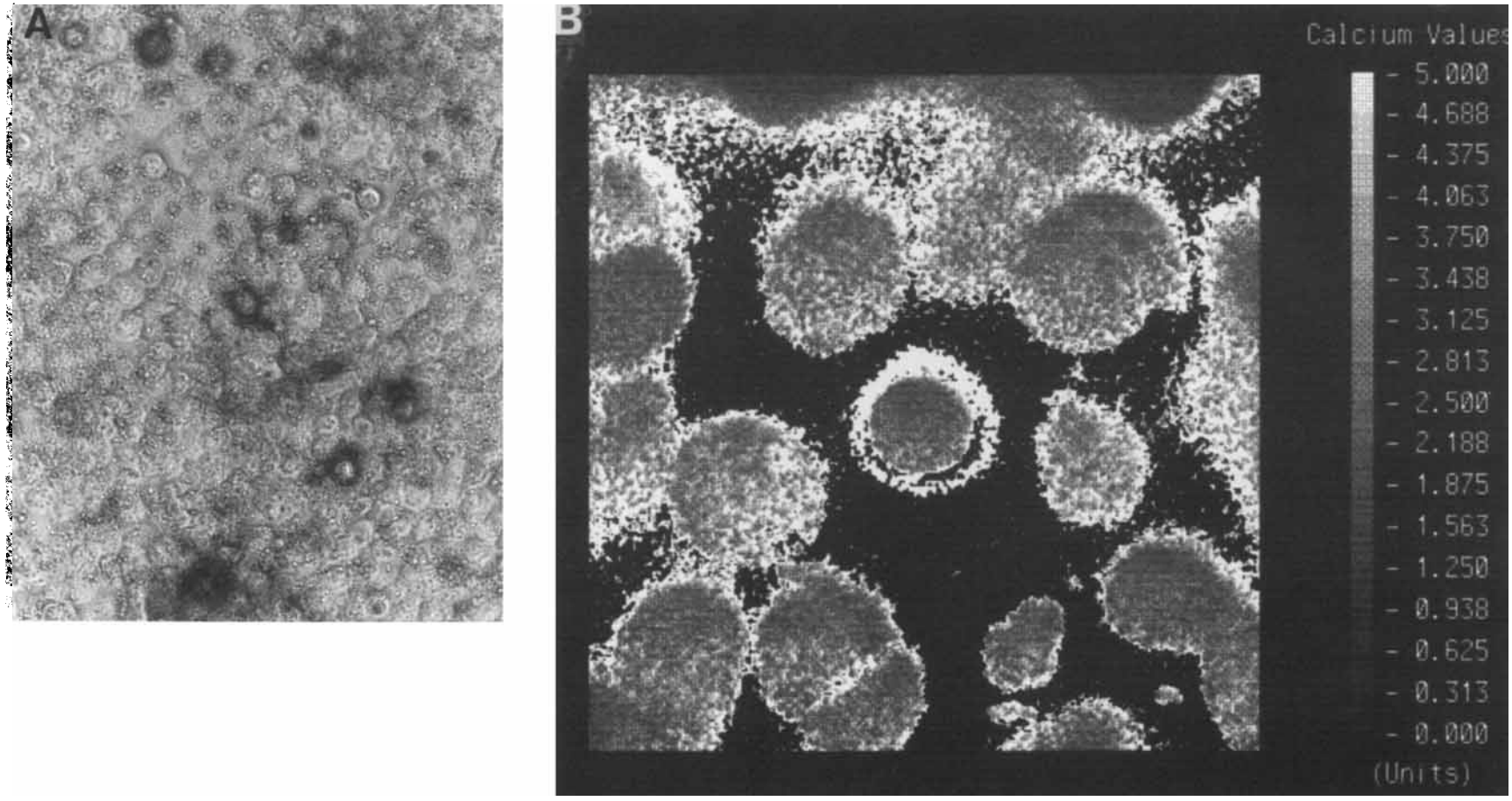

Fig. 6. Phase-contrast light microscopy and confocal laser imaging of Ca^{2+} levels in mineralizing chondrocyte cultures. **A:** Phase-contrast image of live Day 21 DATP5 cultured growth plate chondrocytes showing the opaque (dark) ring that forms around numerous cells in the region where mineralization is just beginning. Although there were major differences in the level of AP activity, the pattern of mineral development appeared identical in both media. These opaque annular structures, seen in both DATP5 and HL-1 cultures at the onset of mineralization, are preceded by the appearance of refractile granules on the cell surface. These refractile granules appear to correspond to the cell surface globules seen by SEM in Figure 5E,F (×333). **B:** Laser confocal images of cellular Ca^{2+} concentrations in Day 18 HL-1 culture showing the intense elevation of Ca^{2+}-ion activity in the pericellular region of a mineralizing chondrocyte (center). The live cultures were loaded with 1 μM levels of the fluorescent Ca^{2+} indicator, Indo 1-AM, for 60 min at 25°C before examination using a Meridian ACAS scanning system (See Materials and Methods). Note that many of the adjacent chondrocytes in the field also have highly elevated $[Ca^{2+}]$ levels in the perimeter near the outer membrane (×1,333).

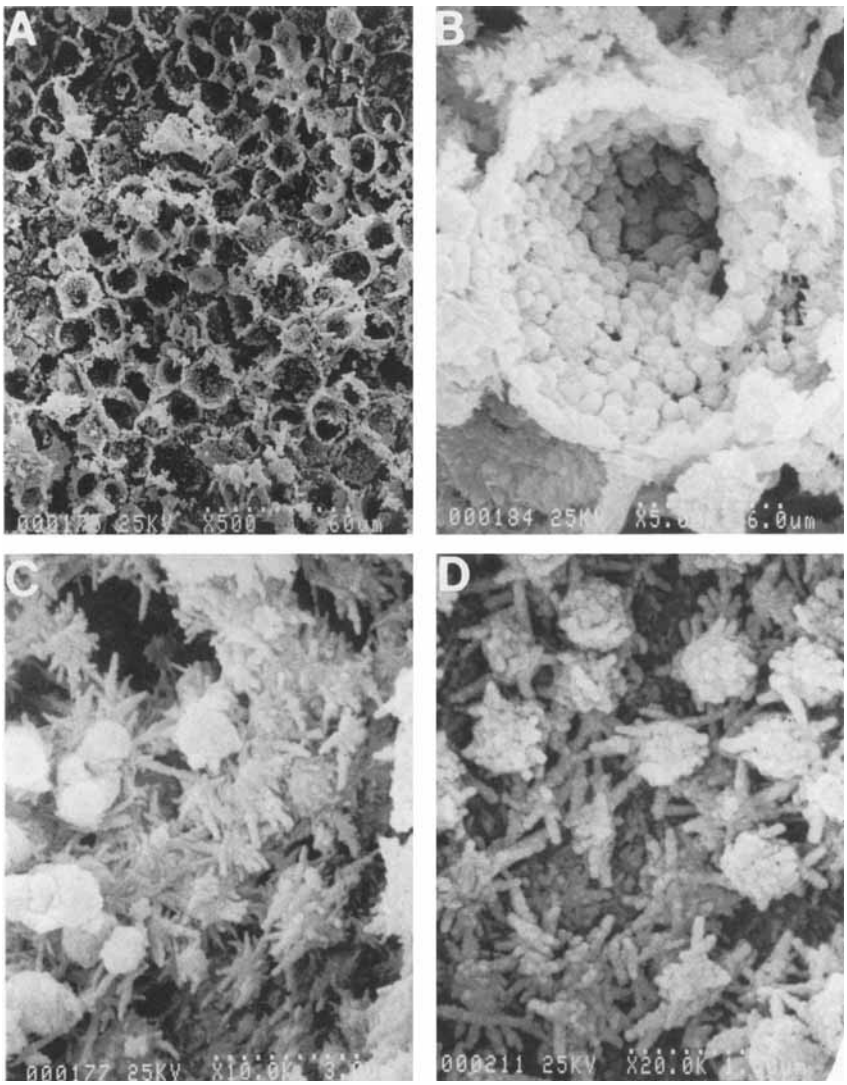

Fig. 7. Scanning electron microscopy of the anorganic residue following treatment of the mineralizing cultures with alkaline sodium hypochlorite. DATP5 cultures of growth plate chondrocytes at day 30 were treated for 30 min at room temperature with 5.25% alkaline (pH 11) sodium hypochlorite to remove organic matter. The residue was rinsed with distilled water, dehydrated, and critical point dried as described in Materials and Methods. Essentially identical structures were seen in HL-1 cultures (not shown). **A:** Low-power field of a region of mineralizing cells showing the well defined cell lacunae (×500). **B:** Higher magnification of the globular mineral deposits forming the cell lacunae (×5,000). **C:** Higher magnification of a region where globular mineral (left) is propagating outward to form needle-like crystallites (×10,000). **D:** Even higher magnification of a region where "amorphous" globular mineral has largely converted to crystallites (×20,000). Note that some globules (right) have a nobby substructure that seems to precede outgrowth of the crystallites.

day 20 and onward, type X (~ 55 kDa) and lesser amounts of type II (~ 120 kDa) collagen began to be abundantly expressed in the media. No other types of collagens were detected.

The protein profile of the *cell/matrix* layer was very similar throughout the entire culture period (Fig. 10A). While there was a large range in the size of the proteins synthesized, particularly prominent bands were evident at MW 102, 78, 56, 55, 45, 41, 36, and 31 kDa. To specifically analyze the collagens, the cell/matrix layer was extracted with 0.5 M acetic acid, digested with pepsin, and then analyzed by SDS-PAGE. Using this approach, quite variable amounts of collagens appeared to be deposited into the cell/matrix layer at different stages of the culture (Fig. 10B). Large amounts of type II and X collagens were detected in the cell matrix layer on days 13 and 17, followed by a period (days 20 to 24) when much less was evident. Very high

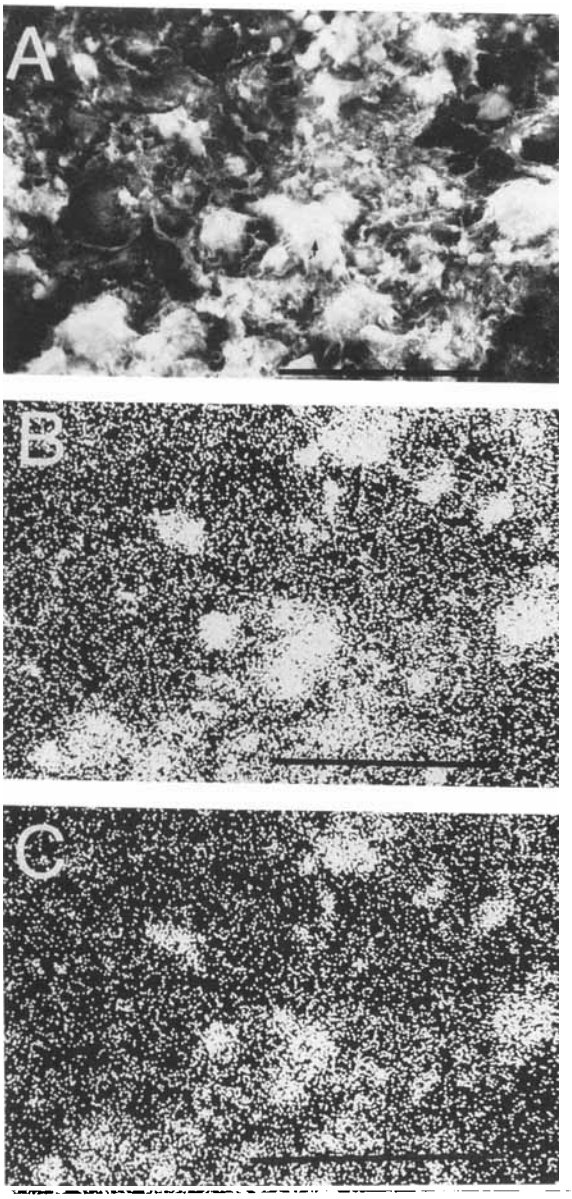

Fig. 8. Scanning electron microscopy of growth plate showing distribution of Ca and P. Chondrocytes were maintained in DATP5 culture medium to Day 24 before fixation, dehydration, and critical point drying. The dried cultures were mounted on a specimen stub and sputter-coated with carbon for conductivity. SEM observation was carried out using a Hitachi S-2500 Delta electron microscope; Ca and P mappings were performed on an EDS-SEM (JEOL T200) microscope (see Materials and Methods) (×1,000, bars ≈ 37 μm). **A:** Scanning electron micrograph of a region of the mineralizing culture. **B:** Ca map of the same region of the culture seen in A. **C:** P map of the identical region seen in A and B. Note the close correspondence of the Ca and P maps, although there is a generally higher Ca background (B). Also note that the "hot spots" correspond to some highly "luminescent" regions of cells evident by SEM in A, but not to others. In these cultures, cells are typically 10–20 μm in diameter.

levels of type X collagen occurred on days 27 and 34. Note that type II collagen also appeared to be absent from the acetic acid/pepsin extract of the cell matrix layer at several points in the long culture period. Despite the variability in the level of 0.5 M acetic acid-extractable type II and X collagen, the levels of pepsin (apparent MW = 38–42 kDa) added to digest the noncollagenous proteins were generally constant from lane to lane, indicating that similar loads of protein had been applied to the gel.

The pepsin-soluble and -insoluble proteins in the cell/matrix layer were analyzed by SDS-PAGE and compared (Fig. 11). As noted above, large amounts of type X collagen were present on days 28–35 in the pepsin-soluble fraction (Fig. 11, left); however, in the pepsin-insoluble fraction, significant amounts of type II collagen were evident from day 11 to 28, but decreased thereafter (Fig. 11, right). Only small amounts of type X collagen were present in the pepsin-insoluble fraction up to day 35, but a significant increase in it and a 32 kDa protein were detected on day 45.

Extraction with 0.1 N HCl was used to release from the cell/matrix layer phospho- and other type proteins associated with the mineral deposits (Fig. 12). Significant amounts of these proteins were first seen on day 13, and thereafter, a large number of proteins was present in these extracts. In particular, bands of ~120, 83, 72, 50–55, 45, 22, and 14 kDa were consistently seen.

In this study we also attempted to determine the level of synthesis of osteonectin and osteopontin by the growth plate chondrocytes during the 48-day culture. The secretion of these proteins into the culture medium and cell/matrix layer was detected by immunoblot analyses using rabbit anti-osteonectin and anti-osteopontin antibodies. Osteonectin (38 kDa) was secreted abundantly into the media from day 24 onward, with a slight decline towards the end of the 48 day culture period (Fig. 13A). However, only small amounts of osteonectin were detected in the cell/matrix layer. Osteopontin (66 kDa) was secreted into the media from day 17 onward, and considerable amounts were detected in the cell/matrix layer from day 27 onward. There were also some higher molecular weight forms, presumably associated with collagen, as well as small amounts of two degradation products (45 and 50 kDa) that immunoreacted with the osteopontin antibodies (Fig. 13B).

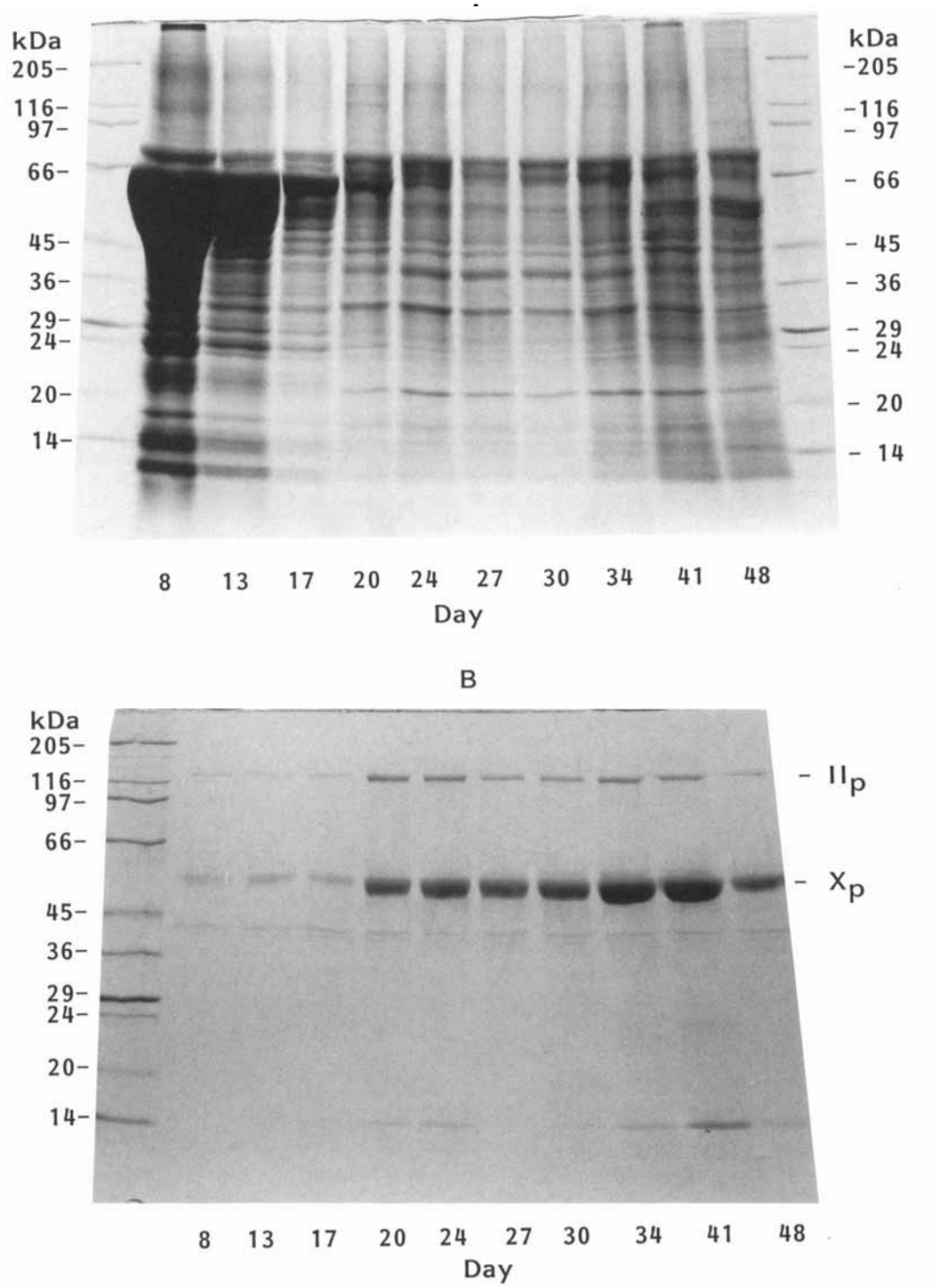

Fig. 9. SDS-PAGE of the general proteins and collagen secreted into the culture medium. **A:** Media (6 ml, HL-1) from 3 culture dishes were combined, and 1 ml of TCA (85% w/v) was added to precipitate the general proteins. After washing with acetone, the pellet was suspended and boiled in Laemmli sample buffer. Protein equivalent of 150 μl of the original medium from the specified day of culture was applied to each lane. **B:** A second set of 3 dishes were pooled and ammonium sulfate was added to 30% saturation to precipitate the collagens. The harvested precipitate was dissolved in 0.5 ml of 0.5 M acetic acid, and then digested overnight at 4°C after addition of 40 μl of pepsin (2 mg/ml in 0.25 M acetic acid). Then, after adjusting the pH to 7, the reaction mixture was centrifuged to remove the insoluble residue and a sample of the clear supernatant was boiled in Laemmli sample buffer. Protein equivalent to 150 μl of the original medium from the specified day of culture was applied to each lane of the gel. II_P = pepsin fragment of type II collagen; X_P = pepsin fragment of type X collagen. MW markers are shown on the outside lanes as indicated.

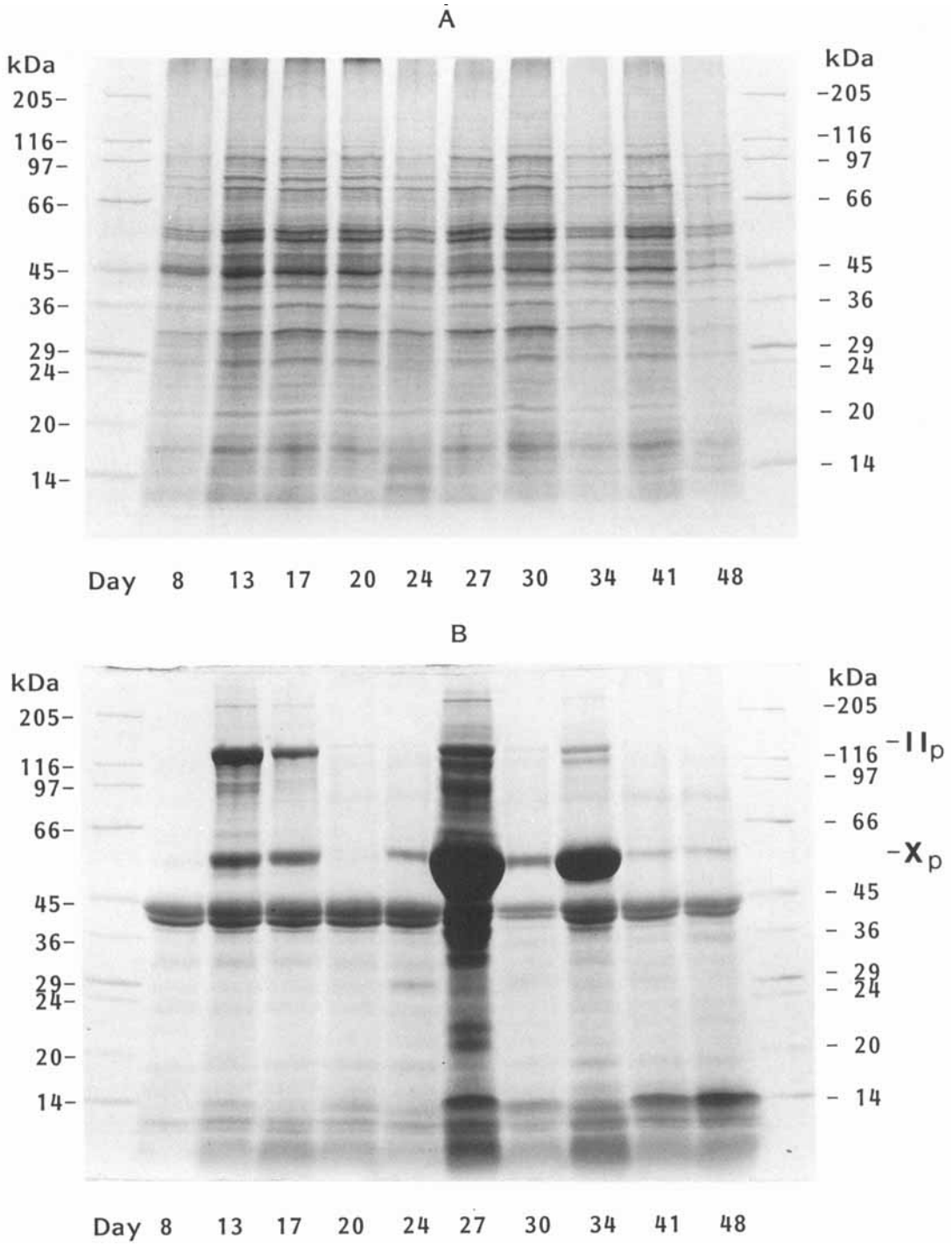

Fig. 10. SDS-PAGE of the general proteins and collagen in the cell/matrix layer of chondrocyte cultures. **A:** General proteins. Material from the harvest of the cell/matrix layer of each of 3 culture dishes (HL-1) was sonicated in 1 ml of TMT buffer and then centrifuged to sediment the insoluble material. Equivalent samples of the TMT supernatant from each of the 3 dishes were pooled and boiled in Laemmli sample buffer. For Day 17 onward, protein equivalent to one-tenth of the cell/matrix material in one dish (100 µl) was applied to each lane. For Days 8 and 13, when the cultures were just becoming established, one-fifth of the total material (200 µl) was applied to the gel to enable better visualization of the proteins. **B:** Collagens. Material from the harvest of the cell matrix layer of each of a second set of 3 culture dishes was suspended in 0.5 ml of 0.5 M acetic acid containing protease inhibitors and incubated overnight at 4°C to extract the collagen. These incubates were sonicated to yield a uniform suspension, and then digested with pepsin (80 µg) at 4°C overnight. After centrifugation to remove the insoluble residue, 0.8 ml of the pooled supernatant was precipitated with TCA, washed with acetone, and then boiled in Laemmli sample buffer for SDS-PAGE (see Materials and Methods). Cell/matrix material equivalent to 1.6 culture dishes (0.8 ml/1.5 ml total for 3 dishes) was applied to each lane of the gel. Doublet bands at MW = 38–42 kDa are residual pepsin. II_P = pepsin fragment of type II collagen; X_P = pepsin fragment of type X collagen. MW markers are shown on the outside lanes as indicated.

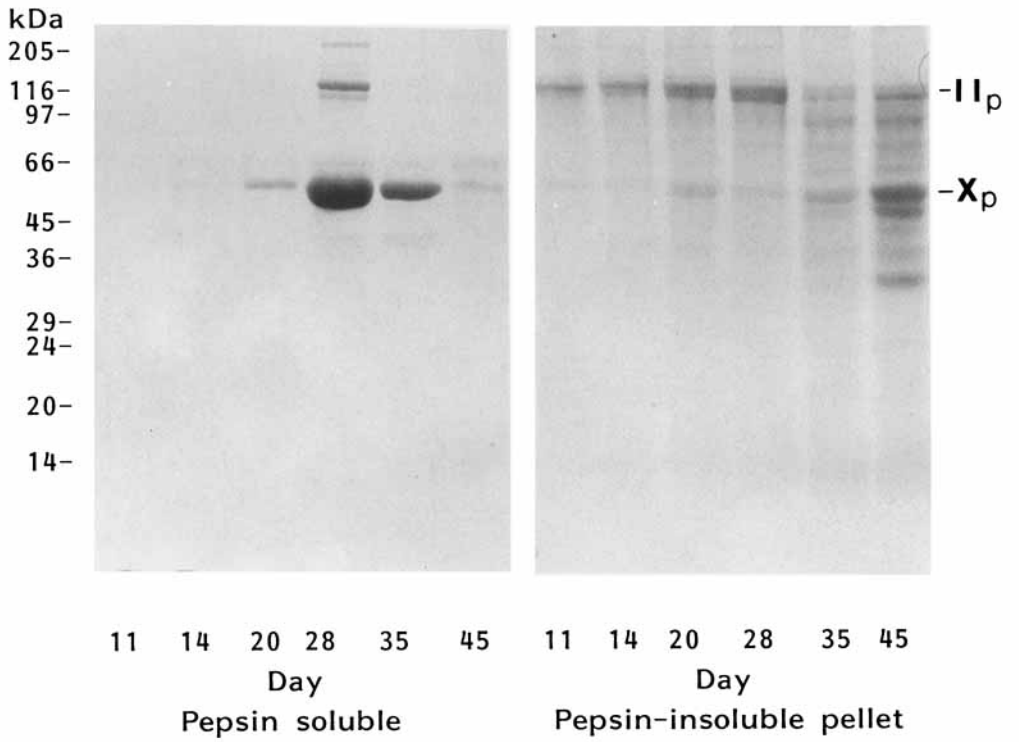

Fig. 11. SDS-PAGE analysis of the pepsin-soluble and pepsin-insoluble collagens of the cell/matrix layer of chondrocytes. The cell/matrix layer of each of 4 culture dishes at the specified days of the HL-1 culture was suspended in 0.5 ml of 0.5 M acetic acid containing protease inhibitors and incubated overnight at 4°C to extract the collagen. These incubates were sonicated to yield a uniform suspension, and then digested with pepsin (80 μg) at 4°C overnight. After centrifugation to sediment the insoluble residue. The supernatant (pepsin-soluble, **left**) was precipitated by addition of NaCl to 2.75 M, centrifuged, and the pellet boiled in Laemmli sample buffer for SDS-PAGE. The insoluble residue above (pepsin-insoluble, **right**) was boiled in Laemmli sample buffer for SDS-PAGE. Protein equivalent to one-half of one culture dish was applied to each lane of the gels. II_P = pepsin fragment of type II collagen; X_P = pepsin fragment of type X collagen. Molecular weights are indicated on the left side.

DISCUSSION

The epiphyseal growth plate is responsible for the longitudinal growth of long bones; it is also directly involved in endochondral calcification. During normal development, the cells in the upper level of the growth plate rapidly divide, mature, differentiate, become hypertrophic, and initiate mineral deposition. With heavy mineral deposition in the extracellular matrix, the cells become trapped in their lacunae; however, their fate remains obscure. Programmed cell death (apoptosis) has long been postulated to occur at this phase; however, there is also evidence that some of these cells may survive and become incorporated into endochondral bone [Hunziker et al., 1984; Farnum and Wilsman, 1987]. Several reports have suggested that some hypertrophic chondrocytes may not only survive, but undergo transformation taking on the phenotypic properties of osteoblasts [Cancedda et al., 1992; Kahn and Simmons, 1977; Lian et al., 1993].

In hypertrophic cartilage, specific expression of type X collagen, and a CH 21 low molecular weight protein by the chondrocytes are unique features [Schmid and Linsenmayer, 1985; Cancedda et al., 1988]. Alkaline phosphatase activity also becomes elevated going from the zone of proliferation to the zone of hypertrophy [Follis, 1949]. Proteoglycan aggregates are decreased in size [Buckwalter et al., 1987; Lian et al., 1993], and levels of type II collagen become reduced during hypertrophy [Alini et al., 1992], coincident with the onset of calcification. In addition to these changes, interactions between the cell and the extracellular matrix are now also believed to influence chondrocyte calcification.

In this investigation, we examined the course of development, differentiation, and mineralization of primary cultures of growth plate chondro-

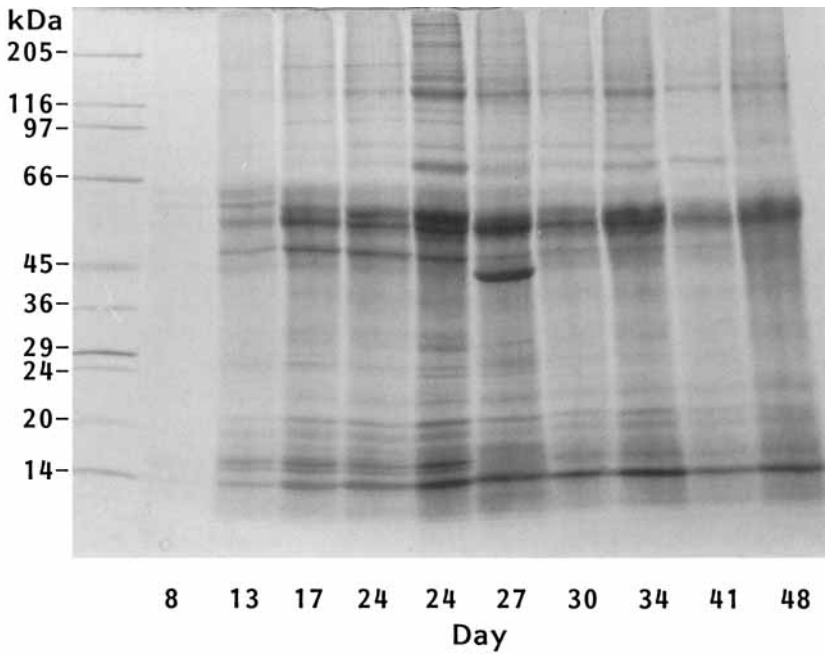

Fig. 12. SDS-PAGE analysis of the mineral-associated proteins of the cell/matrix layer of chondrocytes. The cell/matrix layer of each of 3 culture dishes at the specified days of the HL-1 culture was extracted with 1 ml of TMT buffer as described in the legend to Figure 10, and the insoluble residue sedimented by centrifugation. To the pellet from the TMT extraction of the cell/matrix layer of each culture dish was added 1 ml of 0.1 N HCl and each was incubated overnight to dissolve the mineral and release any mineral-associated proteins. The 0.1 N HCl suspensions were centrifuged and 0.5 ml aliquots from each supernatant were pooled, TCA precipitated, acetone washed, and boiled in Laemmli sample buffer for SDS-PAGE. Protein equivalent to one-half of one culture dish was applied to each lane of the gel.

cytes isolated from 6–8 week old chickens. Synthesis of new collagen and proteoglycan by the cultures was monitored by measuring the rates of incorporation of [^{3}H]proline and [^{35}S]sulfate, respectively, into these matrix macromolecules. The rate of collagen synthesis into the medium and cell/matrix layer gradually increased up to day 17, and was maintained at a relatively constant rate thereafter in the 27 day culture (Fig. 1).

Collagen levels in the media and cell/matrix were monitored more specifically by using SDS-PAGE analysis after pepsin digestion and TCA precipitation. Here it became evident that both type II (~ 120 kDa) and X (~ 55 kDa) collagens (pepsin-digested size) were being secreted into the *medium* throughout the culture period (Fig. 9B). However, when the *cell/matrix layer* was analyzed for deposition of collagens by extraction with 0.5 M acetic acid followed by pepsin digestion, a variable pattern of waxing and waning of the pepsin-resistant collagens became evident on SDS-PAGE. There were very high levels of pepsin-resistant type X collagen in the matrix on days 27 and 34, with lesser levels on days 13 and 17. Levels of pepsin-resistant type II collagen also followed this pattern. Thus, at times, the pepsin digestion appeared to extensively degrade the collagens in the cell matrix layer. The reason for the susceptibility of the collagens to pepsin is unclear, but may be the result of nicking by stromal collagenase [Dean et al., 1989]. This would be consistent with the findings of Alini et al. [1992] that type II collagen becomes broken down in the hypertrophic region of the growth plate. There was a significant fraction of the type II and X collagens that could not be extracted by acetic acid-pepsin treatment (Fig. 11B). This may be partly due to collagen cross-linking, or interactions with proteoglycans that render collagen resistant to pepsin extraction [Wardale and Duance, 1993]. These phenomena obviously merit further study.

While the rate of proteoglycan secretion into the medium was high until day 21, the rate of deposition into the cell/matrix layer declined progressively after day 12 (Fig. 2). Nevertheless, the accumulation of proteoglycan increased progressively until day 30, remaining relatively constant for the remainder of the 48 day culture period (Fig. 3). Thus, whatever caused the breakdown in collagen did not seem to influence the

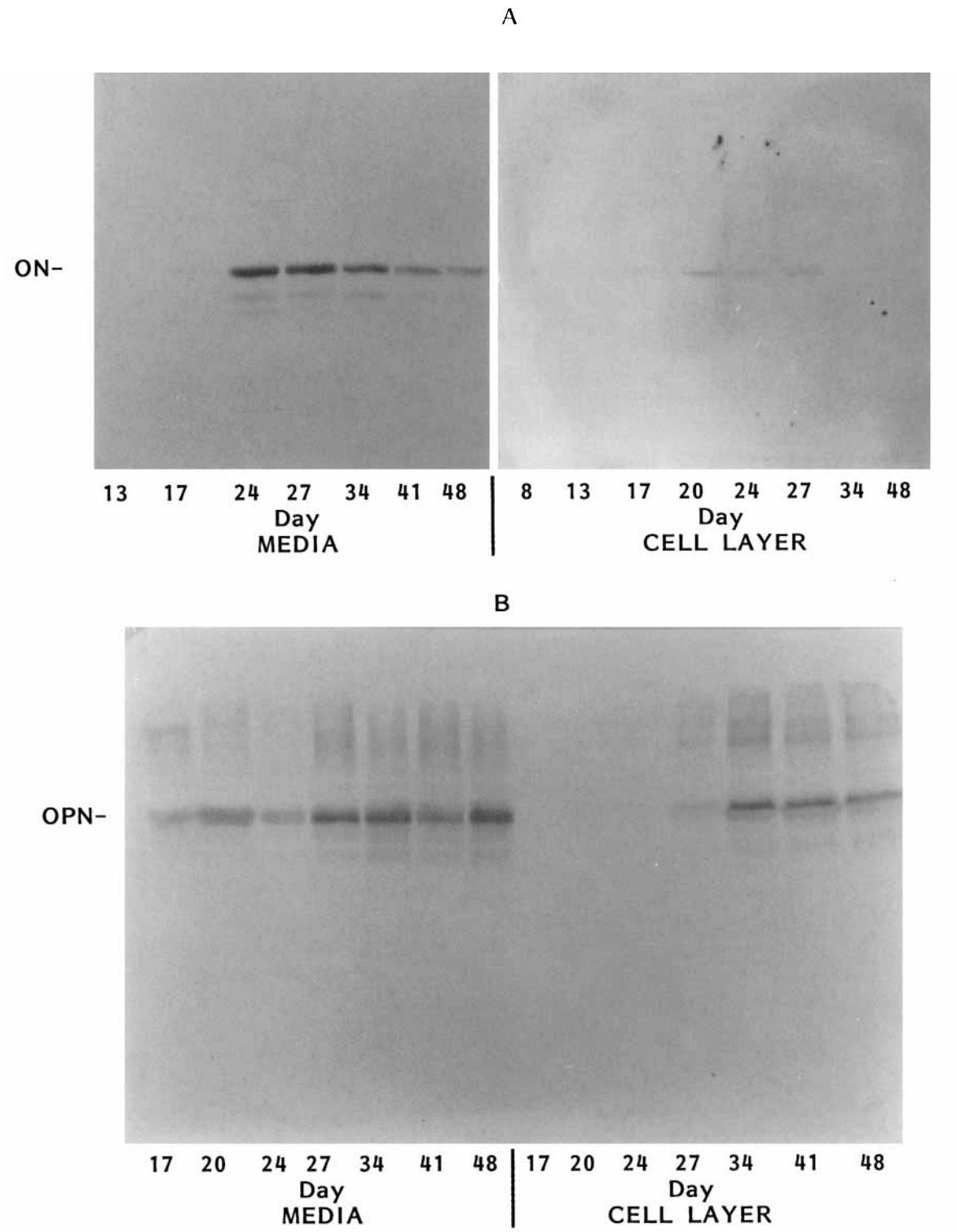

Fig. 13. Expression of osteonectin and osteopontin during the culture of the growth plate chondrocytes. Both the HL-1 medium and the cell/matrix layer were analyzed for the presence of osteonectin (**A**) and osteopontin (**B**). Proteins in the culture medium were precipitated with TCA; proteins from the cell/matrix layer were extracted with either 0.1 N HCl (**A**) or sonication in TMT buffer (**B**). These extracts were subjected to SDS-PAGE as described in Figures 10 and 11, transferred to nitrocellulose paper, and immunodetected with the respective antibodies as described in Materials and Methods. Protein equivalent to 7.5% of the culture medium from one dish (150 μl) was used per lane of the immunoblot. Protein equivalent to 25% of the cell/matrix layer of one dish (250 μl) was used for each lane of the osteonectin blots; that equivalent to 8% of the cell/matrix layer of one dish (80 μl) was used for each lane of the osteopontin blots. ON = osteonectin; OPN = osteopontin.

level of proteoglycans. This finding is in close agreement with the results of zonal analyses of these matrix constituents in growth plate tissue in vivo [Alini et al., 1992]. This indicates that the turnover of the proteoglycans and acetic acid-soluble collagens in the extracellular matrix are not closely coupled, either in this cell culture system or in vivo.

Onset of substantial Ca^{2+} and Pi accumulation in the cell/matrix layer was first evident on day 17, in both HL-1 and DATP5 media, increasing steadily thereafter (Fig. 4). It is possible that in addition to apatite formation, some Ca^{2+} may be bound to matrix proteoglycans [Dziewiatkowski, 1987; Hunter et al., 1988] or perhaps to type X collagen [Kirsch and von der Mark, 1991].

In addition to the quantitative analysis of Ca^{2+} and Pi deposition, deposition of mineral in the cultures was also followed by various microscopic and ultrastructural methods (Figs. 5–8). SEM analysis of the calcifying cultures revealed that on some cells in the underlying layers of chondrocytes there were numerous cell surface blebs 0.4–2.0 μm (mean = 0.70 ± 0.27 μm) in diameter (Fig. 5). These structures seem to correspond to the opaque ring seen by phase contrast microscopy around cells associated with early mineral deposition in the chondrocyte cultures (Fig. 6A).

This idea was further reinforced by the laser confocal imaging studies on the mineralizing cultures which showed a striking "ring of fire" indicative of the very high levels of Ca^{2+} in the perimeter of the calcifying chondrocytes (Fig. 6B). These images of intensely high inner cell surface-associated Ca^{2+} closely correspond to those obtained with slices of freshly prepared growth plate cartilage [Wuthier, 1993]. Further supportive evidence for Ca and P accumulation in premineralizing cells was gained from two-dimensional elemental mapping of the mineralizing cultures which showed selective and coincident loading of certain cells prior to the massive extracellular deposition of mineral (Fig. 8).

Images of the mineral deposits were obtained after treatment with alkaline NaOCl to remove cellular and matrix organic matter (Fig. 7). At low magnification (Fig. 7A), each cell appears to have been encased by a wall of mineral; the bulk of the space obviously was originally occupied by the cells. At higher magnification (Fig. 7B), it becomes evident that the wall is constructed of a syndesis of calcospherites, globoid clusters of mineral, 0.4–1.0 μm (mean = 0.78 ± 0.23 SD μm) in diameter. Further magnification (Fig. 7C,D) reveals that these globules are closely associated with clusters of needle-like crystallites ~0.1 μm in diameter and ~1.0 μm long. The needle-like structures are almost 100 times larger than single hydroxyapatite crystals found in biological tissues; they appear to be aggregates of apatite microscrystals originally associated with collagen fibrils. Similar mineralized structures have been seen in freeze-fracture images of calcified cartilage [Borg et al., 1981].

The crystallite clusters seem to arise from the globules; some globular structures appear to be in transition to the more needle-like form (Fig. 7D). Based on their localization and similar size, the spherules (calcospherites) appear to be the anorganic remnants of the Ca^{2+}-rich blebs released from the surface of the mineralizing cells (see Fig. 5E,F). It is probable that the cell surface blebs (globules) originally contained noncrystalline mineral ions complexed with acidic lipids [Boskey et al., 1980; Wuthier and Gore, 1977] and proteins [Genge et al., 1989]. Since the NaOCl solution (Chlorox) used to treat the samples was highly alkaline (~pH 11), it is possible that after removal of the organic constituents, globular ACP-like remnants remained. ACP is stable under alkaline conditions, i.e., pH ≥ 8 [Wuthier et al., 1985]; the pH must be lowered to ~7.4–7.8 to permit transformation of ACP complexes to apatitic mineral [Valhmu et al., 1990].

Calcospherite-like structures have been seen at the calcification front of woven bone, circumpulpal dentin, and mineralizing cartilage of several species [Boyde and Sela, 1978; Mishima et al., 1991; Ornoy and Langer, 1978; Lester and Ash, 1980]. These globules are likely the initiation sites of mineral formation in the cultures, and probably correspond to the material recently identified as the nucleational core complex responsible for mineral induction by matrix vesicles [Wu et al., 1993]. While mineralization in vivo is first associated with matrix vesicles [Anderson, 1969], just as it is with the cell-surface globules in these cultured chondrocytes, there is a significant discrepancy in size. In the cell cultures, these blebs (0.70 ± 0.27 μm diameter) are significantly larger than matrix vesicles seen in vivo (0.1–0.3 μm diameter). A likely explanation for this is that the extracellular matrix formed in vitro is not as condensed, and allows larger blebs to form during the cellular

events that seems to be associated with the onset of mineral deposition.

Regarding the relation between type X collagen and mineral formation by the cultures, it has been shown that transcription of the type X collagen gene is increased dramatically during hypertrophy prior to mineralization [Phyllis-Luvalle et al., 1992]. Type X collagen has been shown to be localized in a capsule-like configuration around hypertrophic cells [Phyllis-Luvalle et al., 1992; Bashey et al., 1991; Kirsch and von der Mark, 1992]. As noted earlier, confocal imaging shows localization of Ca^{2+} in the pericellular region [Wuthier, 1993], consistent with that of type X collagen. Previous studies have shown that type X collagen is tightly associated with the cell membrane (matrix vesicles) [Wu et al., 1989], and binds to several proteins associated with the chondrocyte plasma membrane, namely alkaline phosphatase, link protein, and hyaluronic acid binding region (HABR), and annexin V [Wu et al., 1991]. Thus, this spacial arrangement may be due to the ability of type X collagen to bind Ca^{2+} [Kirsch and von der Mark, 1991], and it is probable that this conjunction of Ca^{2+}, the cell membrane, type X collagen, and proteoglycan contributes to the signal for mineralization to proceed.

Other noncollagenous matrix proteins were also investigated. Osteonectin, a 38 kDa Ca^{2+} and type I collagen-binding protein present in bone matrix [Termine et al., 1981; Lane and Sage, 1994], has been thought to be involved in bone formation. Osteonectin also has been found in the matrix of nonmineralizing zones of growth plate cartilage [Jundt et al., 1987], but accumulates only in the mineralizing zone [Pacifici et al., 1990]. Our mineralizing cultures synthesized osteonectin into the culture medium, and to a lesser extent into the cell/matrix layer (Fig. 13A).

Also detected in our mineralizing cultures was a 66 kDa protein, cartilage osteopontin, seen in the media from days 27 to 48 (Fig. 9A), and confirmed to be present in both the media and cell/matrix layer by immunoblot analysis (Fig. 13B). This phosphoglycoprotein, discovered in bone by Franzén and Heinegård [1985], also has been found in cartilage by Gerstenfeld et al. [1990] in a form with similar cDNA sequence to bone osteopontin. The consistent finding of osteopontin in mature growth plate chondrocyte cultures [Gerstenfeld et al., 1990; Moore et al., 1991] indicates that osteopontin expression is a phenotypic characteristic of hypertrophic chondrocytes. Osteopontin is found associated with hydroxyapatite at sites of mineralization [Lian et al., 1993] and is postulated to regulate mineral crystal growth. Another possible role of this protein is that of an anchor of cells to bone via the RGD sequence [Reinholt et al., 1990].

Another protein, with apparent MW of ~80 kDa under reducing conditions, was consistently detected in the media throughout the culture period (Fig. 9A), as well as in acid extracts of the cell/matrix layer (Fig. 12) from day 17 onward. This protein is similar to one reported previously to be expressed during chondrocyte hypertrophy and mineralization [Cancedda et al., 1992]. This protein has not been identified, but it is destroyed by pepsin digestion.

Small amounts of a protein similar in size to CH21 [Cancedda et al., 1988] were detected in the culture medium (Fig. 9A) and in the cell/matrix layer (Fig. 10A). In addition, the major acid-extractable protein from the mineralizing cell/matrix layer was a set of intense bands with MW in the range of 54–58 kDa (Fig. 12). These proteins are also destroyed by pepsin digestion. Since these proteins are notably enriched in the acid extract from mineralized tissue, it is probable that they were associated with the mineral phase. What their identity is, and whether they function to stimulate or regulate mineral deposition, remains to be established.

Finally, while there is growing evidence that hypertrophic chondrocytes can undergo further differentiation to bone-like cells [Lian et al., 1993; Cancedda et al., 1992; Strauss et al., 1990], the level of expression of proteins characteristic of osteoblastic phenotype (e.g., osteocalcin) by these cultures was only about one-hundredth that observed for mature avian osteoblast cultures [Lian et al., 1989]. This suggests that additional signals from humoral factors are necessary for this transition to occur.

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