The Calcification of Cartilage Matrix in Chondrocyte Culture: Studies of the C-Propeptide of Type II Collagen (Chondrocalcin)

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Abstract. We have shown that when chondrocytes are isolated by collagenase digestion of hyaline cartilage from growth plate, nasal, and epiphyseal cartilages of bovine fetuses they rapidly elaborate an extracellular matrix in culture. Only growth plate chondrocytes can calcify this matrix as ascertained by incorporation of $^{45}Ca^{2+}$, detection of mineral with von Kossa's stain and electron microscopy. There is an extremely close direct correlation between $^{45}Ca^{2+}$ incorporation in the first 24 h of culture and the content of the C-propeptide of type II collagen, measured by radioimmunoassay, at the time of isolation and during culture. Moreover, growth plate cells have an increased intracellular content of the C-propeptide per deoxyribonucleic acid and, during culture, per hydroxyproline (as a measure of helical collagen) compared with nasal and epiphyseal chondrocytes. In growth plate chondrocytes 24,25-dihydroxycholecalciferol (24,25-[OH]$_2$D$_3$), but not 1,25-dihydroxycholecalciferol alone, stimulates the net synthesis of the C-propeptide and calcification; proteoglycan net synthesis is unaffected. Together, these metabolites of vitamin D further stimulate C-propeptide net synthesis but do not further increase calcification stimulated by 24,25-(OH)$_2$D$_3$. These observations further demonstrate the close correlation between the C-propeptide of type II collagen and the calcification of cartilage matrix.

THE calcification of cartilage matrix in the growth plates of developing bones represents a fundamental requirement for bone growth and development. Recently, we identified a molecule with a 35,000 mol wt subunit in the fetal bovine epiphysis (4) that is present in the extracellular matrix in developing cartilages (19). When cartilage calcifies this molecule is focally deposited exactly where and when calcification occurs (19, 20); we called this molecule chondrocalcin (19). Subsequent sequencing analysis of chondrocalcin has revealed that it is the C-propeptide of type II collagen (26). Whether or not the C-propeptide is an active participant in the calcification process remains to be seen.

We now describe comparative studies of this C-propeptide in cultures of chondrocytes isolated from calcifying fetal growth plate cartilage, and noncalcifying nasal and epiphyseal cartilages of the same fetus. We describe a culture system for the study of calcification and demonstrate that only growth plate cartilage matrix calcifies in culture, that calcification is closely correlated with the content of the C-propeptide of type II collagen, and that calcification and net synthesis of the C-propeptide are both stimulated by vitamin D.

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Materials and Methods

Isolation of Tissues

Bovine fetuses were obtained within 30 min of slaughter of pregnant cows from a local abbatoir (Abbatoir Soulanges, Les Cedres, Quebec). They were immediately transported to the laboratory. Fetal age was determined by measurement of tibial or femoral length (17); it ranged from 101 to 218 d. Femora, tibiae, and humeri were aseptically removed. Longitudinal incisions were made through each epiphysis from the articular surface to the bony metaphysis with a fine-toothed metal saw. The metaphysis was separated from the lower hypertrophic zone of the growth plate at its natural fracture face just below the last transverse septum of the lower hypertrophic zone. The primary growth plate was carefully dissected away from the non-calcifying cartilagenous epiphysis; since there is no clearly defined boundary in younger fetuses, growth plate slices up to ∿2-mm distant from the metaphyseal junction were isolated. In older animals, isolation of growth plate was more precise since the secondary center of ossification is well developed and enlarges with increasing age, with the primary and secondary growth plates sandwiched between it and the junction with the metaphysis. Perichondrium and periosteum were removed circumferentially. Nasal septal cartilage and epiphyseal cartilage, remote from the primary and secondary centers of ossification, were removed for study at the same time. All tissues were placed in DME at room temperature and cut into small fragments ∿1-3 mm^3.

    Isolation of Chondrocytes. The basal solution used to isolate chondrocytes was also the culture medium. It consists of DME containing 10% FCS, 20 mM Hepes buffer pH 7.4, 100 U/ml of benzylpenicillin, 100 μg/ml streptomycin sulfate, 2.5 μg/ml Fungizone, 50 μg/ml ascorbic acid, and 5 mM sodium β-glycerophosphate. For chondrocyte isolation 0.25% collagenase (type 1A; Sigma Chemical Co., St. Louis, MO) and 0.01% DNase 1 (Sigma Chemical Co.) were added. Cartilage was digested in an incubator at 37°C

with constant gentle stirring on a styrofoam pad (to prevent overheating) with a teflon-coated bar until extracellular matrix had been completely removed. This took from 4–6 h. Undigested cartilage was removed by passing the digestion mixture through three layers of nylon mesh (size 35 × 35 μm; Nitex, Swiss Weaving Mills, Zurich). Cells were separated by centrifugation at 100 g for 5 min at room temperature. They were washed twice by centrifugation in the culture medium. Cells were counted with a hemocytometer chamber in the presence of 0.5% trypan blue in 0.85% sodium chloride (Flow Laboratories, Inc., McLean, VA). The viability was between 93–97%. The inclusion of serum in the digestion medium was found to be essential for the retention of good viability.

Cell Culture. Cells were cultured in the complete enzyme-free tissue culture medium at a density of 2×10^6 cells in 1 ml per well of multiwell tissue culture plates (24 well, 1.5 cm diameter; Costar, Data Packaging Corp., Cambridge, MA) and maintained in a humidified incubator at 37°C in 5% CO_2 in air. Media were changed every third day. For each experimental determination triplicate cultures were established and means ± SD were determined.

Metabolites of Vitamin D. 1,25-dihydroxycholecalciferol (1,25-[OH]$_2$D$_3$)[1] and 24,25-dihydroxycholecalciferol (24,25-[OH]$_2$D$_3$) were generous gifts from Dr. M. R. Uskovic, Hoffmann-La Roche (Nutley, NJ). They were always used at a final concentration of 1×10^{-10}M, singly or in combination. They were dissolved in ethanol to form a stock solution and added to culture medium at a final concentration of 0.04% ethanol (vol/vol); ethanol alone at this concentration was present in control cultures.

Detection of Calcification. Incorporation of ^{45}CaCl$_2$ (New England Nuclear, Lachine, Quebec, Canada) into cells and cartilage matrix was measured in cultures by the addition of 0.25 μCi/ml at time zero and whenever the culture medium was changed. At the end of the incubation, culture medium was discarded and the intact cell layers were rinsed twice for 1 h at room temperature with fresh culture medium without serum. This medium was discarded. Cultures were air dried under vacuum (~1 h) at room temperature. They were solubilized by the addition of 0.5 ml of 90% formic acid for 30 min at 70°C before being mixed with 5 ml of scintillant (Ready Solvent; Beckman Instruments, Inc., Palo Alto, CA). Counting efficiency was found to be unaffected by the presence of this amount of formic acid.

DNA and Uronic Acid Assays. Cell layers were first digested at 37°C for 18 h with the bacterial collagenase (as used for cell isolation) at 0.5 mg/ml in 0.2 Tris-HCl, pH 7.0 containing 1 mM CaCl$_2$. Digestion then continued for a further 12 h at 37°C by the addition of 5 mM EDTA (to inactive collagenase) and 1 mM dithiothreitol with 1 mg/ml papain. Each culture digest was then divided into two equal parts: one was centrifuged at 100 g for 5 min and the supernatant was retained for uronic acid assay (2); the other half was used for the fluorimetric assay of DNA (25).

Hydroxyproline assay. This was as described (3). Only cell layers were analyzed.

Radioimmunoassay for the C-Propeptide of Type II Collagen

Tissue Extraction. Culture medium and cell layers were assayed separately. Cell layers were first dried under vacuum overnight at room temperature then extracted with 100 μl 4 M guanidine hydrochloride, containing 0.1 M potassium acetate, pH 5.8 and the proteinase inhibitors phenylmethylsulfonyl fluoride, EDTA, pepstatin, and iodoacetamide (22) at 4°C for 24 h.

Radiolabeling of C-Propeptide. 0.1 mg of C-propeptide isolated as described (4), in 100 μl of 50 mM Tris-HCl, pH 7.6 containing 150 mM sodium chloride was added to 0.5 mCi Na^{125}I (New England Nuclear) in 10 μl 0.4 M phosphate buffer, pH 7.4 and 10 μl chloramine T at 600 μg/ml in 50 mM Tris-HCl buffer, pH 7.6. The mixture was vortexed gently for 2 min at room temperature. Then 100 μl sodium metabisulfite was added as 1.2 mg/ml in the Tris-HCl buffer. Free iodine was removed by chromatography in the presence of 200 μl sodium iodide (10 mg/ml) in the Tris-HCl buffer containing 2 mg/ml of BSA. The column used was a siliconized 10-ml pipette containing a 10-ml bed volume of Sephadex G-25 (Pharmacia, Montreal, Quebec). Siliconization was necessary since the C-propeptide binds to untreated glass and polystyrene surfaces. Hence polypropylene pipettes and tubes were used for sample storage.

Radioimmunoassay. A solution phase inhibition assay was used in which the binding of rabbit antibody to radiolabeled C-propeptide is competed for by known amounts of unlabeled C-propeptide or an unknown amount of

1. *Abbreviations used in this paper:* 1,25-(OH)$_2$D$_3$ and 24,25-(OH)$_2$D$_3$, 1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol, respectively.

C-propeptide (to be assayed). The immune complex that is formed is then bound to protein A-bearing *Staphylococcus aureus*, removed by centrifugation, and counted. The buffer was composed of 7.5 mM potassium dihydrogen phosphate, 143.4 mM disodium hydrogen phosphate at pH 8.0, containing 2.5% BSA, 5% sodium deoxycholate, 2.5% NP-40, and 0.05% sodium azide. 10 μl of tissue extract in 4 M guanidine hydrochloride diluted 10-fold with buffer, or 10 μl undiluted culture medium, or 10 μl of purified C-propeptide in buffer were added to 50 μl of rabbit antiserum to the C-propeptide (R117; references 4 and 19) previously diluted with buffer so that it binds 40–50% of the total (10,000 cpm) radiolabeled C-propeptide added in 50 μl of buffer. After mixing, the solution was left overnight at 37°C. 50 μl of protein A as a formalin-killed preparation of *Staphylococcus aureus*, Cowan strain I was added (10% suspension in water diluted 2.5-fold with buffer; Zymed, Cedarlane Laboratories, Hornby, Ontario) and mixed. Total counts were determined. After 20 min at room temperature 2 ml of buffer were added. After centrifugation at 5,000 g for 10 min, supernatants were aspirated, and pellets were counted. A standard curve was constructed for all assays. Polypropylene tubes (12 × 75 mm; Fisher Scientific, Montreal) were used throughout together with polypropylene pipettes. All assays were performed in triplicate. Nonimmune binding of C-propeptide was determined by substituting immune for similarly diluted nonimmune rabbit serum. Counts of nonimmune binding were deducted from those for immune binding and the percentage inhibition of binding was determined. The presence of dilute guanidine hydrochloride and proteinase inhibitors was shown to have no effect on antibody binding in this assay. A typical inhibition curve is shown in Fig. 1. Unless otherwise stated, results of all biochemical and radioimmunoassays represent the means ± SD of triplicate determinations on each of triplicate cultures.

Histology

Cell layers were fixed in a mixture containing freshly prepared formaldehyde (2%) and glutaraldehyde (2%) in 0.1 M cacodylate buffer, pH 7.4 at 4°C for 1 h (19). After embedding in resin (JB4; Polysciences, Inc., Warrington, PA), according to the manufacturers recommendations, 4-μm sections were prepared, stained with von Kossa's reagent (19), and counterstained with 0.1% basic fuchsin in 70% ethanol. Permanent mounts were prepared in Permount (Fisher Scientific).

Electron Microscopy

For morphological examination, cultures were fixed as described above for histology and then postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4. Tissue was dehydrated in graded ethanols and embedded in Spur resin (Polysciences, Inc., Warrington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Phillips 400 electron microscope.

Results

Histology, Immunohistochemistry, and Electron Microscopy of Cells and Cell Cultures

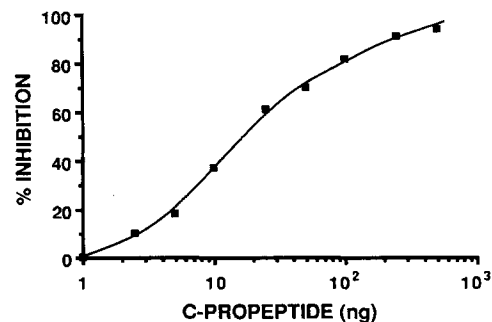Examination of electron micrographs of freshly isolated cells

Figure 1. Standard inhibition curve for the radioimmunoassay of the C-propeptide. ng indicates the total amount of C-propeptide per assay tube. The values represent the means of triplicate determinations. Determinations were made on cell layers and culture media as indicated.
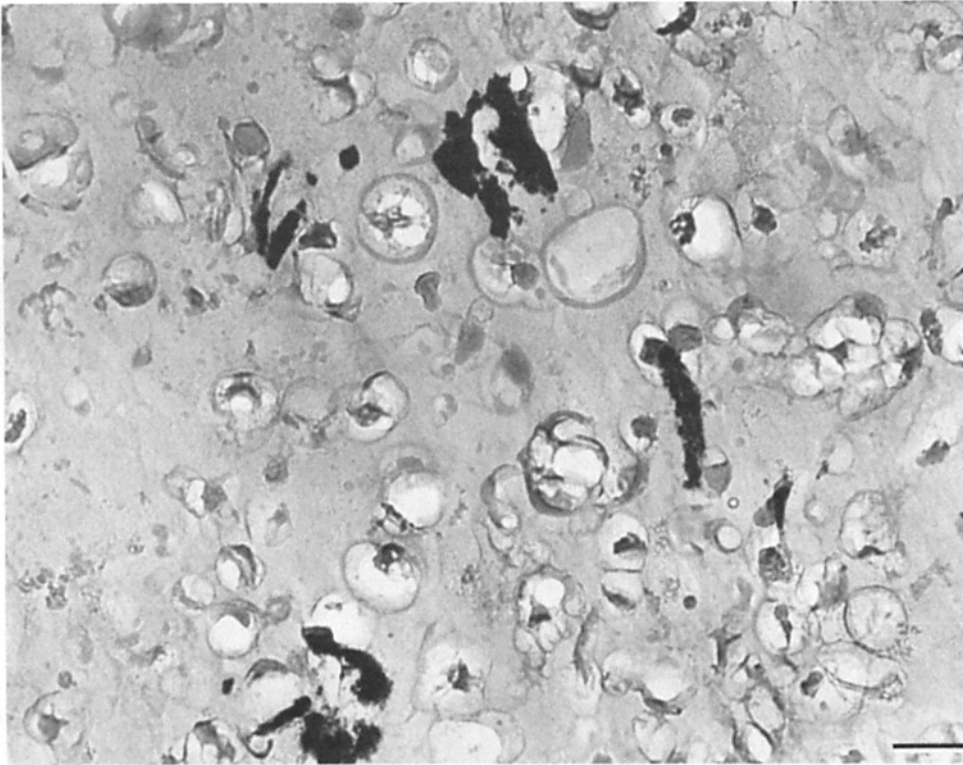
Figure 2. Section of a 5-d-old growth plate chondrocyte culture stained in JB4 resin with von Kossa's stain for calcium phosphate. Bar, 20 μm.
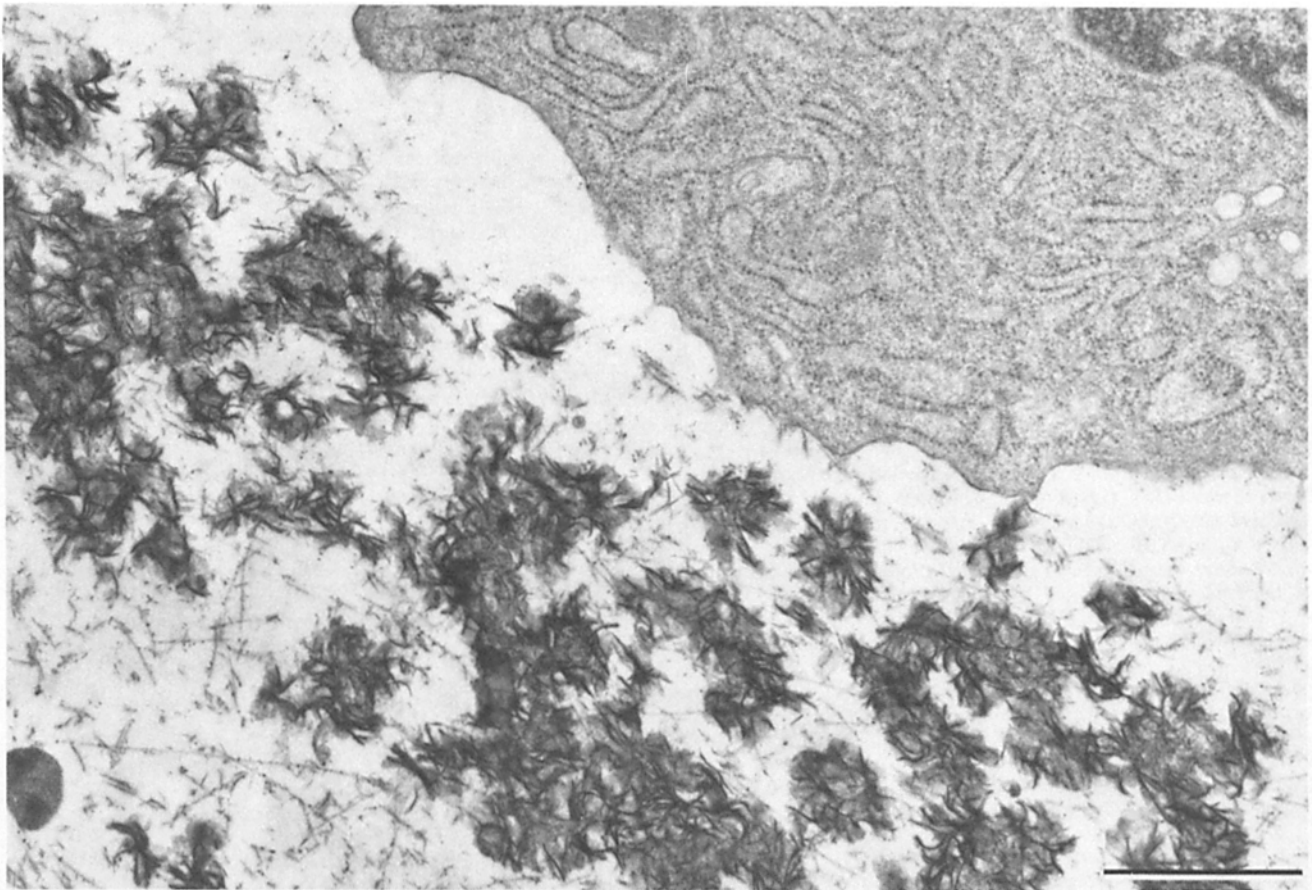

Figure 3. Electron micrograph of a 6-d-old growth plate chondrocyte culture. It shows part of a hypertrophic chondrocyte and, in the adjacent extracellular matrix, typical mineralization sites of the kind observed in growth plate cultures that stain intensely with von Kossa's stain (Fig. 2). Bar, 1 μm.
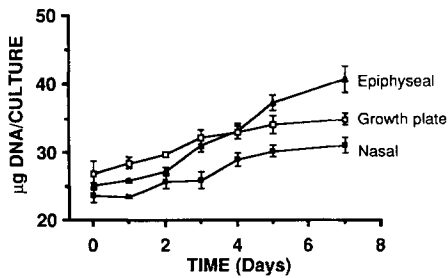
Figure 4. DNA contents of cultures of growth plate, nasal, and epiphyseal chondrocytes of a 134-d-old fetus studied in Figs. 5–7.

from growth plate, nasal, and epiphyseal cartilages revealed the presence of isolated healthy looking chondrocytes that were free of morphologically recognizable cartilage matrix (data not shown). In culture, all chondrocytes rapidly produced an extracellular matrix in which the cells were embedded. The appearance of a growth plate chondrocyte culture is shown in Fig. 2. A "mat" of cartilage was formed that was firm enough for it to be removed intact with forceps from the culture vessel after 3 d. Hypertrophic cells were generally larger. Staining with von Kossa's reagent revealed the presence of mineral deposits (Fig. 2); these were only observed in growth plate cultures. They were of irregular shape and scattered throughout the extracellular matrix. Sites of calcification were clearly recognizable by electron microscopy in cultures of growth plate chondrocytes by their crystalline appearance and considerable electron opacity (Fig. 3). All these experiments were repeated several times with similar results.

Proliferation of Chondrocytes in Culture and the Synthesis of the C-Propeptide by Chondrocytes Isolated from Growth Plate, Nasal, and Epiphyseal Cartilages

Fig. 4 shows that in 7-d-old cultures of chondrocytes isolated from epiphyseal, nasal, and growth plate cartilages, the DNA content increases progressively, this being greatest for
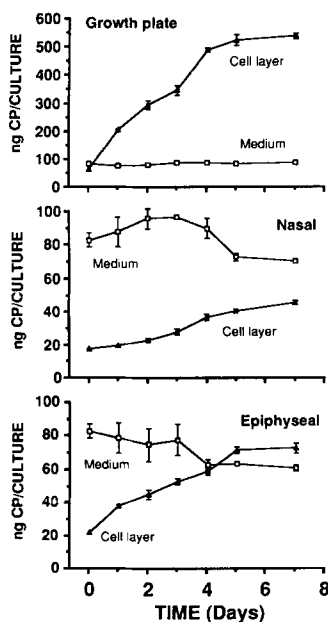

Figure 5. C-propeptide contents of cultures of growth plate, nasal, and epiphyseal chondrocytes isolated from the 134-d-old fetus studied in Figs. 4, 6, and 7.

Table I. C-propeptide Contents of Chondrocytes Freshly Isolated from the Growth Plate, Nasal, and Epiphyseal Cartilages

Fetal age	Growth plate	Nasal	Epiphyseal
days	ng C-propeptide/ µg DNA	ng C-propeptide/ µg DNA	ng C-propeptide/ µg DNA
134	3.4 ± 0.2	0.8 ± 0.1	0.9 ± 0.1
140	5.8 ± 0.3	1.0 ± 0	1.6 ± 0.1

epiphyseal chondrocytes. Several comparative studies were made of the net synthesis of the C-propeptide in these cultures, all of which produced similar results. In Fig. 5, a typical set of data for a 134-d-old fetus is shown. The FCS used in this study already contains significant amounts of the C-propeptide (time 0, Fig. 5) (0.82 µg/ml serum) as suggested by an earlier study (4). During the culture of growth plate cells, the total C-propeptide content in the cell layer progressively increased while the total content in culture medium remained essentially unchanged (Fig. 5). A smaller increase in the total C-propeptide content of the cell layer was also observed in cultures of nasal and epiphyseal chondrocytes (Fig. 5). The content of C-propeptide in culture media of nasal and epiphyseal cells exhibited a small decrease after several days in culture. The C-propeptide content of freshly isolated growth plate chondrocytes was always greater per cell than that of nasal and epiphyseal chondrocytes (Fig. 5); this was also the case when expressed per DNA (Table I). The total C-propeptide contents of cultures (cell layer plus medium) per DNA is shown for all three cultures in Fig. 6. Growth plate chondrocytes accumulated more C-propeptide than other cultures and this was retained primarily in the cell layer (Fig. 5). The amount of immunoreactive C-propeptide rapidly increased in the first 4 d in growth plate cultures whereas a small overall decrease per DNA was observed in nasal and epiphyseal cultures (Fig. 6).

Incorporation of $^{45}Ca^{2+}$ in Cell Cultures. The binding of $^{45}Ca^{2+}$ to cell layers of cultures was used as an index of calcification, representing the formation of entrapped $^{45}Ca^{2+}$ in forming insoluble mineral. Results for all three types of cultures from the 134-d-old fetus are shown in Fig. 7 *top*. Compared with cultures of nasal and epiphyseal chondrocytes, growth plate cell layers exhibited a much greater accumulation of $^{45}Ca^{2+}$/µg DNA, which was apparent after only 12 h and increased thereafter. This relationship of calcification to cellular C-propeptide content was further analyzed by examining freshly isolated growth plate chondrocytes from fe-
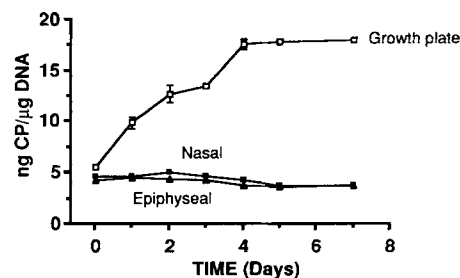

Figure 6. Total C-propeptide contents (cells plus medium) per DNA of growth plate, nasal, and epiphyseal chondrocytes of the 134-d-old fetus studied in Figs. 4, 5, and 7.
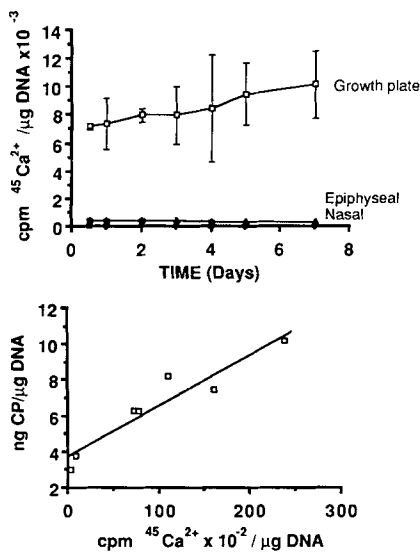
Figure 7. (top) The incorporation of $^{45}Ca^{2+}$ per DNA content in cell layers of cultures of growth plate, nasal, and epiphyseal chondrocytes isolated from the 134-d-old fetus studied in Figs. 4–6. *(bottom)* The correlation between C-propeptide content of freshly isolated growth plate chondrocytes and calcification in the first 24 h. For further details see Table II.

tuses of various ages and recording the $^{45}Ca^{2+}$ incorporation during the first 24 h of culture. Table II shows that with increasing fetal age, the C-propeptide content of growth plate chondrocytes increased in parallel with $^{45}Ca^{2+}$ incorporation. The correlation coefficient of $r = 0.95$ for paired samples demonstrates the extremely close correlation between C-propeptide content and calcification (Fig. 7 *bottom*).

Synthesis and Accumulation of Uronic Acid and Hydroxyproline

Uronic acid as a measure of chondroitin sulfate and hence proteoglycan content was also determined in the cell layers. In all cultures of the same 134-d-old fetus, the uronic acid

Table II. C-Propeptide Contents of Freshly Isolated Growth Plate Chondrocytes from Fetuses of Different Ages and the Total Incorporation of $^{45}Ca^{2+}$ in the First 24 h of Culture

Fetal age	ng C-propeptide/μg DNA at 0 h	cmp $^{45}Ca^{2+}$/μg DNA
days		
107	3.0 ± 0.1	210 ± 4
119	2.3 ± 0.01	ND
124	3.9 ± 0.2	838 ± 4
134	2.5 ± 0.1	ND
137	6.3 ± 0.2	7,412 ± 185
140	6.3 ± 0.1	7,862 ± 20
152	8.2 ± 0.1	10,822 ± 80
210	7.4 ± 0.2	16,120 ± 150
210	9.8 ± 0.6	ND
218	10.1 ± 0.2	23,750 ± 500

The results represent the means ± standard deviations of C-propeptide and of $^{45}Ca^{2+}$ incorporation in triplicate cultures. There are two fetuses each of 210 d of age. Regression analysis of paired samples revealed that the correlation coefficient ($r = 0.95$; $y = 3.765 + 0.028x$) is significant by Students' t test ($P < 0.005$). This plot is shown in Fig. 7 *b*.
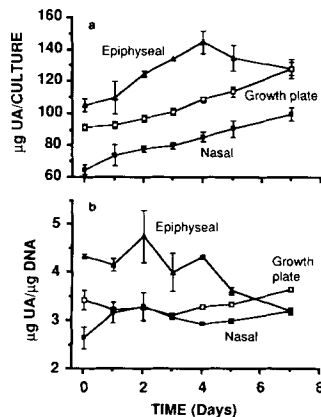

Figure 8. (a) The total uronic acid contents of cultures of growth plate, nasal, and epiphyseal chondrocytes isolated from the 160-d-old fetus. *(b)* The uronic acid contents of these cultures per DNA content.

content was increased and was greatest (as was growth rate) in epiphyseal cultures (Fig. 8 *a*). On a DNA basis some decline per cell content was observed in epiphyseal cultures with time but little change was seen in other cultures (Fig. 8 *b*). Similar amounts of hydroxyproline (as a measure of helical collagen) in the cell layer per unit DNA were found in cultures of nasal and growth plate chondrocytes, but more was present in epiphyseal cultures. With time, the hydroxyproline content exhibited a reduction in all cultures (Fig. 9 *a*). The content of C-propeptide in the cell layer in relation to hydroxyproline content in a 160-d-old fetus revealed that the C-propeptide content was always greater in growth plate cultures at all times during the experiment (Fig. 9 *b*). To determine the molar ratios of C-propeptide to helical collagen, hydroxyproline was determined as representing 11.2% of the total weight of helical collagen, which has a molecular weight of ∼300,000 (14). The molecular weight of the C-propeptide was taken as 105,000 (6). With this information, the molar ratios of helical collagen to C-propeptide at 12 h were calculated, from the data shown in Fig. 9 *b*, as follows: 883:1 (growth plate), 2,301:1 (nasal), and 1,805:1 (epiphyseal). At
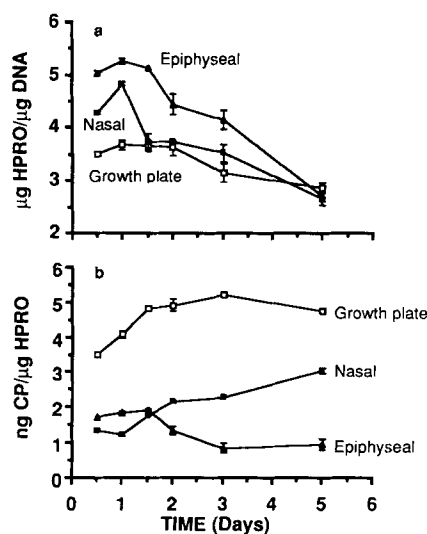

Figure 9. (a) The hydroxyproline contents per DNA of cultures of growth plate, nasal, and epiphyseal chondrocytes isolated from a 160-d-old fetus. *(b)* Radioimmunoassay of the C-propeptide in cell layers of growth plate, nasal, and epiphyseal chondrocytes of the 160-d-old fetus per hydroxyproline content.
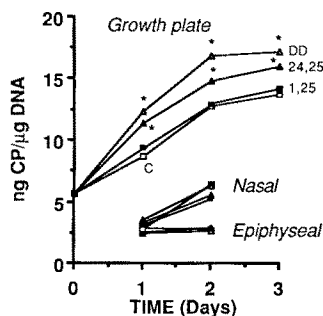
Figure 10. Total C-propeptide contents (cells plus medium) per microgram DNA of growth plate, nasal, and epiphyseal chondrocytes isolated from a 124-d-old fetus and cultured in the absence (c) and presence of 24,25-(OH)₂D₃ (24, 25), 1,25-(OH)₂D₃ (1,25) and the two metabolites in combination at the same concentrations (DD). Standard deviations are not shown (<5% of mean) but significant differences (student's t test analyses, P < 0.001) are indicated (*).

Figure 11. Incorporation of $^{45}Ca^{2+}$ per microgram DNA in cultures of growth plate and nasal chondrocytes isolated from the same 124-d-old fetus referred to in Fig. 10 and cultured with vitamin D metabolites as in Fig. 10. Standard deviations are not shown (<5% of mean) but significant differences (student's t test analyses) are indicated: *P < 0.001; **P < 0.05.

5 d these ratios were 654:1 (growth plate), 1,021:1 (nasal), and 3,287:1 (epiphyseal). Thus we can only account for a very small proportion of the C-propeptide, presumably synthesized as part of the procollagen molecule. This is probably a result of its degradation to fragments which are not detected by our antibodies. The increased content of C-propeptide in growth plate cultures may therefore be due to increased synthesis and/or reduced degradation of the molecule, possibly as a result of its association with mineral (4, 19). Whether there are any differences in the synthesis and post-translational processing of procollagen and the C-propeptide in growth plate chondrocytes remains to be established. In spite of the small amount of C-propeptide present in these cultures, the results together demonstrate that growth plate chondrocytes contain and accumulate increased amounts of C-propeptide with respect to helical collagen. Moreover, the contents of C-propeptide closely correspond to $^{45}Ca^{2+}$ incorporation, used here as a biochemical index of calcification.

The Effect of Vitamin D on the Net Synthesis of C-Propeptide and $^{45}Ca^{2+}$ Incorporation in Cell Cultures

It is well established that vitamin D deficiency is associated with an arrest of cartilage calcification in the growth plate. Although fetuses were removed from vitamin D–sufficient cows, we decided to determine whether two metabolites of vitamin D, namely 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃, had any influence on the metabolism of our cell cultures. Addition of 24,25-(OH)₂D₃ caused a small but significant (P < 0.001) increase in net C-propeptide synthesis in growth plate cultures but no significant changes were observed in nasal and epiphyseal cultures (Fig. 10). On its own 1,25-(OH)₂D₃ had no effect on either cell population. Together these metabolites produced a significant enhanced accumulation of C-propeptide in growth plate cultures compared to that observed with 24,25-(OH)₂D₃ alone.

24,25-(OH)₂D₃ produced a significant increase (P < 0.001) in $^{45}Ca^{2+}$ incorporation in growth plate but not nasal cultures, but addition of 1,25-(OH)₂D₃ alone or in combination had no effect on $^{45}Ca^{2+}$ incorporation in growth plate and nasal cultures except a less significant (P < 0.05) stimulatory effect on growth plate cells at day 3 of culture (Fig. 11). Although these stimulatory effects of 24,25-
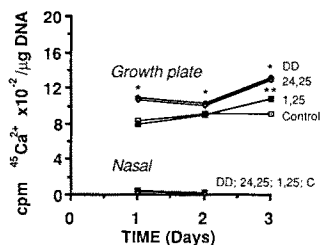
(OH)₂D₃ alone or in combination with 1,25-(OH)₂D₃ were small, they were reproducible in three experiments each with a different fetus. The lesser effect of 1,25-(OH)₂D₃ on $^{45}Ca^{2+}$ incorporation shown in this experiment was not reproducible. To determine whether these metabolites influenced the proteoglycan contents of cultures, we examined uronic acid contents. In repeat experiments there was no reproducible significant influence by either metabolite singly or in combination on uronic acid contents of nasal and growth plate cultures. A typical experiment is shown in Fig. 12.

Discussion

We have shown previously with immunohistochemistry at the light and ultrastructural level that the calcification of cartilage matrix is intimately associated in space and time with the focal concentrations of the C-propeptide of type II collagen, previously called chondrocalcin (19). The present biochemical and immunochemical studies confirm and extend these observations.

In this investigation we have successfully established an in vitro system for the study of matrix synthesis by isolated chondrocytes and, in particular, the calcification of cartilage matrix by chondrocytes isolated from growth plate cartilage. By comparison with cultures of chondrocytes isolated from noncalcifying cartilages, the characteristics of this calcifying system have been identified. The usefulness and sensitivity of $^{45}Ca^{2+}$ incorporation as an index of natural calcification is clearly demonstrated with respect to morphologically and histochemically detectable calcification. The striking direct correlation between C-propeptide content and calcification is readily apparent, further implicating this molecular species in the process of calcification.
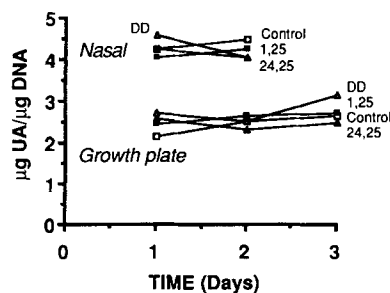

Figure 12. Uronic acid contents per microgram DNA of culture of growth plate and nasal chondrocytes isolated from the same 124-d-old fetus referred to in Figs. 10 and 11 and cultured with vitamin D metabolites as in Fig. 10. Standard deviations are not shown; there are no significant differences.

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The increased intracellular content of the C-propeptide in growth plate chondrocytes and its selective accumulation in cultures of these cells raises the question whether there may be a different synthesis and/or processing of the C-propeptide in these chondrocytes. The C-propeptide of type II procollagen is known to play an essential role in the assembly of the triple helix (10, 21, 23). In fibril assembly the C-propeptide is thought to be removed by a C-propeptidase that has been described as having properties of both metallo and aspartate proteinases (7, 9, 12). Hence, since the C-propeptide is normally derived from the procollagen molecule, an inevitable question is how can the C-propeptide selectively accumulate in growth plate chondrocytes. Immunoelectron microscopic examination of growth plate chondrocytes has revealed that the C-propeptide is similarly present in large intracellular vacuoles (19) which are in fact dilatations of the endoplasmic reticulum in the "swollen" hypertrophic cells (Lee, E., and A. R. Poole, manuscript in preparation). Whether this apparent accumulation represents that of procollagen or of the C-propeptide is presently under investigation. In cultures the extracellular accumulation would be favored by the incorporation and entrapment of the C-propeptide in calcification sites that was observed earlier (19). The C-propeptide binds strongly to hydroxyapatite (4) and hence may accumulate in mineralization sites in a passive manner. Other studies, however, have revealed that this binding may be very important since addition of the C-propeptide accelerates mineral growth in culture in cartilage matrix (Hinek, A., L. Rosenberg, and A. R. Poole, manuscript in preparation).

In vivo studies of rachitic animals have demonstrated that 24,25-$(OH)_2D_3$, rather than 1,25-$(OH)_2D_3$, may be required for normal endochondral bone formation (1, 16) and that in vitro this metabolite may be necessary for the transport and release of calcium in endochondral ossification (18). In addition, growth plate chondrocytes selectively bind 24,25-$(OH)_2D_3$ but not 1,25-$(OH)_2D_3$ (5, 8). In contrast, specific 1,25-$(OH)_2D_3$ binding to osteoprogenitor cells and osteoblasts has been reported (11, 13, 15); this metabolite also stimulates osteocalcin synthesis by bone cells (24). The observed specific stimulation of C-propeptide synthesis and $^{45}Ca^{2+}$ accumulation in growth plate cultures treated with 24,25-$(OH)_2D_3$ supports the observations that this metabolite has a specific effect on growth plate chondrocytes and provides direct evidence that this metabolite plays an important role in the calcification of cartilage and that this is directly correlated with the C-propeptide content of the tissue. The relative lack of effect of 24,25-$(OH)_2D_3$ on proteoglycan synthesis is of interest since it demonstrates the selectivity of the effect on the C-propeptide and calcification. The reason for the synergistic effect on the C-propeptide of the two metabolites in combination remains to be elucidated. The lack of a detectable effect of either of these metabolites on nasal chondrocytes points to the molecular as well as cellular specificities of their effects.

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