Calcif Tissue Int (1992) 50:175–183

Calcified Tissue International

Expression of Collagen, Osteocalcin, and Bone Alkaline Phosphatase in a Mineralizing Rat Osteoblastic Cell Culture

Pascal Collin,[1] Jean Raphaël Nefussi,[1] Antoinette Wetterwald,[2] Véronique Nicolas,[1] Marie-Laure Boy-Lefevre,[1] Herbert Fleisch,[2] and Nadine Forest[1]

[1]Laboratoire Biologie-Odontologie, S.D.I. CNRS 6326, Institut Biomédical des Cordeliers, 15-21 rue de l'Ecole-de-Médecine, 75270 Paris Cedex 06, France; and [2]Department of Pathophysiology, University of Bern, Murtenstrasse 35, CH 3010 Berne, Switzerland

Received December 4, 1989, and in revised form October 31, 1990

Summary. Rat calvaria bone cells isolated by collagenase digestion form a bone-like matrix which mineralizes *in vitro* in the presence of β-glycerophosphate, in less than 2 weeks. The purpose of this work was to investigate, in this mineralizing rat osteoblastic cell culture, the synthesis of collagen, osteocalcin, and bone alkaline phosphatase (ALP). The results obtained indicate (1) After 15 days in culture, the extracellular-matrix contains collagen type I, V, and to some extent type III. Metabolic labeling at day 14, during the phase of nodules mineralization as well as new nodules formation, shows that collagen types I and type V are synthesized; (2) During the phase of cell growth, no osteocalcin could be detected in the medium, however, at the point of nodule formation, the osteocalcin level reached values of 3.55 ± 1.39 ng/ml, followed by a 30-fold increase after nodules became mineralized. At day 14, after metabolic labeling, *de novo* synthesized osteocalcin was chromatographed on an immunoadsorbing column. With urea-SDS PAGE the apparent molecular weight was determined to be 9,000 daltons. (3) Specific activity of ALP was found to be 10 nmol/min/mg of proteins at cell confluence. At day 15, when nodules are mineralized, this activity was increased by 40-fold. The Michaelis constant was 1.58 10^{-3} M/L. ALP was inhibited by L-homoarginine and levamisole but not by L-phenylalanine. ALP was shown to be heat sensitive at 56°C with two slopes of inhibition. On SDS-PAGE, apparent molecular weight of ALP showed one band at 116,000 daltons (d) when extracted at cell confluence and two bands at 116,000 and 140,000 d when extracted at the 15th day of culture. ^{32}P-labeled subunit of the enzyme migrated as one band at 75,000 d. Sialic acid content was demonstrated by neuraminidase treatment either on the dimeric form or on the ^{32}P-labeled subunit. These data indicate that ALP expressed in this culture is bone specific. The results of the present study show that this mineralizing rat osteoblastic cell culture system synthesizes collagen type I, V, and traces of type III, osteocalcin, and bone ALP isoenzyme. Medium osteocalcin was detected during nodule formation and increased during mineralization. Increase in ALP activity as well as the presence of an additional form of ALP occurred in the mineralization phase. Therefore, this culture may be a useful model for studying the functions of bone-specific proteins during the process of mineralization.

Key words: *In vitro* mineralization – Collagen – Osteocalcin – Bone alkaline phosphatase.

The mechanisms of bone matrix formation and mineralization are still poorly understood. Many processes have been found to occur, among them the synthesis of various matrix proteins, enzymes, and proteoglycans. To advance our understanding of their role in bone metabolism, many attempts have been made to isolate osteoblasts that will retain their specific function of bone-forming cells in culture. Thus, *in vitro* models have been developed using bone cells that produced abundant fibrous intercellular substances that form a dense network and subsequently nodules that undergo mineralization [1–10]. With the culture system currently used in our laboratory, we have demonstrated that rat bone cells form a bone-like matrix which mineralizes when cells are cultured in the presence of β-glycerophosphate [6]. Electron transmission and scanning microscopy observations have shown that the tissue formed *in vitro* is similar to the one found *in vivo* [6]. Autoradiographic and cinematographic studies have shown that the kinetic of this matrix formation and mineralization follow a time sequence similar to the one observed *in vivo* [11]. Furthermore, osteoblastic cells under these conditions synthesize and release proteoglycans into the extracellular-matrix similarly in localization, organization, and morphology, as found *in vivo* [12]. As the functions of bone-specific proteins are as yet unknown, the present work was therefore undertaken to investigate in this mineralizing osteoblastic cell culture (1) the synthesis of collagen, osteocalcin, and alkaline phosphatase (ALP); (2) the isoenzyme form of ALP; (3) and the modulation of their expression with respect to the different phases of maturation of the culture. We demonstrate that collagen type I, V, and trace of type III, osteocalcin, and bone ALP are expressed in this mineralizing osteoblastic cell culture. Osteocalcin secretion and ALP activity are modulated during the four major steps of the culture, namely, cell growth/confluence, cluster formation, nodule formation, and mineralization. ALP expressed during the mineralization phase exhibits an additional structural form not detected at cell confluence.

Materials and Methods

Cell Isolation and Culture Procedure

Calvariae bone cells from 21-day-old fetal Sprague Dawley rats were isolated according to the modified procedure outlined elsewhere [6]. Briefly, central parts of the parietal and frontal bone with their endosteum and periosteum were incubated for 2 hours at 37°C in phosphate-buffered saline (PBS) containing 0.25% collagenase (Sigma type 1). Cells released from the bone fragments were washed several times in PBS, counted, seeded (2.10^4 cells/cm^2), and cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10 mM β-glycerophosphate (Sigma, St.

Louis, MO), 50 mg/ml ascorbic acid (Sigma), 10% fetal calf serum (FCS) (Boehringer-Mannheim), 50 U/ml streptomycin (Gibco), and 50 mg/ml penicillin (Gibco). Cells were cultured for 2 weeks in a humidified atmosphere of 5% CO_2 in air at 37°C. The medium was changed every 48 hours. Observations of cell cultures were performed using an inverted phase-contrast microscope (Leitz Diavert).

Isolation and Biochemical Analysis of Labeled Collagen

Metabolic labeling of collagen was performed from days 14–15 by the addition of fresh medium containing 10 μCi of 2,3 ^{3}H proline/ml (Ci/mmol). Labeling was terminated by adding acetic acid to a final concentration of 0.5 M. Cells were then homogenized and dialyzed against 0.5 M acetic acid for 24 hours. To extract and purify newly synthesized collagen, samples were treated with 1% (w/w) pepsin. Collagen was then analyzed by 7.5% SDS PAGE [13]. The same procedure was used to analyze nonlabeled collagen. Fluorography was carried out after electrophoresis. To assess for the presence of reducible α1 (III) chains, samples were submitted to the interrupted SDS-PAGE [14].

Osteocalcin in Culture Medium

The presence of osteocalcin in the culture medium was detected by an enzyme-linked immunosorbent assay (ELISA) in an inhibition assay under nonequilibrium condition as described by Stronsky et al. [15]. Briefly, equal amounts of sample or standard and antiosteocalcin rabbit antiserum (diluted 1:4000) were preincubated overnight at 4°C. They were then transferred to a microtiter plate (Immunoplates I, Nunc, Roskilde, Denmark) coated with 5 ng/ml of purified rat osteocalcin. Bound antiosteocalcin antibodies were detected with peroxidase-labeled sheep antirabbit Ig antibodies (New England Nuclear, Boston, MA, USA). The detection limit of the assay was 0.3 ng osteocalcin/ml and the interassay variation was 8.5%.

Metabolic labeling of newly synthesized osteocalcin was performed from days 14 to 15 by adding fresh medium containing 10 μCi of 2–3 ^{3}H proline/ml (Ci/mmol) and 2% FCS. Aliquots of the culture medium were then chromatographed on an immunosorbant column prepared with rabbit anti-rat osteocalcin antibodies coupled to sepharose. Unbound material was removed with PBS. Osteocalcin was eluted with gly-HCl, pH 2.5 (0.1 M), neutralized with 1 M Tris-buffer, pH 8.5, and subjected to ELISA. Fractions containing osteocalcin were pooled, dialyzed, lyophilized, dissolved in sample buffer containing 9 M urea, and subsequently subjected to urea-SDS-PAGE as described by Swank and Munkres [16]. Labeled molecular weight standard proteins (Boehringer-Mannheim) were run on the same gel.

Characterization of Alkaline Phosphatase

ALP was extracted by sonication with 20 times 0.5 sec/35 w pulses in a solution containing 0.1% NP_{40}, 1 mM $MgCl_2$. Cell debris was removed by centrifugation at 3,000 *g* for 10 minutes. ALPase activity was assayed by the method of Bessey et al. [17] in which one unit of enzyme activity is defined as 1 nmol of p-nitrophenyl-phosphate hydrolyzed/minute at 37°C, pH 10.2. Protein concentrations were determined by the method of Lowry et al. [18] using bovine albumin as standard. The km value was determined using the Lineweaver-burk, (Hanes and Eadie-Hofstee, unpublished data) representations. Thermal inactivation experiments were conducted at 56°C and inhibition studies were performed with L-homoarginine (HAR), L-phenylalanine (PHE), and levamisole (LEV). The time and concentration of each inhibitor required to produce 50% inhibition were determined.

Apparent Molecular Weight. ALP was extracted from the cell-matrix layer after the 3rd and 15th day of culture by sonication in 5 mM tris-HCl buffer, pH 7.5, containing 140 mM NaCl, 14 mM KCl, 1 mM $MgCl_2$, 0.2% NP_{40}, followed by centrifugation at 3,000 *g* for 10 minutes. After SDS-PAGE, ALP was detected by staining the gel, first in 0.1% 5-bromo-3-indolyl phosphate and then with coomassie blue. Molecular weight standard proteins (Sigma) were run on the same gel.

^{32}P-Labeling of ALP Subunit. To facilitate the detection of the ALP subunit in SDS-PAGE, enzyme preparation was labeled with ^{32}P-orthophosphate according to Milstein [19]. Two hundred microliters of enzyme (0.2 U) was incubated for 10 minutes at 0°C with 100 μl of 1 M sodium acetate (pH 5.0), 10 μl of 1 mM potassium phosphate (pH 7.4), and 100 μCi of carrier-free ^{32}P-phosphoric acid in 0.02 M HCl (New England Nuclear, Boston, MA). The same procedure was applied to the intestinal ALP (Sigma). The incubation was terminated by the addition of 25 μl of 3 M HCl and 2 ml of cold acetone. Precipitated proteins were washed twice by suspension in acid acetone (2 vol 0.2 M HCl: 8 vol acetone) and subsequent centrifugation. The pellet was then dried by an acetone wash, followed by gassing with a gentle stream of dry nitrogen gas.

Desialylation. This procedure was performed by mixing 1 vol of enzyme solution (0.5 ml) with an equal vol of 10 mM $NaHPO_4$/citrate buffer, pH 5.0, containing 2 U of neuraminidase, and the resulting solution was then incubated for 60 minutes at 37°C. Desialylation was monitored by electrophoresis on a 5% disc polyacrylamide gel at 130 V for 15 hours. ALP was located after specific staining using 0.1% 5-bromo-3-indolyl phosphate. Desialylation was also monitored after ^{32}P labeling of ALP subunit by electrophoresis on a 7.5% SDS-PAGE, followed by autoradiography.

Results

Bone Cell Culture

Under these culture conditions, bone cells form a matrix that mineralizes in less than 2 weeks. The process leading to this matrix formation and mineralization can be separated into four major steps, illustrated by Figure 1. The first phase (a) starting from day 1 to days 4/5 corresponds to a proliferative phase. During this period, cell proliferation occurs with the formation of several cell layers. The second phase (b), (days 5–7) is determined by the observation of a cell shape modification; cells are more refringent and round than the background cell layer, forming some clusters of functional osteoblastic cells. The third phase (c) (days 7–9), is associated with an active secretion of proteins by the osteoblastic cells forming a matrix that appeared as refringent material in phase contrast (nodule formation). The last phase (d) (days 9–15) corresponds to matrix mineralization as well as formation of new cell clusters.

Collagen Expression

As shown in Figure 2 (a), the major type of collagen present in the extracellular matrix layer of a 15-day-old mineralizing osteoblastic cell culture is type I, with some type V collagen. Type III collagen is not detected after late reduction (b+), compared with the nonreduced sample (b−). However, type III is faintly detected if a higher amount of collagenous extract is applied with the same procedure, although this does not appear clearly on the photograph (c+ versus c−). Under these conditions, α1 (III) chains migrated between α1 (I) and β chains.

In order to detect osteoblastic activity during nodule mineralization as well as new nodule formation, metabolic labeling was performed. The fluorograph in Figure 3 shows that (1) collagenous proteins are synthesized at this time period and (2) the bulk of the radiolabeled collagen is type I, with some collagen type V.

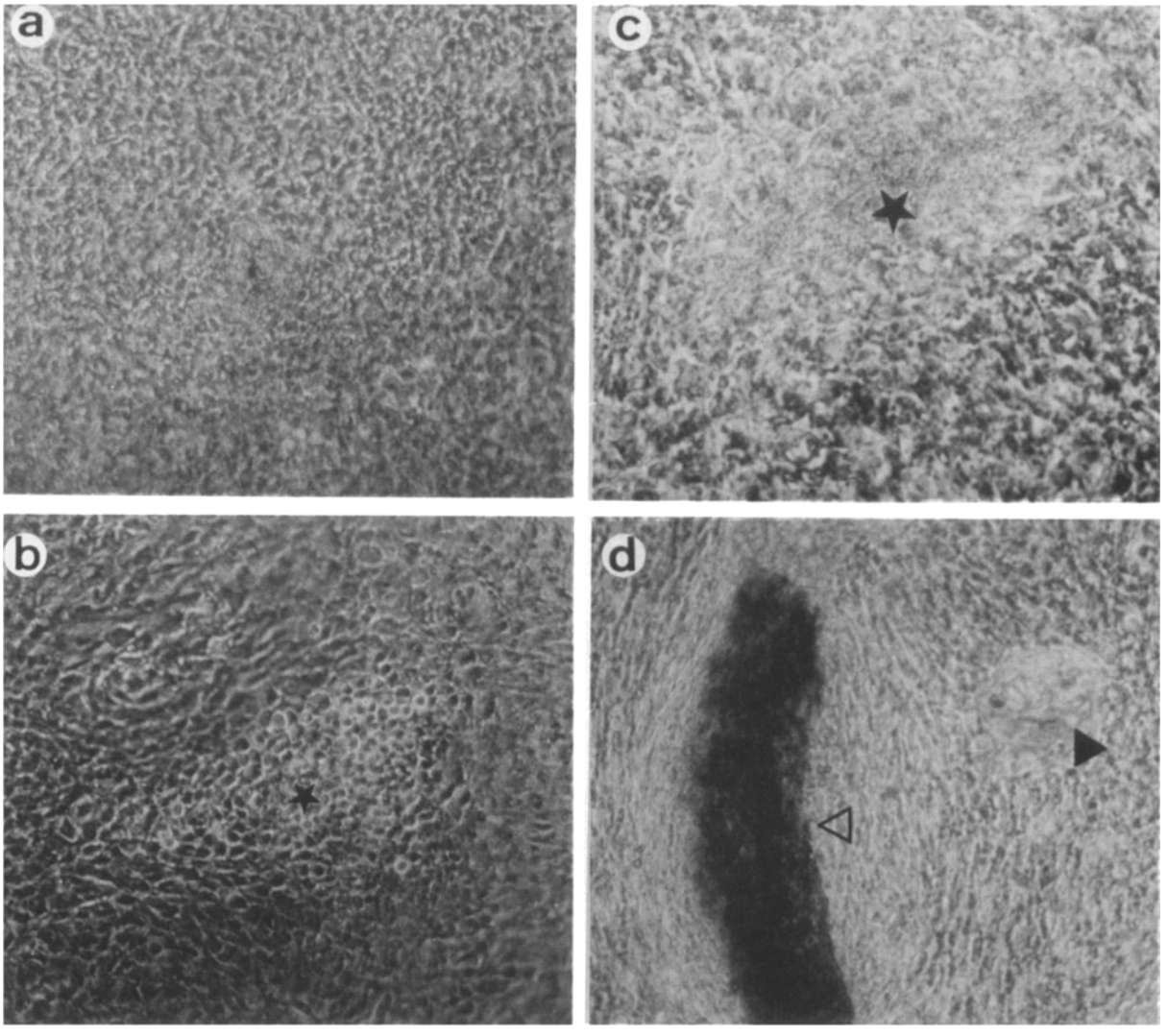

Fig. 1. Phase-contrast microscope study of the different phases of mineralizing rat osteoblastic cell culture. **(a)** First phase: cell growth until the cells have reached confluency (×320). **(b)** Second phase: appearance of cell clusters where cells have a different morphology compared with the background cell layers (small star) (×320). **(c)** Third phase: nodule formation as determined by the formation of a matrix seen as refringent material in phase contrast (large star) (×320). **(d)** Fourth phase: nodules mineralization (open arrowhead) as well as formation of new cell clusters followed by matrix formation (black arrowhead) (×320).

Osteocalcin Synthesis

The amounts of osteocalcin (bone Gla-protein, BGP) secreted into the medium during the four steps of cell culture maturation are illustrated in Figure 4. During cell growth (phase 1) and cluster formation (phase 2), no osteocalcin was found in the medium. However, at the onset and during nodule formation phase 3, osteocalcin was detected in the medium at a concentration of 3.55 ± 1.39 ng/ml. During the next step (phase 4), corresponding to the mineralization of the nodules as well as new nodule formation, osteocalcin level reached maximal values of 107.9 ± 24.7 ng/ml. Thereafter, osteocalcin concentration appeared to decline. This pattern of osteocalcin synthesis was highly reproducible (Fig. 4, cultures A and B). However, the level of osteocalcin concentration in the medium during the last phase is subject to variations.

To further examine the synthesis of osteocalcin, metabolic labeling was performed during the mineralization process (phase 4). Figure 5 illustrates the results obtained after electrophoresis of the culture medium before (a) and after (b) purification by immunochromatography. The fluorograph shows that at this time point osteocalcin is synthesized, secreted, and released in the medium within 24 hours. The radiolabeled osteocalcin was found to migrate as one band, with an apparent molecular weight of 9,000 d.

Characterization of ALP

The specific activity of ALP determined in a crude extract prepared from a 3-day and 15-day-old mineralized culture was found to be 10 and 400 nmol/minute/mg of proteins, respectively. These values represent a 40-fold increase of enzyme activity between phase 1 and phase 4. During phase 4, the km of ALP, using p-nitrophenylphosphate, was determined to be 1.58 mM/liter (Fig. 6). Hanes and Eadie-Hofstee plots were linear in the region of km, which is in agreement

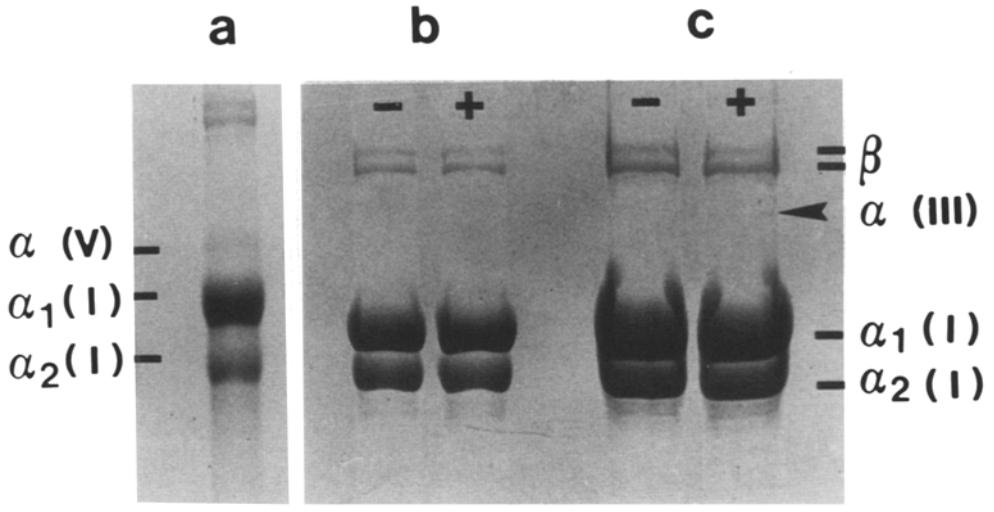

Fig. 2. SDS-polyacrylamide gel analysis of collagen obtained from a 15-day-old culture (phase 4) when nodules are formed and mineralized. **(a)** Coomassie blue staining of pepsin-solubilized collagen after 7.5% SDS-PAGE in nonreduced condition. Collagen was prepared from a 90 mm Petri dish, as described in Materials and Methods, and dissolved (4 mg/ml) in sample buffer. **(b)** Coomassie blue staining of pepsin-solubilized collagen (20 μl) after interrupted electrophoresis on a 7.5% polyacrylamide gel in the absence (−) or the presence (+) of 2-mercaptoethanol. **(c)** As in **b**, with 50 μl of collagen solution. Black arrowhead indicates the position of α1 (III) chains.

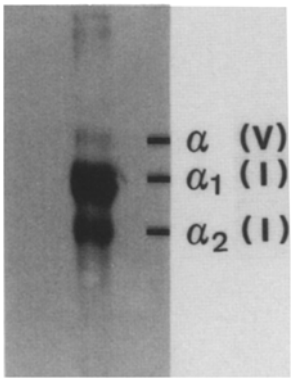

Fig. 3. Fluorograph of radiolabeled collagen. At day 14, culture was labeled with 10 μCi of 2,3^{3}H proline/ml for 24 hours. After extraction, 6 10^4 cpm were applied on a 7.5% SDS gel. Fluorography was carried out after electrophoresis. Gel was exposed for 72 hours at −70°C to Kodak X-Omat AR film.

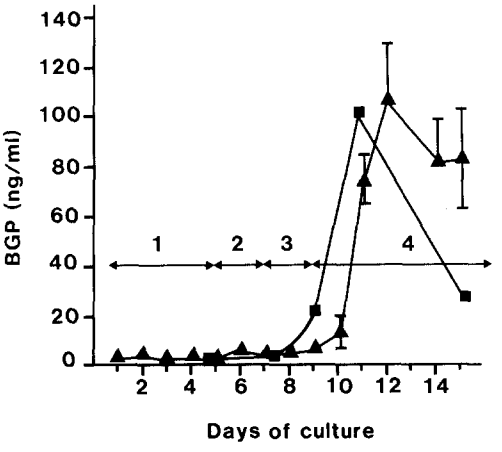

Fig. 4. Osteocalcin (BGP) concentration in the medium of mineralizing rat osteoblastic cell culture. Osteoblastic cells were cultured on a 6 × 9.62 cm^2 multiwell plate. Medium was removed every 24 hours for osteocalcin measurement and subsequently replaced by fresh medium. Osteocalcin concentration was determined by ELISA as described in Materials and Methods. Each point represents the mean of six culture dishes which were run together for culture A (▲). For culture B (■), osteocalcin concentration was determined in a 35 mm culture dish. Each data is the average of duplicate determinations. Results are expressed as mean ± SD. 1, 2, 3, 4 represent the four major steps of maturation of culture A as shown in Figure 1.

with one isoenzyme form (data not shown). The inhibition of ALP activity by different inhibitors is shown in Figure 7. L-homoarginine (ARG) caused a strong inhibition (Fig. 7a), and the concentration of ARG required to produced a 50% inhibition was 9 mM. With L-phenylalanine, only a weak inhibition was obtained whereas a 50% inhibition was never reached even with 15 mM (Fig. 7a). As shown in Fig. 7b, levamisole produced a marked inhibition on ALP activity. ALP was very thermolabile at 56°C, and 50% inactivation occurred after 90 seconds (Fig. 8). Semi-log representation of this inactivation pattern showed two slopes of inhibition (Fig. 8, insert) compared with the intestinal ALP which showed only one slope of inhibition. The enzymatic activity of ALP, extracted from a 3-day-old culture, migrated on SDS-PAGE as a single band either in the absence (Fig. 9a, lane 1) or the presence of p-hydroxymercuriphenylsulfonic acid, a phospholipase C inhibitor (Fig. 9a—lane 2). Two single bands were detected (Fig. 9b, lane 1) when ALP was extracted at day 15 when nodules have become mineralized. No ALP enzymatic activity could be detected on the gels when samples were treated with 1% 2-mercaptoethanol (Fig. 9b, lane 2) or after heat treatment at 100°C (Fig. 9b, lane 3). The apparent molecular weight of ALP at day 3 (phase 1) was found to be 116,000 d either in absence (Fig. 9c, lane 1) or in presence of phospholipase C inhibitor (Fig. 9c, lane 2). ALP extracted at day 15 expressed two bands at 116,000 and 140,000 d for the lower and higher band, respectively (Fig. 9d). To further examine the structure of the enzyme, ^{32}P-labeled ALP was submitted to electrophoresis. Figure 10 shows that ALP monomer migrated in one band with an apparent molecular weight of 75,000 d (lane 2), compared with the intestinal ALP which migrated at 68,000 d (lane 1). These results indicate that ALP contains a 75 kD monomer. To established whether ALP, at day 15, contains sialic acid as does bone ALP *in vivo*, desialylation was performed by neuraminidase treatment. Figure 11a shows that neuraminidase altered the electrophoretic mobility of ALP on native polyacrylamide gel (lane 1) compared with the control (lane

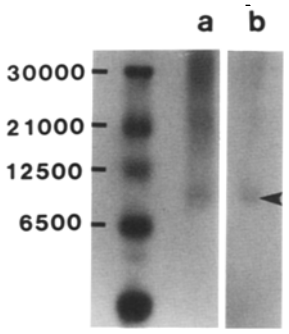

Fig. 5. Urea-SDS polyacrylamide gel electrophoresis fluorograph of labeled osteocalcin. Osteoblastic cells were cultured in 90 mm culture dishes. At day 14 (phase 4), when nodules are mineralized, culture was labeled with 10 μCi of ^{3}H proline/ml in 2% FCS. After 24 hours, medium was chromatographed on an immunoadsorbant column as described in Materials and Methods. Lane (a) represents an aliquot of labeled proteins before immunoadsorption; (b) corresponds to the eluated osteocalcin from the column. As molecular weight standards, ^{14}C methylated carbonic anhydrase 30 kD, soybean trypsin inhibitor 21 kD, cytochrome C 12.5 kD, aprotinin 6.5 kD (Amersham) were used.

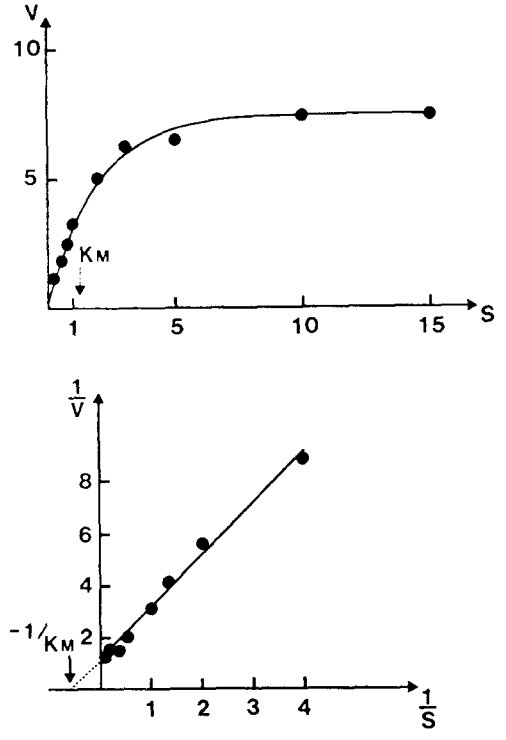

Fig. 6. Effects of substrate concentration on ALP activity from a 15-day-old culture (phase 4). Experiments were conducted on 90 U/ml enzyme activity. Activity was measured at pH 10.5 at 37°C with p-nitrophenylphosphate as substrate. Substrate concentrations were between 0.5 and 15 mM. Each point represents the mean of triplicate samples. Upper graph: substrate concentration curve of ALP. Note that the enzyme exhibited hyperbolic substrate kinetics with a km of 1.25 10^{-3} M/liter. Lower graph: Lineweaver-Burk's plot. The calculated km was 1.72 10^{-3} M/liter. Using the Eadie-Hofstee's and Hanes representation, the calculated km was 1.6 10^{-3} M/liter and 1.78 10^{-3} M/liter, respectively. The mean km value given in the text represents the average of these four determinations.

2). These data indicate a difference of charge between the treated and the nontreated ALP. Figure 11b shows that the neuraminidase-treated ^{32}P-labeled monomer (lane 2) migrates at a lower position than the nontreated enzyme (lane 1) on 7.5% SDS-PAGE. These results suggest that bone ALP expressed in this culture contains sialic acid.

Discussion

Type I collagen is the predominant collagen isoform found in the cell matrix-layer during phase 4 of this mineralizing rat osteoblastic cell culture, thus confirming the previous results obtained by Nefussi et al. [6] using the immunofluorescence technique. This observation is consistent with Epstein and Munderloh [20] and Miller [21] who have shown that *in vivo,* bone matrix contains type I collagen. Similar results have also been reported by other laboratories using osteoblastic cell cultures [3, 5, 7, 8, 22–27]. The presence of type V collagen has been described in intact calvaria [28] as well as in osteoblastic cell cultures [3, 5, 23, 25]. Type V collagen is also found in the composition of the extracellular matrix. The detection of a small amount of type III collagen on collagen typing gel is in accordance with previous studies [3, 5, 7, 23]. This low level of type III collagen in our cultures may have been due either to a small number of contaminating fibroblastic cells or to bone cells that have been shown to synthesize type III collagen [24–26]. In some studies, however, collagen type III mRNA but not collagenous protein were detected [27, 29]. In addition, synthesis of types I and V collagen at day 14 indicates that this osteoblastic cell culture is still producing collagenous proteins at this time point. Whether this occurs within new nodules or in the nodules already formed during phase 4 [12] remains to be evaluated.

Earlier studies with bone organ culture derived from chick embryo [30] and calf [31], as well as primary cultures of osteoblastic cells derived from fetal rat calvariae [27, 32] or from human trabecular bone [33], have demonstrated *de novo* formation of osteocalcin. In addition, osteocalcin has been shown to be a specific extracellular matrix product of osteoblasts derived either from clonal osteosarcoma cell line [34, 35] or from human bone cells [36, 37]. Presently, the precise function of osteocalcin is still unknown. In this study, high osteocalcin concentrations were found in the medium from mineralizing rat osteoblastic cell culture at the phase of nodule mineralization, as recently described with chicken osteoblastic cells [8]. Therefore, this culture provides an experimental system for studying the role of osteocalcin during mineralization. The osteocalcin secretion was obtained without any prior treatment with $1,25(OH)_2D_3$ which has been shown to increase the synthesis of osteocalcin [38]. Thus, it seems that osteocalcin secretion is not only regulated by $1,25(OH)_2D_3$, but also by other factors, as recently suggested by Lian et al. [39]. Therefore, such factors should be present in mineralizing osteoblastic cell cultures.

The experiments presented here were also directed at determining whether a temporal program of osteocalcin expression might be associated with osteoblastic differentiation, matrix formation, and nodule mineralization. As it was found that osteocalcin is absent in the medium during cell growth/confluence or cluster formation and is present in the medium during nodule formation, our data would support the idea that induction of osteocalcin synthesis is related to osteoblastic differentiation.

Osteocalcin is a potent inhibitor of hydroxyapatite formation *in vitro* [40]. However, we found that osteocalcin is released in large amounts into the medium, when nodules start to mineralize, which could be interpreted that osteocalcin might favor nodule mineralization *in vitro*. The correlation between osteocalcin synthesis and mineralization seems to be in agreement with previous investigations on the appearance of osteocalcin in the developing rat [41, 42].

To assess whether osteocalcin was continuously synthesized at the end of phase 4, metabolic labeling experiments

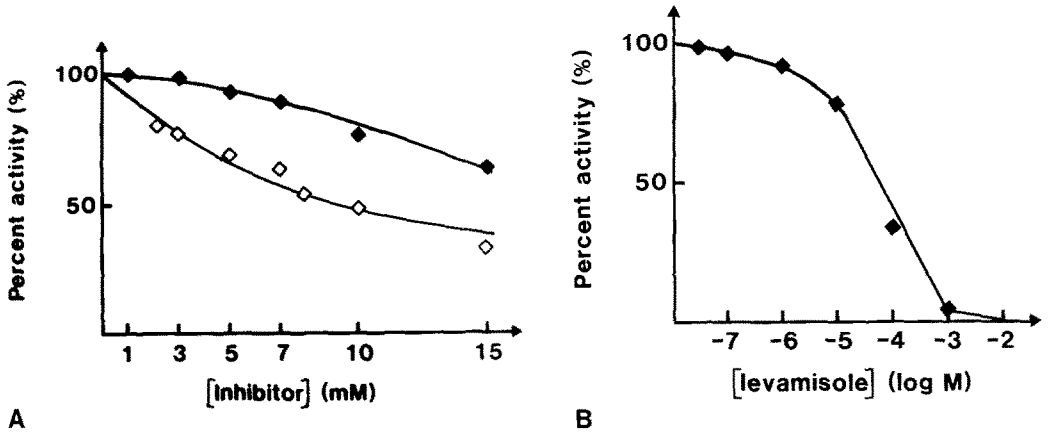

Fig. 7. Effect of L-phenylalanine, L-homoarginine, and levamisole on ALP activity. ALP was extracted from a 15-day-old culture (phase 4) and assayed immediately for p-nitrophenyl phosphatase activity in the presence of inhibitors at various concentrations. Assays were carried out either on 25 or 50 µl aliquots of 90 U/ml enzymatic activity for either 5 or 10 minutes at 37°C in carbonate buffer. (a) Effect of L-phenylalanine (♦) and L-homoarginine (◇) on ALP activity. (b) Effect of levamisole (♦) on ALP activity. Data are expressed as mean percentage of basal activity.

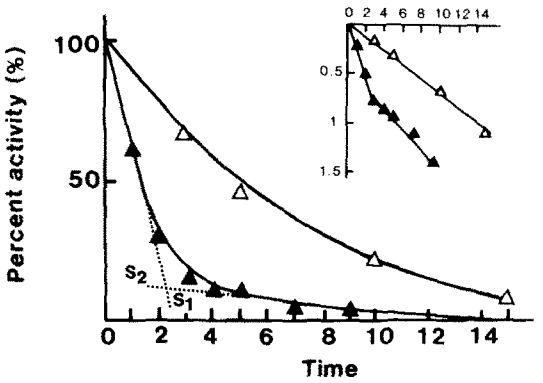

Fig. 8. Heat inactivation of ALP. ALP was extracted from a 15-day-old culture (phase 4). (▲). Aliquots (25 or 50 µl) of 90 U/ml enzymatic activity were incubated at 56°C for the indicated time periods expressed in minutes, followed by rapid cooling. ALP activity was measured in carbonate buffer as described in Materials and Methods. The same procedure was applied to intestinal ALP (△). Data are expressed as mean percentage of basal activity. Semi-log representation of these data is shown within the dotted lines. Note the presence of two slopes for bone ALP. The linear regression analysis of these two slopes gives two distinct values: $\alpha 1 = +0.02$ and $\alpha 2 = -0.377$. Only one slope is detected with the intestinal ALP.

were performed. Our data have shown that osteocalcin is synthesized and found in the medium at day 15. This indicates that active osteoblasts are present at day 15 in this culture, and osteocalcin is synthesized, secreted, and released in the medium within 24 hours. Radiolabeled osteocalcin, on urea-SDS PAGE, migrated at a position of 9,000 d. As it was shown in a previous report [43], electrophoretic mobility of osteocalcin did not fit with its molecular weight, determined from amino-acid sequence (6,000 d). Thus, we cannot conclude whether the osteocalcin represents the osteocalcin precursor (9,000 d from amino-acid sequence) or the mature form. This, however, does not preclude the possibility that osteocalcin has been modified by glycation [44] or decarboxylation [45] which could also modify the electrophoretic mobility.

Bone ALP is known to be related to the bone-liver-kidney group and clearly distinct from the intestinal and placental form [46–50]. Partial amino-acid sequence [51] and cDNA sequences have been recently reported [52, 53]. To assess whether ALP expressed in this mineralizing rat osteoblastic cell culture was a bone isoenzyme, enzymatic and structural studies were performed on day 15, when nodules are mineralized. The present data show that ALP activity was very high, comparable to values reported previously [54, 55]. Furthermore, the value for the Michaelis constant is similar to that reported for bone ALP [56, 57]. Inhibition profile obtained by specific inhibitors and the rapid heat inactivation are in agreement with previous reports on bone ALP either extracted *in vivo* [56, 58, 59] or *in vitro* [57, 60, 61]. In this study we also demonstrated that ALP monomer migrate as one band with an apparent molecular weight similar to that reported in previous studies [58, 59]. Furthermore, the dimeric form of the enzyme is disrupted by 2-mercaptoethanol treatment which could indicate that ALP is composed of two subunits linked by disulfide bridges. Finally, we have shown that ALP was modified by neuraminidase treatment indicating that sialic acid is linked to ALP. This result is consistent with previous studies showing that ALP expressed by human osteosarcoma cells contained sialic acid [59] whereas placental and intestinal ALP lack it [47]. Therefore, these results suggest that ALP expressed in this culture is a bone-isoenzyme. Studies performed on ALP expressed during phase 4 have shown linearity in the region of km and only one ^{32}P-radiolabeled monomer. Therefore, it is reasonable to suggest that only one isoenzyme form is expressed in this culture during the last phase of this culture.

Although the function of skeletal ALP *in vivo* is unknown, the enzyme is thought to be involved in bone formation and calcification [62–64]; the latter might be due to the pyrophosphatase activity of the enzyme [65]. ALP activity has been shown to coincide with the onset and subsequent development of skeletal elements in the fetal limb [66]. However, induction of ALP by extraskeletal implants of bone matrix has been shown not to correlate with the mechanism of calcification [67, 68]. In the present experiment, we demonstrated a 40-fold increase of ALP activity between cell growth (phase 1) and mineralization (phase 4); it therefore seems possible that ALP is involved in the maturation pro-

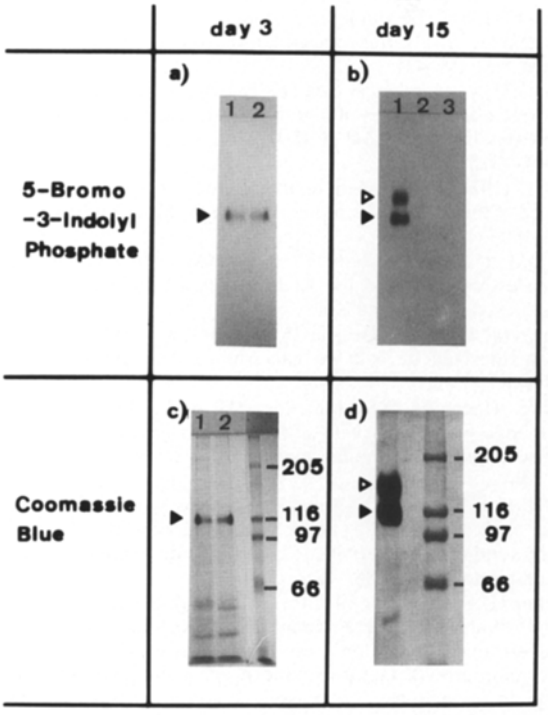

Fig. 9. SDS-polyacrylamide gel electrophoresis of ALP from a mineralizing rat osteoblastic cell culture. ALP was extracted either from a 3-day-old culture, at cell confluence (phase 1), in the absence or presence of p-hydroxymercuriphenylsulfonic acid 2.5 mM (**a, c:** lane 1 and lane 2, respectively) or from a 15-day-old culture (**b, d**) when nodules are mineralized (phase 4). Samples of enzyme solution were mixed in sample buffer and electrophoresed on a 7.5% SDS polyacrylamide gel. In **b**, ALP was either not treated before electrophoresis (**b**, lane 1) or treated either with 2-mercaptoethanol (*b*, lane 2) or by heating at 100°C for 60 seconds (**b**, lane 3). Gels were stained with 5-bromo-3-indolyl phosphate (**a, b**) and then with coomassie blue (**c, d**). Black arrowheads indicate the "higher" form and white arrowheads the "lower" form for ALP. Molecular weight standard proteins were run on the same gel in **c** and **d:** myosin (205 kD); β-galactosidase (116 kD); phosphorylase b (97 kD); bovine albumin (66 kD), and ovalbumin (45 kD).

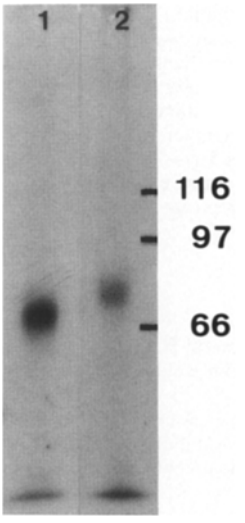

Fig. 10. SDS-polyacrylamide gel electrophoresis autoradiograph of ^{32}P-labeled alkaline phosphatase subunit. Lyophilized ^{32}P-labeled subunit of ALP from the culture (lane 2) as well as ^{32}P-labeled intestinal ALP (Sigma) (lane 1) were mixed in sample buffer and electrophoresed on a 7.5% SDS polyacrylamide. Molecular weight standard proteins were run on the same gel. Gel was stained with coomassie blue, followed by autoradiography for 72 hours at −70°C (Kodak X-Omat AR film).

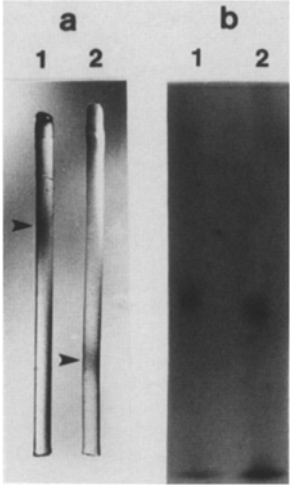

Fig. 11. Effect of neuraminidase treatment on ALP of a 15-day-old mineralizing rat osteoblastic cell culture. **(a)** 5% disc PAGE of ALP after (lane 1) and before (lane 2) treatment with 2 U of neuraminidase. After electrophoresis, gels were stained with 5-bromo-3-indolyl phosphate. Arrowheads indicate the position of ALP in the gel. **(b)** 7.5% SDS-PAGE of ^{32}P-labeled ALP subunit before (lane 1) and after (lane 2) treatment with 2 U of neuraminidase. After electrophoresis, the gel was exposed to Kodak X-Omat AR film for 72 hours at −70°C.

cess. However, whether this induction of ALP activity occurred during cluster/nodule formation or at the onset of mineralization remains to be evaluated.

ALP is a membrane-glycoprotein [64] and is known to be covalently linked to the membrane by a phosphatidylinositol-glycan structure [69, 70]. Because we found that bone ALP enzymatic activity extracted during phase 4 of the mineralizing osteoblastic cell culture exhibits two bands with two different apparent molecular weights and that biochemical studies have shown the presence of only one isoenzyme form, our data suggest that (1) the two bands with ALP activity observed on SDS gel are the same bone isoenzyme (2) and the band in the higher position corresponds to the bone ALP (lower band), with an additional structure that is not removed by nonionic detergent. Such a structure modifies the electrophoretic mobility of ALP on SDS polyacrylamide gel. The presence of these two forms was suggested by heat inactivation study at 56°C which shows two different heat sensitivities. We have previously reported [71] that nonspecific phospholipase C and endoglycosidase F treatment transformed the "higher" band of ALP into a "lower" band. Such covalent membranous linkage of bone ALP has been demonstrated using specific PI-PhLC, either on rat [72] or on human osteosarcoma cells [73, 74]. Therefore, we suggest that the higher form of ALP, expressed at day 15, is covalently linked to a phospholipid-glycan structure on the cell membrane.

During cell growth/confluence (phase 1), only the band in the lower position has been detected in the cell-matrix layer. This is the first demonstration that shows a difference between the structure of bone ALP expressed at cell confluence and during *in vitro* mineralization. It is presently unclear why the band in the higher position is not detected during this step. This is not due to the release of the higher form into the lower form by phosphatidylinositol phospholipases C during the extraction because the same profile was obtained in the presence of inhibitors, which have been shown to block the PhLC activity [75]. However, this could have been due to the presence of another pathway of secretion that does not involve covalent linkage to the membrane. A similar feature has been reported for the intestinal ALP [76]. Therefore, this culture provides an experimental system that can be used to study the role of these bone-associated proteins in the mineralization process.

Acknowledgment: The authors wish to thank Dr. J. W. Poser, Procter and Gamble for providing the osteocalcin antibodies and C. Plas for reviewing this manuscript. We also thank N. Martin, M. Bolle for their technical assistance, E. Marie-Rose and K. Marlin for secretarial assistance, and C. Stieger for assistance with the illustrations. This work was supported by the University of Paris VII, the Dental association of Garanciere and the Swiss National Science Foundation (Grant 3.894.0.88). This study represents part of a dissertation by P. Collin for the degree of "Doctorat d'Etat."

References

1. Binderman I, Duksin D, Harell A, Katzir E, Sachs L (1974) Formation of bone tissue in culture from isolated bone cells. J Cell Biol 61:427–439
2. Nijweide PJ, van Iperen-van Gent AS, Kawilarang-de Haas EWM, van der Plas A, Wassenaar AM (1982) Bone formation and calcification by isolated osteoblastic cells. J Cell Biol 93:318–323
3. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S (1983) In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96:191–198
4. Ecarot-charrier B, Glorieux FH, van der Rest M, Pereira G (1983) Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. J Cell Biol 96:639–643
5. Whitson SW, Harrisson W, Dunlap MK, Bowers DE, Fisher LW, Robey PG, Termine JD (1984) Fetal bovine cells synthesize bone-specific matrix proteins. J Cell Biol 99:607–614
6. Nefussi JR, Boy-Lefevre ML, Boulekbache H, Forest N (1985) Mineralization in vitro of matrix formed by osteoblasts isolated by collagenase digestion. Differentiation 29:160–168
7. Bellows CG, Aubin JE, Heersche JNM, Antosz ME (1986) Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcif Tissue Int 38:143–154
8. Gerstenfeld LC, Chipman SD, Glowacki J, Lian JB (1987) Expression of differentiated function by mineralizing cultures of chicken osteoblasts. Dev Biol 122:49–60
9. Bhargava U, Bar-lev M, Bellows CG, Aubin JE (1988) Ultrastructural analysis of bone nodules formed in vitro by isolated fetal rat calvaria cells. Bone 9:155–163
10. Lomry A, Marie PJ, Tran PV, Hott M (1988) Characterization of endosteal osteoblastic cells isolated from mouse caudal vertebrae. Bone 9:165–175
11. Nefussi JR, Pouchelet M, Collin P, Sautier JM, Develay G, Forest N (1989) Microcinematographic and autoradiographic kinetic studies of bone cell differentiation in vitro: matrix formation and mineralization. Bone 10:345–350
12. Nefussi JR, Septier D, Collin P, Goldberg M, Forest N (1989) A comparative ultrahistochemical study of glycosaminoglycans with cuprolinic blue in bone formed in vivo and in vitro. Calcif Tissue Int 44:11–19
13. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature (London) 227:680–685
14. Sykes B, Puddle B, Francis M, Smith R (1976) The estimation of two collagens from human dermis by interrupted gel electrophoresis. Biochem Biophys Res Commun 72:1472–1480
15. Stronski SA, Bettschen-Camin L, Wetterwald A, Felix R, Trechsel U, Fleisch H (1988) Biphosphonates inhibit 1,25-dihydroxyvitamin D_3-induced increase of osteocalcin in plasma of rats in vivo and in culture medium of rat calvaria in vitro. Calcif Tissue Int 42:248–254
16. Swank RT, Munkres KD (1971) Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal Biochem 39:462–477
17. Bessey OA, Lowry OH, Brock MJ (1946) A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J Biol Chem 164:321–329
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin-phenol reagent. J Biol Chem 193:265–275
19. Milstein C (1964) The amino acid sequence around the reactive serine residue in alkaline phosphatase from escherichia coli. Biochem J 92:410–421
20. Epstein EH Jr, Munderloh NM (1975) Isolation and characterization of CNBR peptides of human $\alpha1(III)_3$ collagen and tissue distribution of $\alpha1(I)_2$ and $\alpha1(III)_3$ collagens. J Biol Chem 250:9304–9312
21. Miller EJ (1976) Biochemical characteristics and biological significance of the genetically distinct collagens. Mol Cell Biochem 13:165–190
22. Scott DM, Kent GN, Cohn DV (1980) Collagen synthesis in cultured osteoblast-like cells. Arch Biochem Biophys 201:384–391
23. Wiestner M, Fisher S, Dessau W, Müller PK (1981) Collagen types synthesized by isolated calvarium cells. Exp Cell Res 133:115–125
24. Aubin JE, Heersche JNM, Merrilees MJ, Sodek J (1982) Isolation of bone cell clones with differences in growth, hormone responses, and extracellular matrix production. J Cell Biol 92:452–461
25. Hata RI, Hori H, Nagai Y, Tanaka S, Kondo M, Hiramatsu M, Utsumi N, Kumegawa M (1984) Selective inhibition of type I collagen synthesis in osteoblastic cells by epidermal growth factor. Endocrinology 115:867–876
26. Guenther HL, Hofstetter W, Stutzer A, Mühlbauer R, Fleisch H (1989) Evidence for heterogeneity of the osteoblastic phenotype determined with clonal rat bone cells established from transforming growth factor-β-induced cell colonies grown anchorage independently in semisolid medium. Endocrinology 125:2092–2102
27. McCarthy TL, Centrella M, Canalis E (1988) Further biochemical and molecular characterization of primary rat parietal bone cell cultures. J Bone Miner Res 3:401–408
28. Rhodes RK, Miller EJ (1978) Physical characterization and molecular organization of the collagen A and B chains. Biochemistry 17:3442–3447
29. Gerstenfeld LC, Chipman SD, Kelly CM, Hodgens KJ, Lee DD, Landis WJ (1988) Collagen expression, ultrastructural assembly, and mineralization in cultures of chicken embryo osteoblasts. J Cell Biol 106:979–989
30. Lian JB, Heroux KM (1980) In vitro studies of osteocalcin biosynthesis in embryonic chick bone cultures. In: Suttie JW (ed) Vitamin K metabolism and vitamin K-dependent proteins. University Park Press, Baltimore, pp 245–254
31. Nishimoto SK, Price PA (1979) Proof that the γ-carboxyglutamic acid-containing bone protein is synthesized in calf bone. J Biol Chem 254:437–441
32. Lian JB, Coutts M, Canalis E (1985) Studies of hormonal regulation of osteocalcin synthesis in cultured fetal rat calvariae. J Biol Chem 260:8706–8710
33. Beresford JN, Gallagher JA, Poser JW, Russel RGG (1984) Production of osteocalcin by human bone cells in vitro. Effects of $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, parathyroid hormone, and glucocorticoids. Metab Bone Dis Rel Res 5:229–234
34. Nichimoto SK, Price PA (1980) Secretion of the vitamin K-dependent protein of bone by rat osteosarcoma cells. J Biol Chem 255:6579–6583
35. Majeska RJ, Rodan SB, Rodan GA (1980) Parathyroid hormone-responsive clonal cell line from rat osteosarcoma. Endocrinology 107:1494–1503
36. Kaplan GC, Eilon G, Poser JW, Jacobs JW (1985) Constitutive biosynthesis of bone gla protein in a human osteosarcoma cell line. Endocrinology 117:1235–1238
37. Auf'mkolk B, Hauschka PV, Schwartz ER (1985) Characterization of human bone cells in culture. Calcif Tissue Int 37:228–235
38. Price PA, Baukol SA (1980) 1,25-dihydroxyvitamin D_3 increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. J Biol Chem 255:11660–11663
39. Lian J, Stewart C, Puchacz E, Mackowiak S, Shalhoub V, Collart D, Zambetti G, Stein G (1989) Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. Proc Natl Acad Sci USA 86:1143–1147
40. Price PA, Otsuka AS, Poser JW, Kristaponis J, Raman N (1976)

Characterization of a γ-carboxyglutamic acid-containing protein from bone. Proc Natl Acad Sci USA 73:1447–1451
41. Price PA, Lothringer JW, Nishimoto SK (1980) Absence of the vitamin K-dependent bone protein in fetal rat mineral. J Biol Chem 255:2938–2942
42. Price PA, Lothringer JW, Baukol SA, Reddi AH (1981) Developmental appearance of the vitamin K-dependent protein of bone during calcification. J Biol Chem 256:3781–3784
43. Gundberg CM, Hauschka PV, Lian JB, Gallop PM (1984) Osteocalcin: isolation, characterization, and detection. Methods Enzymol 107:516–548
44. Gundberg CM, Anderson M, Dickson I, Gallop PM (1986) "Glycated" osteocalcin in human and bovine bone. J Biol Chem 261:14557–14561
45. Price PA, Williamson MK, Lothringer JW (1981) Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. J Biol Chem 256:12760–12766
46. Badger KS, Sussman HH (1976) Structural evidence that human liver and placental alkaline phosphatase isoenzymes are coded by different genes. Proc Natl Acad Sci USA 73:2201–2205
47. McKenna MJ, Hamilton TA, Sussman HH (1979) Comparison of human alkaline phosphatase isoenzymes. Biochem J 181:67–73
48. Seargeant LE, Stinson RA (1979) Evidence that three structural genes code for human alkaline phosphatases. Nature 281:152–154
49. Goldstein DJ, Rogers CE, Harris H (1980) Expression of alkaline phosphatase loci in mammalian tissues. Proc Natl Acad Sci USA 77:2857–2860
50. Harris H (1982) Multilocus enzyme systems and the evolution of gene expression: the alkaline phosphatases as a model example. Harvey Lect 76:95–124
51. Nair BC, Johnson DE, Majeska RJ, Rodkey JA, Bennett CD, Rodan GA (1987) Rat alkaline phosphatase. II. Structural similarities between the osteosarcoma, bone, kidney, and placenta isoenzymes. Arch Biochem Biophys 254:28–34
52. Weiss MJ, Henthorn PS, Lafferty MA, Slaughter C, Raducha M, Harris H (1986) Isolation and characterization of a cDNA encoding a human liver/bone/kidney-type alkaline phosphatase. Proc Natl Acad Sci USA 83:7182–7186
53. Thiede MA, Yoon K, Golub EE, Noda M, Rodan GA (1988) Structure and expression of rat osteosarcoma (Ros 17/2.8) alkaline phosphatase: product of a single copy gene. Proc Natl Acad Sci USA 85:319–323
54. Singh I, Tsang KY (1975) An in vitro production of bone-specific alkaline phosphatase. Exp Cell Res 95:347–358
55. Levine AM, Triche T, Rosenberg SA (1980) Osteosarcoma cells in tissue culture: II. Characterization and localization of alkaline phosphatase activity. Clin Orthop 146:259–268
56. Farley JR, Ivey JL, Baylink DJ (1980) Human skeletal alkaline phosphatase. J Biol Chem 255:4680–4686
57. Majeska RJ, Rodan GA (1982) Alkaline phosphatase inhibition by parathyroid hormone and isopropanol in a clonal rat osteosarcoma cell line. Possible mediation by cyclic AMP. Calcif Tissue Int 34:59–66
58. Nair BC, Majeska RJ, Rodan GA (1987) Rat alkaline phosphatase. I. Purification and characterization of the enzyme from osteosarcoma: generation of monoclonal and polyclonal antibodies. Arch Biochem Biophys 254:18–27
59. Masuhara K, Sugamoto K, Yoshikawa H, Takaoka K, Ono K, Morris DC, Hsu HH, Anderson HC (1987) Purification of bone alkaline phosphatase from human osteosarcoma. Bone Miner 3:159–170
60. Murray E, Provvedini D, Curran D, Catherwood B, Sussman H, Manolagas S (1987) Characterization of a human osteoblastic osteosarcoma cell line (SAOS-2) with high bone alkaline phosphatase activity. J Bone Miner Res 2:231–238
61. Hamada H, Amitani K, Ono K, Sakamoto Y, Kishimoto R, Nakata Y (1979) Osseous alkaline phosphatase from osteosarcoma in mouse and in culture. Cell Mol Biol 25:77–84
62. Robison R (1923) The possible significance of hexosephosphoric esters in ossification. Biochem J 17:286–293
63. Siffert RS (1951) The role of alkaline phosphatase in osteogenesis. J Exp Med 93:415–426
64. McComb RB, Bowers GN, Posen S (1979) Alkaline phosphatase. Plenum, New York
65. Felix R, Fleisch H (1976) Pyrophosphatase and ATPase of isolated cartilage matrix vesicles. Calcif Tissue Res 22:1–7
66. Jaffe NR (1976) Alkaline phosphatase activity, characterization, and subcellular distribution during initial skeletogenesis in the prenatal rat limb. Calcif Tissue Int 21:153–162
67. Firschein HE, Urist MR (1971) The induction of alkaline phosphatase by extraskeletal implants of bone matrix. Calcif Tissue Res 7:108–113
68. Reddi AH, Huggins C (1972) Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. Proc Natl Acad Sci USA 69:1601–1605
69. Cross GAM (1987) Eukaryotic protein modification and membrane attachment via phosphatidylinositol. Cell 48:179–181
70. Low MG (1987) Biochemistry of the glycosyl-phosphatidylinositol membrane protein anchors. Biochem J 244:1–13
71. Collin P, Nefussi JR, Nicolas V, Boy-Lefevre ML, Forest N (1988) Membranous and soluble alkaline phosphatase in a mineralizing osteoblastic cell culture. Evidence for the release of the membranous form by phospholipase C and endoglycosidase F. Calcif Tissue Int (suppl) 42:107b
72. Noda M, Yoon K, Rodan GA, Koppel DE (1987) High lateral mobility of endogenous and transfected alkaline phosphatase: a phosphatidylinositol-anchored membrane protein. J Cell Biol 105:1671–1677
73. Fedde NK, Lane CC, Whyte MP (1988) Alkaline phosphatase is an ectoenzyme that acts on micromolar concentrations of natural substrates at physiologic pH in human osteosarcoma (SAOS-2) cells. Arch Biochem Biophys 264:400–409
74. Nakamura T, Nakamura K, Stinson RA (1988) Release of alkaline phosphatase from human osteosarcoma cells by phosphatidylinositol phospholipase C: effect of tunicamycin. Arch Biochem Biophys 265:190–196
75. Malik AS, Low MG (1986) Conversion of human placental alkaline phosphatase from a high Mr form to a low Mr form during butanol extraction. Biochem J 240:519–527
76. Seetharam S, Ovitt C, Strauss AW, Rubin D, Alpers DH (1987) Fat feeding stimulates only one of the two mRNAs encoding rat intestinal membranous and secreted alkaline phosphatase. Biochem Biophys Res Comm 145:363–368