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Characterization of Human Bone Cells in Culture

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Summary. Cultures of human bone cells were established, maintained, and characterized with respect to several metabolic parameters. These studies were undertaken with a view to using the bone culture system as a means of studying mechanisms of bone metabolism. The donor patients' ages ranged from 1 to 90 years and their disease states included congenital limb anomalies, exostosis, and osteo- and rheumatoid arthritis. Cultures were maintained up to 5 months. The osteoblast-like character of these cells was confirmed with the use of measurements applied to bone cells from other systems. Analyses showed that (a) the cells' appearance resembled that of cultured osteoblasts from other animal sources, b) intracellular cAMP was stimulated by human parathyroid hormone, c) osteocalcin was detected in the medium of all tested bone cell cultures and its production was found to be stimulated by 1,25-dihydroxycholecalciferol, and d) newly synthesized collagen was almost exclusively type I.

In contrast, cultures of human fibrolasts, established in one instance from tissue specimens of the same donor patient, grew faster, reached a higher limiting density, and produced a greater proportion of type III collagen than the corresponding bone cells. Furthermore, fibroblasts did not accumulate osteocalcin in their culture medium.

The conditions described in this report to maintain human bone cells in culture should provide a suitable test system to study the regulation of human bone metabolism.

Key words: Human bone — Cell culture — Osteoblast.

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The use of isolated fetal rat bone cells in culture was first described by Peck and coworkers in 1964 [1]. Since that time, cell culture studies concerning the regulation of bone cell growth and activity have been conducted with the use of cells derived from embryonic or young laboratory animals including rats [2, 3], mice [4], and chicks [5]. In addition, feline [6] or human [7] osteosarcoma cell lines have also been used by some investigators.

The first report of human bone cells in culture focused on the demonstration of nuclear inclusions characteristic of osteoclasts from patients with Paget's disease [8]. These cultures, initiated from tissues specimens of patients with this disease, also exhibited parameters typical of osteoclasts and osteoblasts, including acid and alkaline phosphatase activities and responsiveness to parathyroid hormone. The exact nature of the cells remained conjectural, however, since these cultures probably represented mixtures of cell types. More recently, human bone cells with osteoblast-like characteristics [9, 10] have been used to study the effects of local growth factors [9, 11] and fluoride action [12].

In this study, we describe the successful culture of cells derived from a multitude of specimens of human trabecular bone. Based on extensive biochemical evaluation, we present evidence that these cells have properties characteristic of osteoblasts.

Materials and Methods

Trabecular sections of bone specimens, obtained from patients undergoing necessary surgery or from biopsy or autopsy, were dissected, minced, and rinsed several times with Tyrode's solution. Bone cultures were initiated either by using pieces of trabecular bone as explants or with the use of cells liberated by sequential digestion with a mixture of collagenase and trypsin according to the method of Cohn and Wong [13]. The medium used for culture initiation and maintenance was Ham's F-12 (Gibco), pH 7.6, supplemented with glutamine (58.5 μg/ml),

$MgSO_4$ (200 μg/ml), antibiotics (100 U penicillin and 100 μg streptomycin sulfate per milliliter), and fetal bovine serum at a final concentration of 12%. Culture medium was replaced two times per week. If present, explant pieces were removed when confluency of the resulting monolayer was approached. Cell passage was carried out by incubating monolayers for 5–10 min in calcium- and magnesium-free Tyrode's solution containing 0.25% trypsin and replating the cells in the fresh medium at about one third of their confluent density. Over 80% of the cells reattached within 24 hours. Fibroblast cultures were initiated and maintained in a manner analogous to that used for bone specimens.

Measurement of cAMP Production

The cells' response to parathyroid hormone (PTH) and calcitonin was monitored by measuring intracellular levels of cAMP. PTH[hPTH-(1-34)](3000 U/mg) and human calcitonin (125 U/mg) were obtained from Sigma. Cultures near confluency were incubated in fresh medium containing 1 mM isobutylmethylxanthine alone or in combination with 0.2 to 200 ng/ml of human PTH or with 1 to 10 μg/ml of human calcitonin for 10 min at 37°C. The incubations were stopped by transferring the cultures to ice, washing rapidly with ice-cold Tyrode's solution, and then adding ice-cold ethanol containing 2 mM HCl. After 18 hours at −20°C, the cells were scraped off the plastic surfaces and homogenized in the ethanol for 5 sec with the use of a Polytron homogenizer. The samples were dried at 100°C and cAMP concentrations were measured by radioimmunoassay.

Measurement of Osteocalcin Production

Osteocalcin production was measured during a 45-hour period at 37°C. Monolayer cultures were washed and incubated in serum-free mediun containing 10 mg/ml bovine serum albumin in the absence or presence of 10 nM $1,25(OH)_2D_3$. At the end of the incubation period, the medium was removed and stored at −70°C until it was analyzed by radioimmunoassay [14].

Analysis of Newly Synthesized Collagen

Collagen synthesis was measured after incubating the cultures for 48 hours in Minimal Essential Medium (Gibco) containing fetal bovine serum (10%), sodium ascorbate (50 μg/ml), β-aminopropionitrile (50 μg/ml), and 3,4-^{3}H-proline (25 or 50 μCi/ml). Collagen from the culture medium and cell layer was isolated, treated with pepsin, and, after the addition of carrier type I collagen, analyzed by SDS-PAGE under reducing and nonreducing conditions [15, 16]. The bands of the carrier protein were identified by staining with Coomassie blue. The gels were then sliced into 25 equal segments, each of which was monitored for radioactive content. Collagen distribution was calculated according to the method described by Goldberg et al. [15].

Results

A total of 34 bone specimens from 15 females and 19 male donors, ranging in age from 1 to 90 years, were made available to us. From this total, 21 cultures were successfully established with the use of an explant system and six cultures were initiated successfully from cells isolated by enzymatic treatment of trabecular bone. Ten samples did not produce long-term viable bone cultures. No single property characterized these latter specimens since the donor patients ranged in age from 21 to 90 years, and their disease states included rheumatoid and osteoarthritis as well as non-union of fractures. They also had been on different therapeutic agents prior to surgery. It is of interest, however, that all cultures initiated from donors up to 18 years of age were successful on a long-term basis. The underlying pathology of this latter group of patients was largely congenital skeletal defects. However, the ability to establish bone cultures appeared to be independent of the method of initiation. The cell lines were maintained in culture for up to 5 months and through six passages.

The outgrowth in explant cultures, shown in Fig. 1, resulted in newly formed cell layers spreading over the trabeculae and, in large part, radiating onto the plastic surface of the culture vessel. The morphology of the cells in these explant cultures (Fig. 2) was similar to that of cultured cells that had been liberated from the bone tissue by enzymatic treatment (Fig. 3). The cells were usually mononucleated and multipolar, flattened with time in culture, and appeared most compact when a confluent monolayer was established (Fig. 2). Stratified (Fig. 3) and/or network-like structures (Fig. 4) were evident in cultures maintained for more than 2 months.

Intracellular cAMP accumulation was stimulated by PTH in a dose-dependent manner. Maximal stimulation was observed with 200 ng/ml PTH (50 nM) as shown in Fig. 5. The concentrations of cAMP were significantly higher in the presence of each of the concentrations of hPTH which was tested when compared with controls to which no exogenous PTH had been added. These data were obtained with the use of bone cells derived from initial explant cultures. Similar data have been obtained when bone cultures were initiated with cells isolated from bone specimens by proteolytic digestion.

Although all tested cell lines responded in a similar manner, the precise quantitative response was individually specific. Thus, when exposed to 50 nM PTH for 10 minutes, an increase in the intracellular cAMP of 2- to 73-fold was observed (Fig. 6). In those experiments in which tests were conducted in triplicate, the increased levels of cAMP were significantly higher than control values (patients 30 and 31, $P < 0.001$). Calcitonin (300 nM), on the

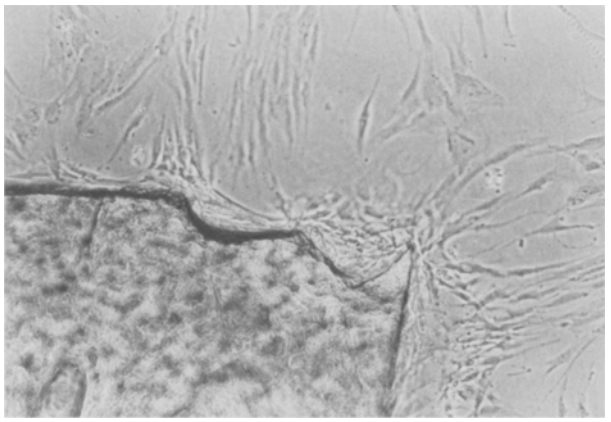

Fig. 1. Phase contrast photomicrographs of bone cells growing out from bone explant specimens derived from a 53-year-old female patient with rheumatoid arthritis. Culture had been established for 23 days. ×75.

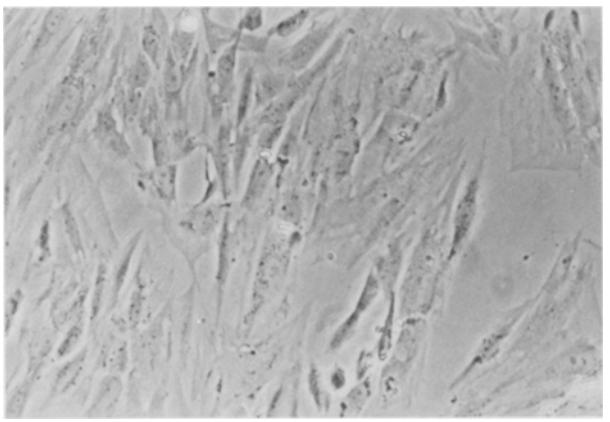

Fig. 2. Phase contrast photomicrograph of bone cells in second passage. Original explant was obtained from donor patient who was a 75-year-old male afflicted with sesamoiditis. Cells are mononucleated and multipolar. ×150.

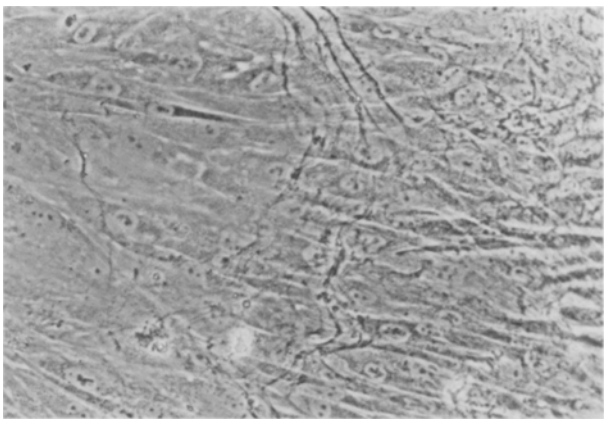

Fig. 3. Phase contrast photomicrograph of a primary culture established with proteolytically isolated bone cells. The culture was established 94 days earlier with cells derived from a bone specimen of a 1-year-old female who had a congenital limb abnormality. Stratified cell layers visible over monolayer base. ×150.

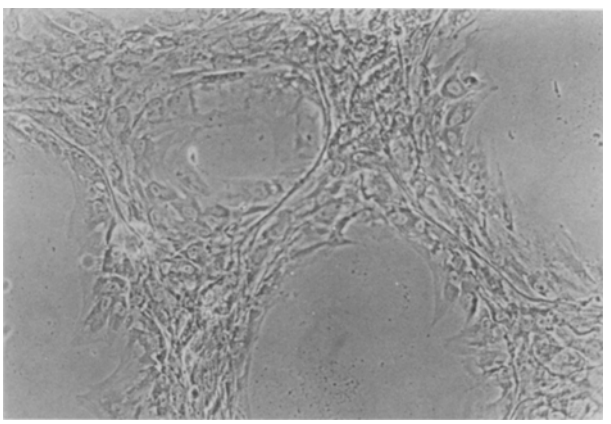

Fig. 4. Same culture as in Fig. 3, showing network formation. Phase contrast, ×75.

other hand, had no effect on cAMP levels in any of the tested cell lines (Fig. 6). A 10-fold higher calcitonin concentration (3 μM) was equally ineffective in altering cAMP levels (data not shown).

Osteocalcin levels in the media of cells cultured in the absence and presence of 10 nm $1,25(OH)_2D_3$ are shown in Fig. 7. Addition of the hormone resulted in a 2- to 26-fold stimulation of osteocalcin production. When analyzed by paired *t* test, a statistically significant increase in osteocalcin production was found in those cultures to which $1,25(OH)_2D_3$ had been added ($P < 0.05$). In a separate experiment, addition of 10 nm $1,25(OH)_2D_3$ to six different cultures produced osteocalcin ranging from 1.0 to 8.6 ng/10^6 cells/45 hours.

Collagen synthesis was monitored in both the medium and cell layer fractions. The bone cultures were incubated with ^{3}H-proline for 48 hours. The types of collagen synthesized were examined by electrophoresis on acrylamide gels. The results shown in Fig. 8 indicate that the major radioactive bands co-migrated with α_1 and α_2 components of standard collagen type I, which had been included as a carrier protein. The small radioactive peak co-migrating with γ-chains of the standard collagen type I was shifted to the α_1 position under reducing conditions. This indicated that it represented α_1 (III)-trimers. Treatment of an unreduced sample with purified bacterial collagenase prior to electrophoresis caused the disappearance of the three major radioactive bands just described. This sub-

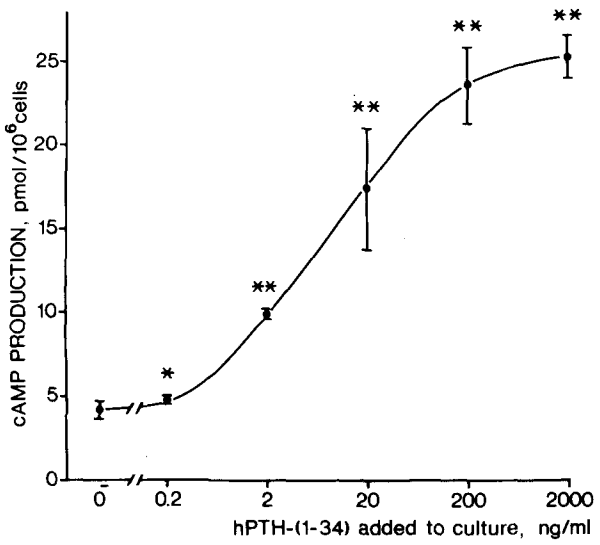

Fig. 5. Stimulation of intracellular cAMP in human bone cells by hPTH-(1-34). Bone cells, in second passage, were obtained from an explant of a bone specimen from a 57-year-old male and were incubated with increasing amounts of PTH as described in Methods. The results represent the mean and SD of triplicate cultures (*$P < 0.05$; **$P < 0.001$ when compared with control).

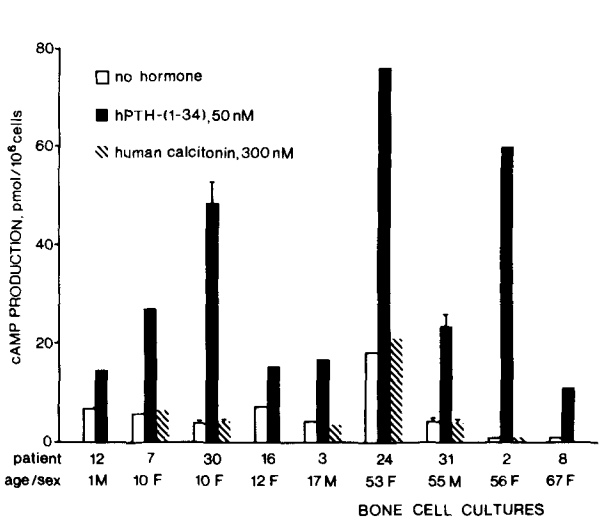

Fig. 6. Effect of hPTH-(1-34) and human calcitonin on human bone cell cultures derived from trabecular explant cultures. Cells were in primary culture except for patients 24 and 31 (secondary cultures) and 30 (tertiary culture). The histogram shows the results with the use of a single culture or with triplicate cultures. In the latter instance, the vertical bar represents SD (see Methods for details).

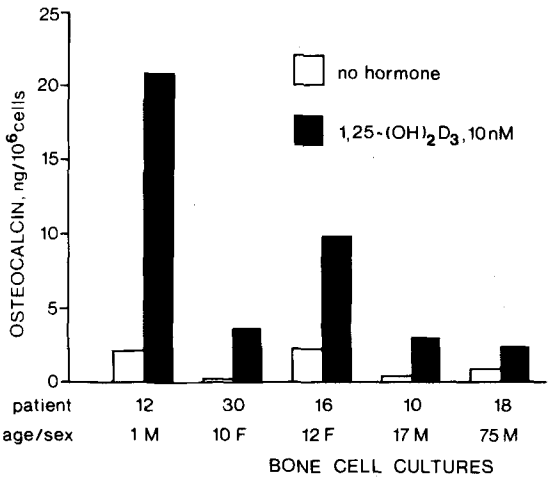

Fig. 7. Effect of $1,25(OH)_2D_3$ on osteocalcin production in human bone cell cultures derived from trabecular explant cultures. Primary (patients 12, 16, and 10) and secondary (patients 18 and 30) cultures were incubated in the absence and presence of 10 nM $1,25(OH)_2D_3$ as described in Methods. The data were analyzed by paired t test. Mean values are significantly different (see text).

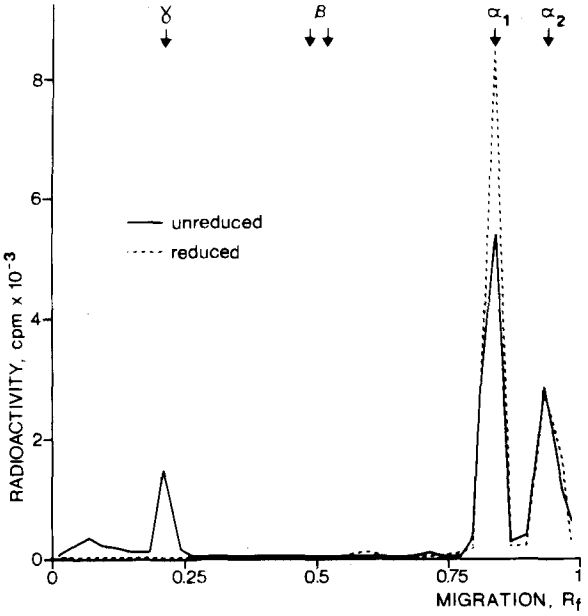

Fig. 8. Collagen synthesis by human bone cell culture derived from an explant culture. The patient donor was a 2-year-old male. Cells were incubated for 48 h in the presence of ^{3}H-proline and analyzed on SDS-PAGE as described in Methods. The positions of component chains of collagen type I from calf skin are indicated by arrows. This representative pattern is from a medium fraction.

stantiated that these components were indeed collagen molecules. Analysis for distribution of radioactive contents in acrylamide gels after electrophoresis of newly synthesized collagen by four bone cell lines showed that 92.3 ± 2.9% of total collagen in the medium and 95.8 ± 3.4% of total collagen in the cell layer was type I collagen (Table 1). The remainder was type III.

Table 1. Collagen synthesis by explant-derived human bone cells in culture

Patient number	Age & sex	Collagen synthesis type I/total (percent)[a]	
		Medium	Cell layer
25	2 M	90.1	97.9
29	25 M	89.4	96.3
24	58 F	95.3	98.2
28	57 M	94.2	90.7
		92.3 ± 2.9[b]	95.8 ± 3.4[b]

[a] Quantification of collagen types was determined by scintillation spectrometry of acrylamide gels after bone cell products synthesized in the presence of ^{3}H-proline were subjected to SDS-PAGE (see Methods for details)
[b] Mean ± SD

Table 2. Metabolic properties of human bone cells in culture

Conditions[a] for culture initiation	Addition to culture medium				
	None	PTH (50 nM)	Calcitonin (300 nM)	None	$1,25(OH)_2D_3$ (10 nM)
	cAMP[b] (pmol/10^6 cells)			Osteocalcin[c] (ng/10^6 cells)	
Explant	6.7	14.5	6.6	2.1	20.8
Population I	2.9	18.8	3.6	3.9	10.2
Population II	3.4	20.9	2.9	3.4	6.2
Population III	4.1	28.7	4.8	4.4	9.0
Population IV	3.8	10.1	4.1	4.4	7.6

[a] Explant culture as well as monolayer cultures of population I–IV were initiated from a bone specimen of a 1-year-old male afflicted with Down's syndrome. Cells comprising populations I–IV were obtained after sequential enzymatic digestion of the bone specimens for 20, 30, 60, and an additional 60 minutes, respectively
[b] cAMP determined after 10 minutes of incubation in the absence or presence of hormones (see Methods for details)
[c] Osteocalcin levels were determined in the medium after incubation in the presence or absence of $1,25(OH)_2D_3$ for 45 hours (see Methods for details)

The response to PTH, calcitonin, and $1,25(OH)_2D_3$ by bone cell cultures initiated either as explants or with enzymatically isolated cell populations from the same patient donor are compared in Table 2. No significant differences were noted in their responses to PTH, calcitonin, or $1,25(OH)_2D_3$, as reflected in intracellular cAMP levels or in osteocalcin production. It was noted, however, that the explant culture gave the strongest osteocalcin response to $1,25(OH)_2D_3$, while the strongest cAMP response to PTH was found in cell population III.

On the other hand, significant differences were observed when bone cells and fibroblasts derived from the same patient were compared. As shown in Table 3, in contrast to bone cells, fibroblast cultures divided more frequently and ceased active growth at a higher cell density. Furthermore, in response to PTH, intracellular cAMP was increased 13-fold in bone cells and only 2.3-fold in fibroblast cultures. This difference was statistically significant ($P < 0.001$). Neither cell type exhibited an increase in cAMP when calcitonin (1 μ/ml) was added to the incubation medium (data not shown).

As also shown in Table 3, osteocalcin production was limited to bone cell cultures. No osteocalcin was detected in the medium of any fibroblast cultures, regardless of the absence or presence of $1,25(OH)_2D_3$. These findings were reproducible when periodic measurements were made over a 45-hour incubation time. No osteocalcin was detected in the medium at any point during the course of the experimental period. The synthesis of collagen by human fibroblasts was also different from that observed for human bone cells. Of the total collagen synthesized by fibroblasts, about 27% were type III and 50% type I. The remainder consisted of two bands migrating in the β-positions and one band migrating slightly slower than α_1-chains. These latter two bands were probably partially processed procollagen type I chains.

Discussion

Bone tissue undergoes remodeling throughout postnatal life. The function is carried out, in large part, by cells resulting from the proliferation and differentiation of precursor cells residing in the soft connective tissue of periosteal and endosteal surfaces and possibly bone marrow [17]. *In vitro,* however, human osteoblasts which normally do not divide *in vivo,* express mitotic activity when placed in suitable culture medium. This re-establishment of mitotic activity when placed into suitable culture conditions is not unique to bone cells. Chondrocytes from human adult cartilage, which also do not divide *in vivo,* will express mitotic activity when placed in an *in vitro* culture environment [18]. In the latter instance, the lag time for initial cell outgrowth from explant pieces was found to increase with increasing age of the patient donor from whom the tissue specimens were obtained (E. R. Schwartz, unpublished observation). Similarly, outgrowth from bone explants of young patients was more rapid than from older donors.

Cells grown in the conditions described herein resembled cultured osteoblasts from animals [13] as well as cultured human bone cells [8]. It has been

Table 3. Properties of fibroblasts and bone cells from the same patient donor[a]

Cell type	Doubling time[b] (hours)	Limiting density[b] (cells/cm^2)	cAMP[c] (pmol/10^6 cells) hPTH (200 ng/ml) −	+	Osteocalcin[d] (ng/10^6 cells) [1,25(OH)$_2$D$_3$ (10^{-8}M)] −	+
Bone cells	120	1.2×10^4	3.7 ± 0.3	48.3 ± 4.4	0.14	3.60
Fibroblasts	48	3.0×10^4	19.7 ± 0.6	44.7 ± 7.6	0	0
			$P < 0.001$[e]			

[a] Patient donor was a 10-year-old female with hip dysplasia. Explant cultures were initiated as described in Methods
[b] Determined for cells in second passage
[c] Intracellular levels determined in triplicate cultures after 10 minute incubation in absence or presence of hPTH
[d] Media levels determined after 45 hour incubation in absence or presence of 1,25(OH)$_2$D$_3$
[e] Determined by paired *t* test

noted that the morphological distinction between different cell types of mesenchymal origin, e.g., osteoblasts, chondrocytes, and fibroblasts, seems to be generally poor when they are cultured in monolayer [19]. Spindle-shaped cells seem to prevail in fibroblast cultures, however [19]. The retraction of cells from several foci to form condensed bands of cells as shown in Fig. 4 was also observed in long-term cultures of human chondrocytes (E. R. Schwartz, unpublished observations). The observed slower growth rate for bone cells, when compared with fibroblasts from the same patient donor, were similar to the findings for human bone cell and fibroblast cultures reported by Beresford et al. [10]. In addition, the observed contact inhibition at a lower density by bone cells when compared with fibroblasts was analogous to the findings with embryonic rat cells [20].

The enhancement of cAMP production by PTH, which was observed in all experimental bone cell cultures, is an acknowledged property of osteoblasts [13]. This response does not appear to be unique to bone cells, however. Goldring and coworkers [21] reported that two out of four experimental human skin fibroblast cultures and one culture of human foreskin fibroblast also responded to PTH. In the studies reported herein, the experimental fibroblast cultures expressed a positive but lesser response to PTH. Whereas the stimulation of cAMP production by PTH may be considered an essential feature of the osteoblast phenotype, the response by fibroblasts is more variable.

Calcitonin is known to inhibit osteoclast activity both *in vivo* [22] and *in vitro* [23]. There is evidence that this inhibition is mediated through an increase in intracellular cAMP [13]. The lack of cAMP response to calcitonin in all of the human bone cell cultures tested indicated the absence of osteoclasts in these cultures. Furthermore, no multinucleated cells were observed in any of the bone cultures including those which were established from cells liberated early during proteolytic treatment of trabecular bone. The isolation of osteoclast-like cells by differential digestion may be specific for use with mice calvaria [13], since application of this method to rat calvaria did not yield isolated osteoclasts [24]. Application of this technique to human bone in these studies also did not yield isolated osteoclast-like cells.

Osteocalcin accounts for about 20% of total noncollagenous proteins in bone. It is produced in culture by the osteoblast-like rat osteosarcoma cell clone ROS 17/2 [25]. Furthermore, only cells that actively formed bone matrix during postnatal development in the rat or chick system synthesized osteocalcin [26]. These data, coupled to the observed correlation between serum osteocalcin levels and bone formation in a human patient population [27], suggested that this GLA-containing protein has a specific role in bone formation and is synthesized by bone forming cells. The absence of any osteocalcin synthesis by two different fibroblast cultures in these studies and its presence in all experimental bone cell cultures, provides additional rationale for the use of this parameter as a measure of osteoblast phenotypic expression. Stimulation of osteocalcin synthesis by 1,25(OH)$_2$D$_3$ in the cultured human bone cells used in the experiments described in this report was analogous to that described for ROS 17/2 cells in culture [28] and in a human patient population [29].

The synthesis of about 94% type I collagen reported here was consistent with findings by Ecarot-Charrier and Glorieux [30] and Whitson et al. [31] for cultured cells from feline and bovine calvaria, respectively. Reduction of the calcium ion concentration in the culture medium to 0.2 mM will apparently increase collagen type I synthesis to nearly

100% (S. W. Whitson, personal communication). Analysis of isolated bone samples has shown that bone contains type I collagen exclusively [32]. Furthermore, only type I collagen has been shown to be synthesized in bone organ cultures [33, 34], and in mouse calvarial osteoblasts cultured for 4 days [35]. In older cell cultures, a small amount of type III collagen was formed, which was interpreted by these authors as an alteration of the phenotype of these cells [35]. On the other hand, cultured skin fibroblasts produce, on the average, 27% type III collagen [36–38]. The observed synthesis of some type III collagen (about 6%) may result either from the presence of bone derived fibroblasts [39] or may reflect the modulation of osteoblasts towards preosteoblastic phenotypic expression in the particular culture environment [40].

In this report, it was demonstrated that cells cultured from many different human trabecular bone specimens consistently displayed osteoblastic properties. The use of these conditions, therefore, provides an experimental system which can be used to study osteoblast functions in normal and diseased states.

Note added in proof: Subsequent to the submission of this manuscript, a publication [Beresford JN, Gallagher JA, Poser JW, Russell 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] appeared which also discusses the hormonal and pharmacologic regulation of osteocalcin production by human bone cells in culture.

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