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CHARACTERIZATION OF HUMAN OSTEOBLASTIC CELLS: INFLUENCE OF THE CULTURE CONDITIONS

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SUMMARY

Human osteoblastic cells were isolated enzymatically from adult human spongy bone and grown in MEM-Ham F12 1:1 medium supplemented with 2% Ultroser (USM). They were subcultured and examined for osteoblast features by morphological, histological, and biochemical approaches. The cells had a characteristic polyhedral morphology and produced a high level of alkaline phosphatase (ALKP). Confluent cultures were uniformly stained for ALKP and flow cytometry analysis with fluorescein diphosphate gave a single peak signal, reflecting a highly positive population, distinct from cultures of fibroblasts. The ALKP activity was stimulated by 1,25 $(OH)_2$ vitamin D_3. CD 44 was strongly expressed in these cultures, although osteoblasts are negative *in vivo* and osteocytes are positive. The main collagen synthesized was type I collagen and osteocalcin was produced after stimulation by vitamin D_3. 10 m*M* βGP induced mineralization and microprobe analysis of the crystals showed a composition close to hydroxyapatite.

Changing the culture conditions to MEM-10% calf serum acted on cell behavior: it reduced the production of these biochemical markers of osteoblasts and the morphology became fibroblastlike with more rapid cell multiplication. The parameter most affected by the change in culture medium was ALKP, which was selected as the determinant criterion for defining an osteoblast culture. ALKP activity was then used to characterize a culture of cells seeded in a collagen gel.

Key words: human osteoblastic cells; collagen lattice; mineral deposition; collagen biosynthesis; alkaline phosphatase; osteocalcin.

INTRODUCTION

Research on bone cells requires suitable *in vitro* model systems. Many culture systems have been developed using osteoblastlike cells of animal or human origin, derived from normal bone or osteosarcomas (2,5,13,24,26,27,29,31). But the use of animal cell lines raises the problem of extrapolation to humans. There are several advantages in using human cells including the capacity to take into account the age and biological status of the patient from which they were derived. The susceptibility of the cells to drugs may also be in the same range as *in vivo*. Human osteosarcoma cells are often used for investigating osteoblast function (28), but these cells have abnormal characteristics, particularly their responses to osteotropic agents. Osteosarcoma cell lines must, therefore, be defined in terms of their functions, such as growth, alkaline phosphatase activity, or ability to synthesize extracellular matrix (9,11,14,16).

These limitations suggest that well-differentiated cells taken from normal adult bone and placed in culture are most suitable for many studies. But this makes it impossible to use the same cell line for several experiments. Therefore, it is necessary to define the main useful osteoblast characteristics of each culture. There are many parameters that can be considered and this study was done to select suitable methods for defining cultures.

The first part of this report is devoted to describing osteoblastic cells isolated from human adult spongy bone using a set of typical markers of mature osteoblasts. The second part analyzes the way the expression of these markers changes with the culture conditions: alkaline phosphatase (ALKP) activity was selected as the most sensitive parameter. Then we described a 3-D model in which cells were seeded into a collagen gel. The osteoblasts were tested for ALKP activity to characterize cell behavior.

MATERIALS AND METHODS

Cell Culture

Osteoblastlike cells were prepared from biopsies of adult human spongy bone removed at surgery. The bone was cut into small pieces and incubated for 30 min in 1 mg/ml collagenase (*Sigma type V*) and 0.25% trypsin in phosphate-buffered saline (PBS). This treatment was repeated and the cells were collected by centrifugation and grown in Earle's minimal essential medium (MEM) Ham F12 (1:1) supplemented with 1 m*M* pyruvate, 2 m*M* glutamine, 5 μ*M* nonessential amino acids, 50 μg/ml ascorbic acid, 50 μg/ml streptomycin, 50 μg/ml penicillin, and 2% Ultroser (USM) (GIBCO BRL). This medium is subsequently referred to as USM. Cells were subcultured by trypsinization and used between the first and sixth passages. In some experiments, cells were subcultured in MEM, 10% calf serum (Sigma), plus the same components as USM, except for Ultroser. This medium is called CSM.

Preparation of Collagen Lattices

The calf skin acid-soluble collagen used in these preparations was purified as previously described (25). Lattices were prepared according to Bell (4). 2×10^5 osteoblastic cells were suspended in a final volume of 5 ml composed of concentrated medium (MEM or MEM-Ham F12 1:1) 10% calf serum or

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2% Ultroser, 1.5 ml (2.4 mg/ml) collagen in 0.1% acetic acid, neutralized with NaOH, immediately poured into 60-mm petri dishes, and left for 2 h to gel. The lattices were then detached from the sides of the dish. The culture medium was changed twice a week.

Cell Proliferation and DNA Assays

Cells were seeded in 24-well culture plates at 10^4 cells/well and proliferation was quantified using the supravital dye Janus green (26). DNA was assayed with bisbenzimide (18) as modified by Bonis (7).

Collagen Analysis

Confluent cells (9 d) were incubated for 24 h in fresh medium containing 50 μg/ml ascorbate and 5 μCi ^{14}C proline (Amersham, 280 mCi/mmol). The medium was removed and the cells were rinsed with PBS. Medium and rinse were pooled and dialyzed exhaustively against acetic acid (sequential baths from 0.1–0.5 mol/l). Collagen synthesis was measured as the ^{14}C hydroxyproline (Hyp) in the dialyzed medium. Samples were hydrolyzed with 6 mol/l HCl for 6 h at 110° C. The amino acids were separated by high pressure liquid chromatography (HPLC) (12) and radioactivity was measured using a Flo-one radioactivity detector (Packard Instruments).

The collagen types were determined by digesting the dialyzed medium with pepsin (8). Briefly, fractions were incubated for 16 h with 1 mg/ml pepsin in 0.5 mol/l acetic acid at room temperature. Protein was precipitated at +4°C with 4.2 mol/l NaCl (final concentration) at 4° C. The pellet was dissolved in 0.5 mol/l acetic acid and reprecipitated by dialysis against six changes of 20 mmol/l Na_2HPO_4. The pellet was dissolved in a small volume of 0.5 mol/l acetic acid and analyzed by SDS-PAGE (19) with or without dithiothreitol. The resulting gels were immersed for 15 min in an enlightning solution (New England Nuclear) and dried. Radioactive bands were revealed by contact with Kodak X Omat AR film and quantified by densitometry.

CD 44 Expression

A monoclonal antibody to human CD 44 (MEM 85, conjugated to FITC) was provided by Caltag Laboratories (San Francisco). 200 000 cells were incubated with anti-CD 44 for 30 min at +4° C, rinsed with PBS, and analyzed by flow cytometry as described for fluorescein emission. Negative controls were obtained by replacing the CD 44 antibody by IgG-FITC.

Alkaline Phosphatase

Histochemical identification of alkaline phosphatase. The azo-dye coupling method of Bancroft and Stevens (3) was used. Briefly, cell cultures were washed three times in cold PBS, fixed in 10% formol in PBS for 10 min, washed three times in PBS, and incubated for 30 min in a freshly prepared solution of 100 mg/ml naphthol ASM (Sigma) dissolved in dimethylformamide and 0.1% Fast Blue RR salt (Sigma) in 50 mmol/l Tris-HCl buffer pH 9.0. Cells were examined under the microscope without counterstaining.

Assay of cellular alkaline phosphatase (ALKP) activity. The alkaline phosphatase activity of confluent monolayer cultures was measured in 24-well culture plates. The medium was removed, and cells were washed three times with 50 m*M* Tris-HCl, 0.15 *M* NaCl, pH 7.5 and shaken for 3 min in 1 ml 10 m*M* p- nitrophenylphosphate (PNP). The PNP solution was removed and the reaction stopped by adding 1 ml 1 *N* NaOH. The optical density (OD) was measured at 405 nm, and results are expressed as OD/min/well.

The activity of lattice cultures was measured in lattices rinsed three times with PBS, digested with 2 mg/ml collagenase Sigma type V at 37° C for up to 30 min (digestion time depended on the age of the lattice). The cells were collected by centrifugation, taken up in 0.5 ml Tris-NaCl, and sonicated on bath at 4° C for 15 s (Sonic and Material Inc., Banbury, Michigan). The cell lysates were centrifugated for 10 min at 2500 rpm and 20 μl of the supernatant was incubated with 1 ml 10 mmol/l 4- nitrophenylphosphate as substrate, 0.5 mmol/l MgCl2, 225 mmol/l NaCl, 1 mol/l diethanolamine, 50 mmol/l Tris-HCl buffer, pH 9.8. The rate of p-nitrophenol release was measured at 37°C at 405 nm. Results are expressed as units per mg supernatant protein. Protein concentrations were determined by the Lowry method (21).

-ALKP by flow cytometry. Confluent human osteoblastic cells or human dermal fibroblasts were harvested with 0.25% wt/vol trypsin at 37° C. The cell pellet was rinsed twice with 50 mmol/l Tris-HCl, 0.15 *M* NaCl pH 7.5 (TN) and 3×10^5 cells were incubated in 0.5 ml TN containing 6.25 μg fluorescein diphosphate (FDP, F 2999 Molecular Probes) for 10 min at 37°C. The reaction was blocked by cooling in an ice bath. The cells were collected by centrifugation, washed twice with TN, and suspended in 0.3 ml TN for analysis. Single cell analysis was performed on a Facstar Plus cell sorter (Becton Dickinson) equipped with an argon laser emitting 250 mW excitation at 488 nm; 5000 cells were analyzed in each sample. Specific green fluorescence due to fluorescein emission was analyzed through a 530–540 nm band pass filter.

Osteocalcin Determination

Cells were grown to confluence in T25 flasks and then cultured for 48 h with 10^{-9} mol/l vitamin D and 10^{-8} mol/l vitamin K. Medium was concentrated by ultrafiltration and osteocalcin was determined by radioimmunoassay (CIS Bio International). USM culture medium was used as control.

Calcium and Phosphorus Determination

Cultures were grown to confluence (15 d) and then treated with 10 m*M* β-glycerophosphate (βGP). Calcium and phosphorus were measured 4 d later: the cell layer was washed three times with 0.15 *M* NaCl and extracted with 5% perchloric acid. Inorganic phosphorus was assayed by the molybdate vanadate reaction (Boehringer Mannheim kit no. 124 974) (32) and calcium was measured by flame ionization spectrometry (Eppendorf) on the supernatant.

Mineral Microanalysis

Coverslips were placed in the wells of a 24-well culture dish; 100 000 cells in 1 ml medium containing 10 m*M* βGP were placed in each well and cultured for 3 wk. For scanning electron microscopy (SEM), cultures were washed three times with PBS, fixed with 1% glutaraldehyde for 30 min, washed three times with PBS, dehydrated through series of acetone baths, lyophilized, sputter coated with gold, and examined in a JEOL JSM 840 scanning microscope. They were also sputter coated with carbon for electron probe microanalysis (at 15 kV).

Results

Characteristics of the Cells From Human Spongy Bone

Morphology. Human osteoblastic cells cultivated in MEM- Ham F12, 2% Ultroser (USM) formed a monolayer of polyhedral cells having an osteoblast phenotype. This appearance was unchanged through six subcultures.

Expression of alkaline phosphatase phenotypic marker. Cultures carried out for six passages in USM produced large amounts of alkaline phosphatase activity: confluent monolayer cultures were uniformly and intensively stained for alkaline phosphatase.

Flow cytometry assays were performed to assess the homogeneity of ALKP activity in the osteoblastic cell cultures. The fluorogenic substrate fluorescein diphosphate (FDP) was used. FDP is nonfluorescent until it is hydrolyzed; the fluorescein produced is readily detected by flow cytometry. Signals due to cell debris were eliminated by analyzing bivariate data plots after acquisition on the basis of forward versus right angle light scatter. Only particles with high forward scatter were assessed. Cells incubated without FDP substrate were used as controls. The fluorescence of osteoblastic cells gave a single peak signal on histogram representation (Fig. 2) reflecting a homogeneous population. The signal obtained with osteoblastic cells was highly fluorescent, and thus easily distinguished from the signal obtained with fibroblasts.

The responsiveness of ALKP to 1,25 $(OH)_2$ D_3 (a gift from laboratoire Hoffmann La Roche, Basel, Switzerland) was also checked. Cultures incubated with 5.10^{-9} *M* 1,25 $(OH)_2$ D_3 for 36 h showed a 1.5-fold increase in ALKP activity.

The distribution of CD 44 is shown in Table 1. 90% of the cells incubated with the highest titer of antibody were fluorescent as detected by flow cytometry. Cultivated fibroblasts were labeled to the same extent by this antibody.

The types of collagen secreted by confluent osteoblasts in 24 h was analyzed by SDS-PAGE (Fig. 3). Cells grown in USM synthesized mainly type I collagen (less than 5% of type III collagen was detected in these cultures).

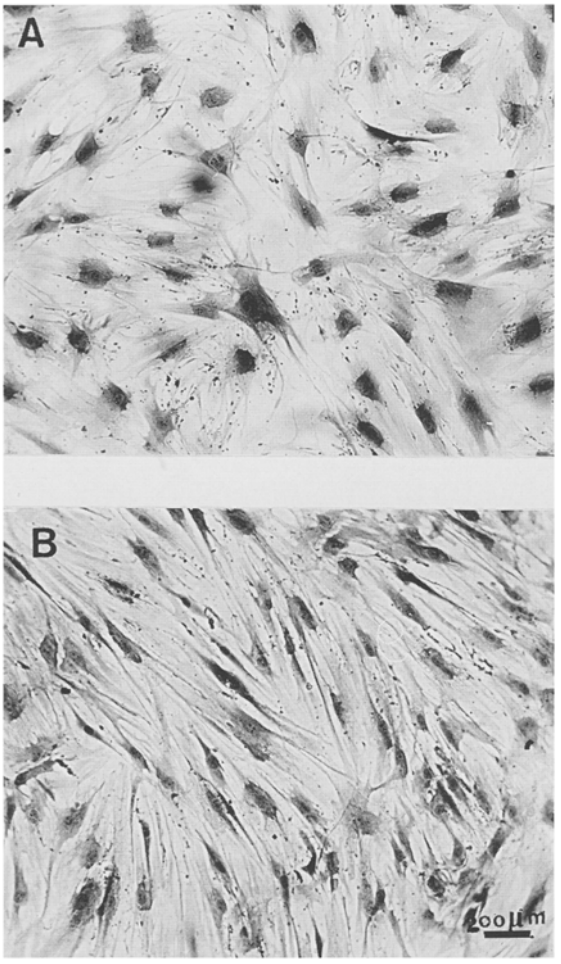

FIG. 1. Morphology of human osteoblast cells: phase contrast micrographs of cells (3 PDL) grown in MEM/Ham F12 medium 1:1 supplemented with 2% Ultroser (*A*), or in MEM supplemented with 10% calf serum (*B*) for 8 d.

whereas human dermal fibroblasts produced 15% type III collagen under the same experimental conditions (Fig. 3).

Osteocalcin in the culture medium was measured after incubating cells for 48 h with vitamin D and vitamin K. Primary cultures produced 120 ng osteocalcin/10^6 cells/48 h, while cells passaged three times gave 33 ng OC/10^6 cells/48 h.

Examination by SEM of cultures grown for 3 wk in the presence of βGP showed crystals, which were analyzed with a micro probe (Fig. 4). The Ca/P atomic ratio was 1.30 ± 0.155 (mean of seven measurements). The theoretical ratio for pure hydroxyapatite is 1.67.

Influence of the Culture Conditions

Cells were grown in calf serum (CSM) to investigate the effect of changing medium on cell behavior. Cells grown in CSM became spindle shaped much like fibroblast cells (Fig. 1 *B*). Cell growth was monitored for 14 d (Fig. 5). There was a 2.2-fold increase in the number of cells when cells were grown with CSM. Collagen synthesis was analyzed in terms of quantity and quality. Osteoblastic cells grown in medium supplemented with 10% serum (CSM) produced 2.6-fold less collagen than cells grown in medium supplemented with Ultroser (USM) (expressed as ^{14}C dpm hydroxyproline), whereas total protein synthesis (expressed as total dpm ^{14}C in the dialyzed medium)

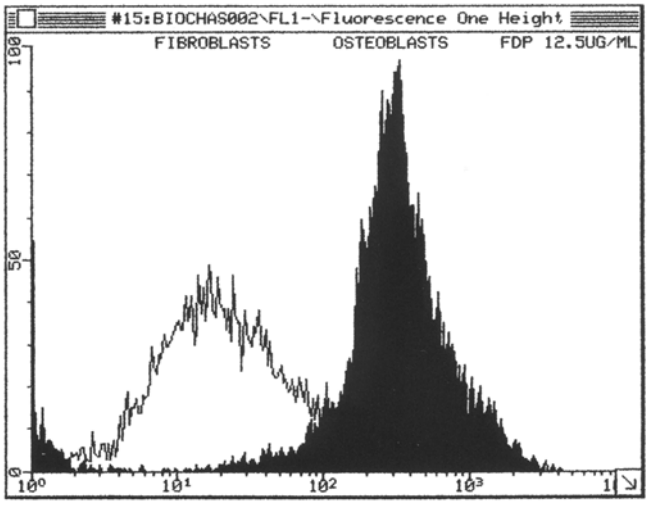

FIG. 2. Flow cytometry of human osteoblastic cells and human fibroblasts stained with fluorescein diphosphate for alkaline phosphatase. Cells were incubated with fluorescein diphosphate (FDP) at 37° C for 10 min. Fluorescence is displayed on a logarithmic scale.

TABLE 1

CD 44 EXPRESSION IN OSTEOBLASTS GROWN IN MONOLAYERS[a]

µg anti-CD 44	% fluorescent cells
0	5
2.5	59
5	68
10	91

[a] Cytoflow analysis was performed after 10 d in culture, when monolayers were subconfluent. 2×10^5 cells were incubated with anti-CD 44 conjugated with FITC for 30 min at +4° C.

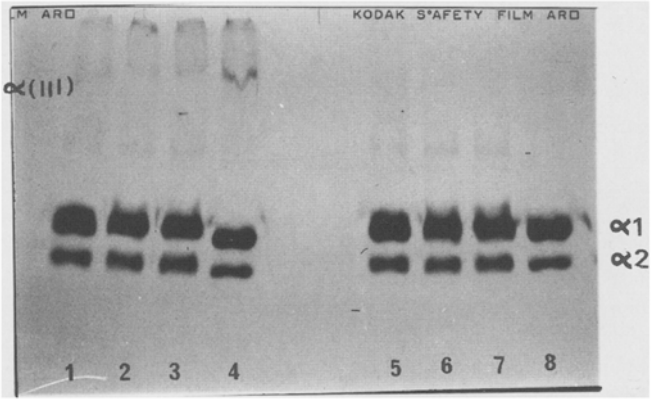

FIG. 3. Type I and type III procollagen production by human osteoblasts: cells were grown to confluence and labeled for 24 h with ^{14}C proline. Collagen secreted into the culture medium was purified and analyzed by SDS-PAGE and autoradiography. Type I and type III procollagens were assayed by densitometric scanning of the fluorogram: *Lanes 1* to *4* without DTT, *lanes 5* to *8* with DTT, *lanes 1–5*: osteoblasts in CSM; *lanes 2–6*: osteoblasts in USM; *lanes 3–7*: osteoblasts in Ham F12:MEM 1:1 without Ultroser; *lanes 4–8*: fibroblasts in CSM.

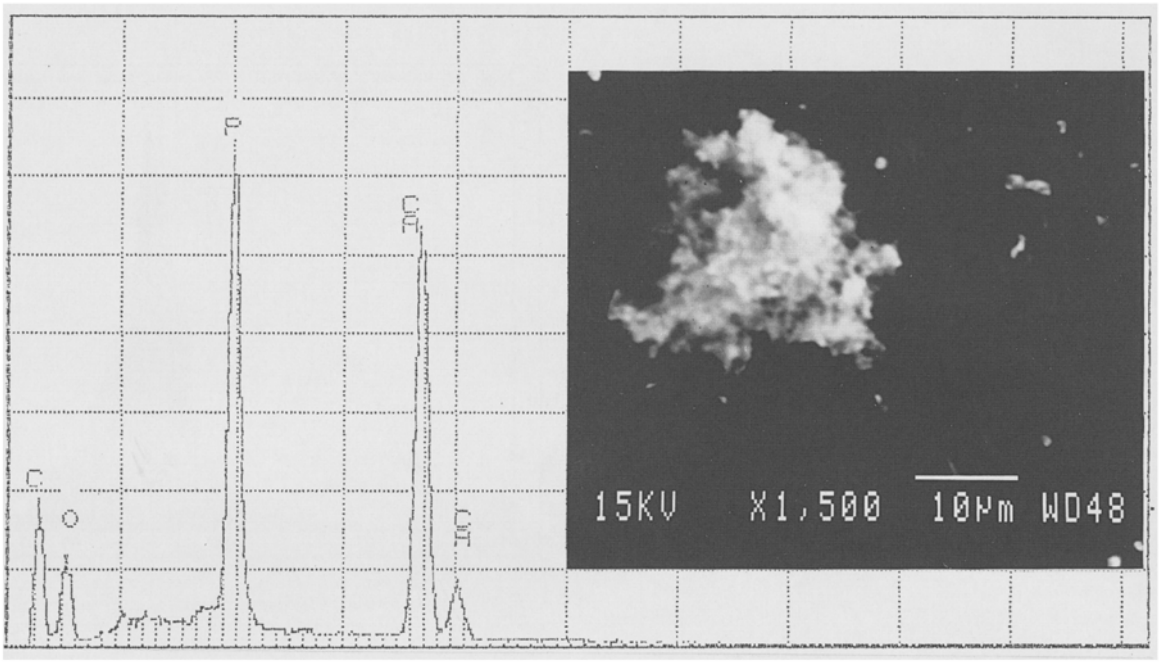

FIG. 4. Scanning electron microscopy and X-ray microprobe analysis of osteoblast cultures grown for 3 wk in USM plus 10 m*M* βGP

was the same in the two systems (Table 2). The proportion of type III collagen was less than 5% for CSM, the same as in USM.

Cells grown in CSM mineralized the extracellular matrix in the presence of 10 mmol βGP as did cells grown in USM. Accumulation of calcium and phosphorus was measured on supraconfluent cultures (Table 3). The Ca/P ratios were the same (1.5) in CSM and in USM, but the quantity of calcium and phosphorus per mg cell protein was 2.2 lower in cells grown in CSM. The composition of the culture medium greatly influenced alkaline phosphatase activity. ALKP was much lower in CSM ($0.012 \pm 2.3 \times 10^{-3}$ OD/min/well) than in USM ($0.270 \pm 6.6 \times 10^{-3}$ OD/min/well). The same occurred when osteoblasts were cultured in a 3-D collagen gel. ALKP activity was constant in cultures grown, while cultures grown in CSM rapidly produced less ALKP (Fig. 6).

DISCUSSION

Cells isolated from adult human spongy bone and cultured in MEM-Ham F12 1:1 containing 2% Ultroser (USM) were examined in regard to several cellular characteristics and phenotypic markers of osteoblasts between 1 and 6 passages. Cells were defined as osteoblastic by their morphology; they formed a layer of polygonal cells, produced mainly type I collagen and a bone- specific noncollagenous protein, osteocalcin, in response to 1,25 $(OH)_2$ D_3. CD 44 is a glycoprotein found on many cell types (10) and has several physiological functions, including cell-cell and cell-matrix adhesion. The expression of CD 44 had been investigated in human bone (15) but, to our knowledge, there have been no *in vitro* studies. Immunohistochemical studies on human bone have shown that osteocytes strongly expressed CD 44, whereas osteoblasts and lining cells do not (15). But our studies indicate that osteoblasts grown in monolayer expressed

TABLE 2

SYNTHESIS OF PROTEINS AND COLLAGEN BY HUMAN OSTEOBLAST CELLS CULTURED IN MEM SUPPLEMENTED WITH 10% CALF SERUM (CSM) OR IN MEM-Ham F12 1:1 SUPPLEMENTED WITH 2% ULTROSER (USM)[a]

	Protein dpm/µg DNA	Collagen dpm ^{14}C Hyp/µg DNA
Culture Medium		
CSM	162 697 ± 562	18 233 ± 62
USM	168 245 ± 2729	48 659 ± 2030

[a] Cells were labeled at confluency (Day 9) for 24 h with ^{14}C proline in the presence of 50 µg/ml ascorbate. Protein and collagen in the culture medium were measured.

TABLE 3

MINERAL DEPOSITION BY OSTEOBLASTS GROWN AS MONOLAYERS IN MEDIUM SUPPLEMENTED WITH ULTROSER (USM), OR CALF SERUM (CSM)[a]

	Calcium µmol/mg protein	Phosphorus µmol/mg protein	Ca/P
USM	3.52 ± 0.33	2.42 ± 0.115	1.45
CSM	1.61 ± 0.52	1.07 ± 0.307	1.50

[a] 10 m*M* βGP was added on Day 15 and assays were performed 4 d later. Proteins were measured in the pellet after perchloric acid precipitation.

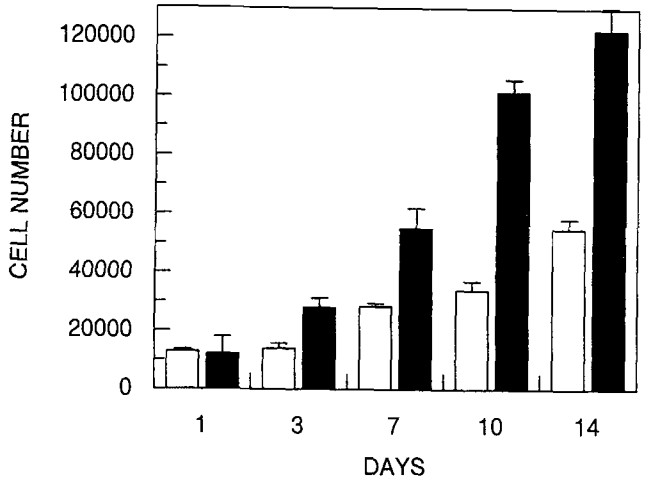

FIG. 5. Growth of osteoblasts cultured as monolayers Ham F12-MEM 1:1, 2% Ultroser (*open bars*), or MEM, 10% calf serum (*dark bars*). Cells were counted by Janus green assay. Optical densities were converted to cell number by reference to a standard curve.

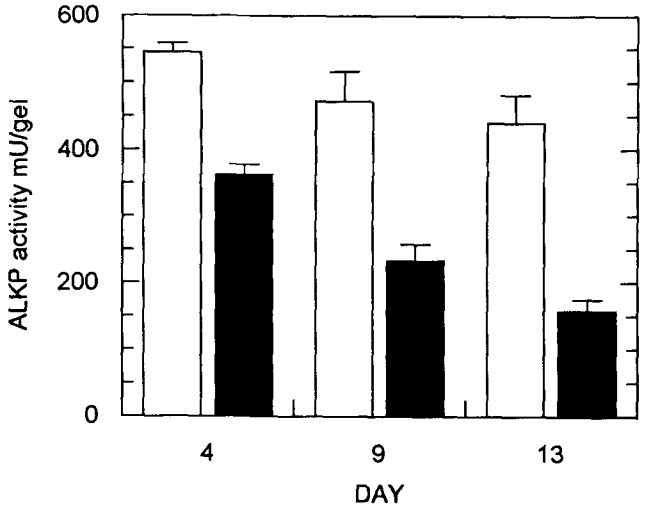

FIG. 6. Alkaline phosphatase (ALKP) activity of human osteoblastic cells in a collagen gel. *Dark bar* = CSM, *open bar* = USM.

CD 44, thus cultivated osteoblasts differ in their expression of the CD 44 membrane receptor than these cells *in situ*. Hence, CD 44 cannot be used as a criterion for defining an osteoblast culture. The mineralization potential is a key feature of the osteoblast phenotype. Cells grown with 10 m*M* βGP formed a mineralized matrix. X-ray microanalysis of the crystals showed a spectrum with a low background and high calcium and phosphorus peaks. The calcium to phosphorus ratio (1.3) was close to that of normal bone (1.63).

High ALKP activity is generally considered to be one of the main markers of the osteoblast phenotype, so our interest was to test ALKP in our osteoblastic cultures. Cells in stationary phase were found to have high ALKP activity by both histochemical and biochemical analyses. But these results reflect only an overall phenomenon. We, therefore, measured ALKP activity using cytometry analysis with fluorescein diphosphate, as it measures the activity in individual cells. The cells formed a single population in terms of their ALKP. This method also distinguishes between cell populations with high ALKP (osteoblasts) and populations with low activity, like fibroblasts. ALKP activity does not always respond to stimulation by the calciotropic hormone, 1,25 $(OH)_2$ D_3, according to published reports. Many studies have demonstrated that this vitamin has direct effects on osteoblast functions, but the *in vitro* results depend on the cell line and how the vitamin is delivered, short-term treatment (24–48h), or continuous treatment over a 15–30-d period (6,20,22,23). Our results indicate that exposure to the vitamin for 48 h significantly increased (50%) the ALKP activity in confluent cultures.

The characteristics of the cells continued to be expressed when the culture conditions were changed, but the composition of the culture medium modulated proliferation and differentiated functions. MEM-Ham F12 2% Ultroser medium (USM) was chosen because it is a defined culture medium, with constant amino acids, vitamins, and other components, unlike serum. When cultures were grown in MEM plus 10% calf serum (CSM), which is widely used in many studies, some characteristics were unchanged, like the collagen type I/collagen type III ratio, but there were also changes towards less differentiated cells. The morphology of the cells were more like that of fibroblasts, cell multiplication increased, while collagen synthesis and mineral deposition per cell decreased. The most striking effect was a considerable decrease in ALKP activity.

As ALKP appeared to be strongly modulated by culture conditions, we considered that this parameter can be selected to describe osteoblasts in different culture models. A 3-D culture model has been developed for studies on tissue reconstruction. This model was first described by Bell (4) and consists of a matrix of native collagen seeded with fibroblasts. The model had been widely used with various kinds of cells, though very few studies have been carried out with osteoblasts (17, 30). To our knowledge, all the experiments have used calf serum in the culture medium, as it contains the elements necessary for contraction of the gel(1). This study shows that the ALKP activity of 3-D cultures in CSM rapidly decreases, whereas cultures in USM have an ALKP activity that remains almost constant throughout the culture. So, in the two culture systems experimented with here, USM is more favorable to ALKP expression.

In conclusion, several parameters had been investigated as a means of defining adult human cultures as having the phenotype of mature and well-differentiated cells that are stable after one to six passages. The ALKP activity is greatly influenced by the culture conditions. This parameter thus gives modulated responses in every case and is readily quantifiable, making it the most useful criterion on which to base the choice of culture models for studies of bone cell function and regulation.

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