Mineralization in serially passaged human alveolar bone cells

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Well-characterized human bone cell cultures have been regarded as a useful tool to study bone control mechanisms and also to analyse bone/biomaterials interactions. In the present study, human alveolar bone cells were cultured in α-minimal essential medium (α-MEM) containing 10% foetal bovine serum (FBS), 50 μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate and either in the presence or in the absence of 10 nM dexamethasone (Dexa). Cultures were characterized concerning cell viability/proliferation, alkaline phosphatase (ALP), acid phosphatase (ACP) and tartraric acid resistant phosphatase (TRAP) activities, and formation of mineralized areas. Cell proliferation increased gradually for approximately 20 days. In the presence of Dexa, cells formed isolated or interconnected multilayered clusters that increased with culture time. Histochemical assays revealed strong positive reactions for ALP and calcium and phosphates deposits, mainly in relation to cells associated with the clusters. High levels of ALP activity (biochemical determination) were observed. Cells cultured in the absence of Dexa showed significantly lower ALP activity and no calcium and phosphates deposits were present. Serially passaged cells kept the proliferation rate constant but a decrease in ALP activity was observed either in the presence or in the absence of Dexa. The ability to form mineralized areas (cultures fed with Dexa) also decreased on serial subculture.

1. Introduction

The utilization of materials for bone replacement and in implantology requires the formation of an appropriate interface bone/material [1–5]. Well-characterized osteoblast-like cell cultures provide a suitable *in vitro* model to study bone regulatory mechanisms and to clarify the biological aspects of bone/materials interactions; complex biological events are analysed in simplified and defined conditions [6–15].

In the last few years, many studies have been performed in human bone cell cultures. This system has advantages over the *in vitro* models of animal cell cultures because it reproduces better the *in vivo* human osteoblastic activity and bone formation. Human bone cell cultures should present several osteoblast features including high levels of alkaline phosphatase (ALP) and ability to mineralize [16–18]. Maintenance of osteoblast phenotype on long–term cultivation (serial subculture) is also essential because large pools of cells are required to perform the experiments and serial cell passage is often necessary [17, 18].

The present study was designed to establish osteoblast-like cell cultures from human alveolar bone and to investigate whether cells maintained the osteoblast phenotype on serial passage. Results concerning cell viability/proliferation, activities of ALP, acid phosphatase (ACP) and tartraric acid resistant phosphatase (TRAP), and formation of mineralized areas in serially passaged cells, are reported. Alveolar bone cells were cultured either in the presence or in the absence of dexamethasone (Dexa).

2. Materials and methods

2.1. Cell culture

Human alveolar bone fragments (obtained from oral surgery procedures, patients aged 20–30 years) were washed extensively with α-minimal essential medium (α-MEM) containing 2.5 mg/ml amphotericin B and 500 μg/ml gentamicin. The fragments were minced into small pieces and seeded as explants into 50 ml culture flasks with α-MEM containing 10% foetal bovine serum (FBS), 0.25 mg/ml amphotericin B and 50 μg/ml gentamicin. Cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C and culture medium was changed every three days.

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Cell migration occurred after approximately 15–20 days. Adherent cells were enzymatically released (0.04% trypsin and 0.025% collagenase) and counted using a hemocytometer counter. Starting on primary culture, cells were serially passaged (on the exponential growth phase) and cultured under the same conditions as those described above. First (1st) to fifth (5th) passage cells were cultured in the presence of 50 μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate and 10 nM Dexa; 1st and 2nd passage cells were also cultured in the absence of Dexa but in the presence of the other two supplements. Cells in subculture were seeded in 35 mm culture dishes (4.5×10^4 cell/ml) and in 96-well culture plates (3×10^4 cell/ml), respectively, for histochemical (7 and 21 days cultures) and biochemical (1, 3, 7, 14, 21 and 28 days cultures) assays. Monitoring of the cultures was done daily using phase contrast inverted microscopy. Cultures were characterized at the end of each culture period.

2.2. Culture characterization

Histochemical assays were performed in fixed cells (1.5% glutaraldehyde in 0.14 M sodium cacodylate, 10 min). ALP positive cells stained brown to black (2 mg/ml sodium-α-naphtyl phosphate and 2 mg/ml Fast blue RR salt in tris buffer, pH 10, in the dark). Calcium and phosphates deposits were visualized, respectively, by the alizarin red S assay (1% alizarin sodium sulfonate and 0.028% ammonium hydroxide, pH 6.5) and von Kossa method (1% silver nitrate and 5% sodium thiosulfate). Mineralized areas stained red (calcium deposition) and black (phosphates deposition).

Cultures stained for alizarin red S assay were analysed in a JEOL JSM 6301F scanning electron microscope equipped with an X-ray microanalysis capability, Voyager XRMA System, Noran Instruments.

Cell viability/proliferation was evaluated by the reduction of the tetrazolium salt MTT by viable cells (50 μg/well for the final 4 h of the incubation period) to form a blue formazan product, measured at 600 nm after solubilization in DMSO.

Activities of ALP, ACP and TRAP were assayed by the hydrolysis of p-nitrophenyl phosphate and colorimetric determination of the product, p-nitrophenol. A microassay was used in the quantification of these enzymes. Cultures grown in 96-well plates were washed with PBS and treated with 1% Triton (100 μl/well, 5–10 min) to lyse the cells; substrate (p-nitrophenyl phosphate) in the appropriate buffer was added to cell lysate (80 μl/well): 2 μmoles in alkaline buffer solution, pH 10.3 (Sigma) for ALP evaluation, 1 μmole in citrate buffer solution, pH 4.8 (Sigma) and 1 μmole in tartrate acid buffer solution, pH 4.8 (Sigma), respectively, for the determination of ACP and TRAP. Hydrolysis was carried out for 30 min at 37 °C and the reaction was stopped with 5 M NaOH (20 μl/well); absorbance of p-nitrophenol was measured at 405 nm in a ELISA reader. Results are expressed as nanomol of p-nitrophenol produced per min per cm^2 (nmol/min cm^2). A microassay was also used in the evaluation of the total protein contents. The protein was determined in 0.1 M NaOH cell lysates according to the method of Lowry using bovine serum albumin as a standard.

Human gingival fibroblasts were prepared for control experiments. Gingival tissue was manipulated and cultured in similar conditions as alveolar bone; 2nd passage cells were cultured and characterized in the experimental conditions described above.

Reagents were purchased at Sigma Chemical Company (St Louis, MO, USA).

Biochemical data were presented as the average of six to eight replicates (mean ± SD). Students *t*-test was applied to determine the statistical significance of the differences observed between groups: *P* values $\leqslant 0.05$ were considered significant.

3. Results

3.1. Cells in culture

Human alveolar bone cells grown in the presence of Dexa formed isolated or interconnected islands of cells dispersed on the culture plate; these groups of cells increased in size with culture time and developed a nodular structure (visible as clear white spots in 21 days cultures). The ability of osteoblastic cells to form nodules gradually decreased from the 1st to the 5th passage. Cells were arranged in many and very intense nodules in the 1st subculture, whereas, cells in the 5th

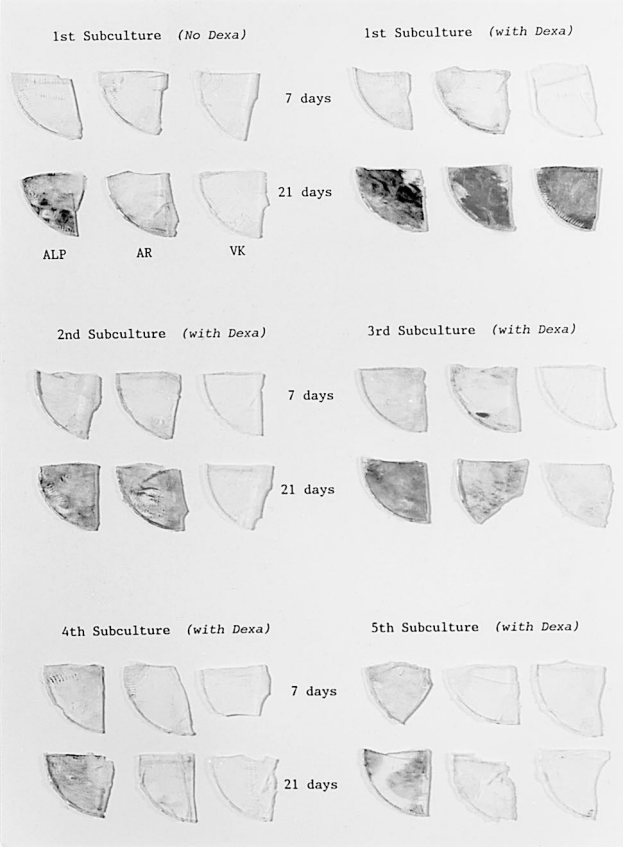

Figure 1 Macroscopic view of human alveolar bone cell cultures stained for ALP, calcium (alizarin red S, A R) and phosphates (von Kossa, V K). Cells were cultured either in the presence of Dexa (1st to 5th subcultures) or in the absence of Dexa (1st subculture) for 7 and 21 days.

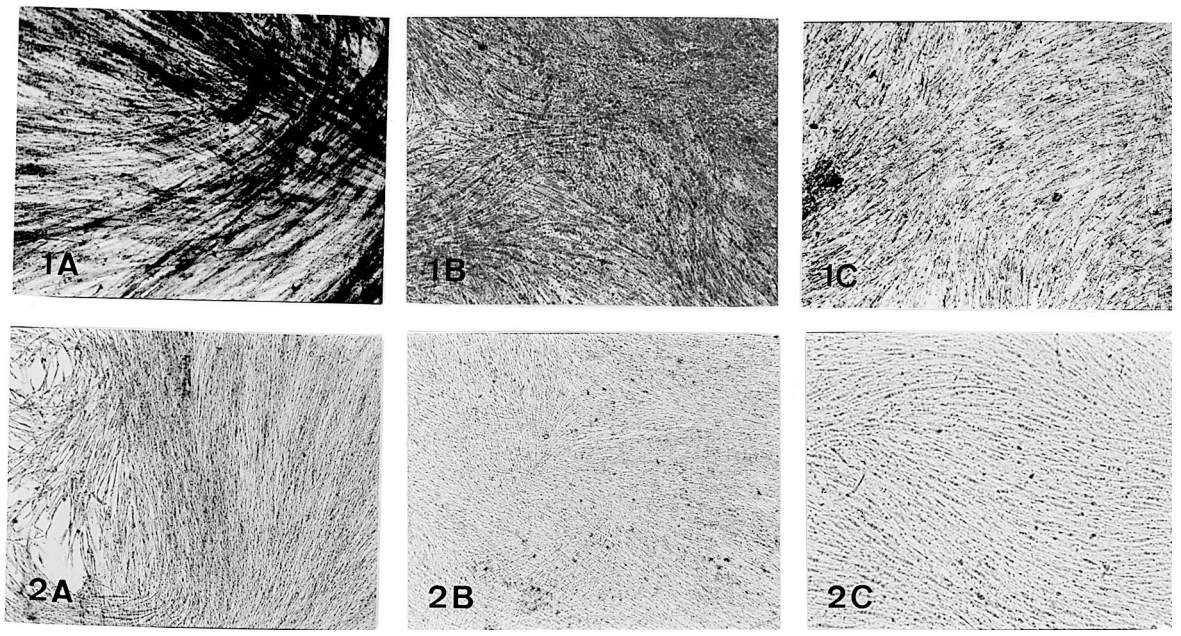

Figure 2 Light micrographs of human alveolar bone cell 1st subcultures stained for ALP (a), calcium (b) and phosphates (c), (× 40). Cells were cultured either in the presence of Dexa (Fig. 2.1) or in the absence of Dexa (Fig. 2.2) for 21 days.

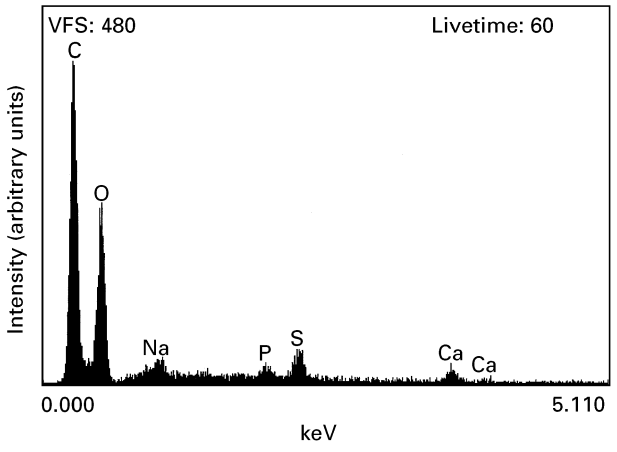

Figure 3 Energy dispersive X-Ray spectrum of mineralized areas showing the presence of calcium (Ca) and phosphorous (P). Alveolar bone cells were cultured for 21 days in the presence of Dexa (1st subculture). The carbon (C) and the oxygen (O) peaks are due to the biological material and the flask substrate. The sodium (Na) and sulfur (S) peaks are due to Alizarin red sulfonate.

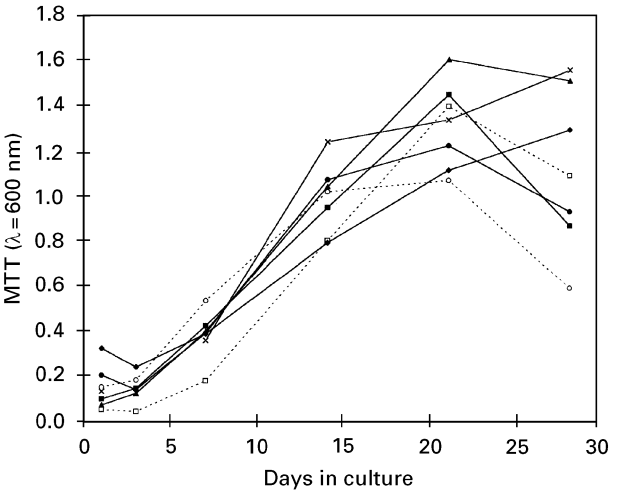

Figure 4 Cell viability/proliferation of human alveolar bone cells cultured for 28 days either in the presence of Dexa, 1st to 5th subcultures (■, 1st; ●, 2nd; ▲, 3rd; ◆, 4th; ×, 5th) or in the absence of Dexa, 1st and 2nd subcultures (□, 1st; ○, 2nd). Date are expressed as the means of 6–8 replicates. The standard deviations were $< 20\%$. *Significantly different from the 1st subculture.

subculture were dispersed in the culture plate and only very few and weak nodules were present. In the absence of Dexa, cultures showed formation of continuous cell multilayers and no nodules were observed. (Data not shown).

3.2. Histochemical studies

The histochemical assays, performed at days 7 and 21 of cell culture, confirmed the above observations. In Dexa treated cultures, cells cultured for 7 days presented a weak positive reaction for ALP and no calcium or phosphates deposition spots were observed; 21-day cultures showed a significant increase in ALP staining and formation of mineralized areas (positive alizarin red S and von Kossa reactions). Staining was mainly associated with the three-dimensional nodules. The intensity of the histochemical reactions was stronger in the 1st subculture and decreased gradually on serial passage. This effect was observed for the presence of ALP and also for the formation of calcium and phosphates deposits and was apparent even on macroscopic observation (Figs 1 and 2). Calcium (Ca) and phosphorous (P) peaks were detected by an energy dispersive X-ray analyser as shown in Fig. 3 for 21-day Dexa–treated 1st subculture.

In the absence of Dexa, cultures showed a positive histochemical reaction for ALP; staining increased with incubation time and was stronger in the 1st subculture (although lower than that observed in Dexa treated cultures). Alizarin red S assay and von Kossa staining did not show formation of calcium and phosphates deposits, even at 21 day cultures (Figs 1 and 2).

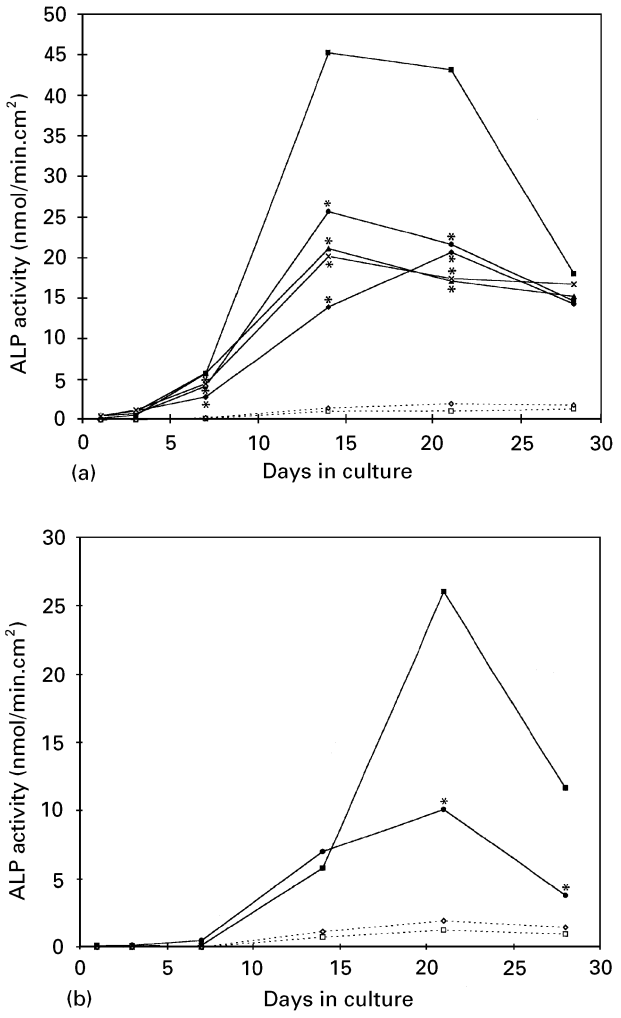

Figure 5 ALP activity of human alveolar bone cells cultured for 28 days either in the presence of Dexa (A), 1st to 5th subcultures (■, 1st; ●, 2nd; ▲, 3rd; ◆, 4th; ×, 5th) or in the absence of Dexa (B), 1st and 2nd subcultures (□, 1st; ○, 2nd). Levels of ACP (––◇––) and TRAP (––□––) found in the 1st subcultures are also shown for comparison; similar values were observed on serial passage. Data are expressed as the means of 6–8 replicates. The standard deviations were less than 20%. *Significantly different from the 1st subculture.

Staining for ALP, calcium or phosphates deposits were not observed in the human gingival fibroblasts cultures, even in the presence of Dexa (data not shown).

3.3. Biochemical parameters

Alveolar bone cells in subculture presented a high growth rate up to day 21. Cell viability/proliferation at day 28 was either similar to the observed at day 21 or lower. This pattern was similar in all subcultures (either in the presence or in the absence of Dexa) and no significant differences were observed in the cell proliferation (Fig. 4). Total protein content increased with incubation time in a way similar to cell proliferation, reaching stationary phase around day 20 (data not shown).

Biochemical determination of ALP activity showed that the levels of this enzyme were influenced by serial passage and also the culture system used (presence or absence of Dexa). In the presence of Dexa, ALP activity was noticeable on day 7, attained maximal levels on day 14 and decreased after day 21. Mineralization occurred in the stationary phase (between day 14 and day 21). In the absence of Dexa, cultures showed significantly lower ALP activity and the enzyme appeared later in the cultures (levels on day 7 were negligible); in addition, the levels observed in the 1st subculture increased mainly from day 14 to day 21 (in the 2nd subculture low levels of ALP were detected). Levels of ALP dropped significantly in the 1st subcultures after 21 days incubation (either in the presence or in the absence of Dexa). ALP activity decreased on serial passage, especially from the 1st to the 2nd subculture. As expected, levels of ACP and TRAP were insignificant in all cultures. These results are summarized in Fig. 5.

Human gingival fibroblast cultures presented insignificant levels of ALP when compared with those observed in the alveolar bone cell cultures (results not shown).

Cultures incubated for periods longer than 21 days presented signs of deterioration, as demonstrated by cell viability/proliferation values and ALP activity (and also cell morphology and matrix stability); this effect was particularly evident in the 1st subcultures.

4. Discussion

Human alveolar bone cells cultured in the presence of 50 μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate and 10 nM Dexa differentiated and proliferated to exhibit osteoblastic features. Contamination by fibroblasts and/or osteoclasts was negligible, as demonstrated by the levels of ACP and TRAP found in the cultures. These culture medium supplements were first reported by Maniatopoulos *et al.* [19] and, since then, used by others to induce *in vitro* mineralization by osteoblastic cells [7, 8, 10–12].

Alveolar bone cells cultured in the presence of Dexa presented high levels of ALP, a marker enzyme of osteoblasts; ALP activity was lower in cells cultured in the absence of Dexa. These results are consistent with previous observations that long-term treatment of bone-derived cells with physiological concentrations of glucocorticoids induce differentiation into cells which display osteoblastic phenotype [20–24].

Dexa treated cultures showed the formation of randomly located multilayered cell three-dimensional nodules, whereas, in the absence of this hormone continuous cell multilayers were observed. The formation of these nodules appeared to be necessary for *in vitro* mineralization of alveolar bone cells. Similar results were observed in other culture systems [7, 8, 10–12, 19, 25, 26]. Mineral deposits, demonstrated by the positive staining of von Kossa and alizarin assays, coincided with the three-dimensional nodules and therefore were present in Dexa treated cultures only. ALP has long been associated with biological calcification and, in alveolar bone cell cultures, mineralization occurred following the maximal ALP activity. This is in agreement with results reported in the literature for other culture systems [25, 27].

Serially passaged alveolar bone cells maintained the proliferation rate constant. ALP activity and the ability to form mineralized areas (Dexa treated cultures) decreased on serial passage. The loss of the osteoblast phenotype on serial passage has been previously reported [18, 28]. In the study described in [18] the authors observed that human osteoblast-like cell cultures derived from spongy bone (other than alveolar bone) dedifferentiated in a culture medium containing 10% FBS but not when a serum substitute was used. Additional studies are required to specifically examine whether the loss of osteoblastic phenotype observed in human alveolar bone cell cultures can be avoided in a FBS deprived culture medium.

The specificity of the examined parameters were confirmed by performing the experiments in human fibroblast cell cultures used as negative controls.

In summary, human alveolar bone cell cultures reported in this study expressed osteoblastic features, namely, a dexamethasone induced increase in ALP and the ability to form mineralized areas. However, loss of the osteoblast phenotype was observed on serial passage which limits the use of this culture system. Bearing in mind that human alveolar bone cell cultures would provide the basis of a biocompatibility assay for dental materials, further studies will be realized in order to overcome this problem. The results obtained also point out the importance in the definition of the experimental conditions in studies involving bone cell cultures.

Acknowledgements

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