Pharmacological Research, Vol. 42, No. 4, 2000
doi:10.1006/phrs.2000.0697, available online at http://www.idealibrary.com on IDEAL

LONG-TERM EFFECTS OF PARATHYROID HORMONE, 1,25-DIHYDROXYVITAMIN D_3, AND DEXAMETHASONE ON THE CELL GROWTH AND FUNCTIONAL ACTIVITY OF HUMAN OSTEOGENIC ALVEOLAR BONE CELL CULTURES

M. ADELINA COSTA and M. HELENA FERNANDES*

Faculdade de Medicina Dentária, Rua Dr Manuel Pereira da Silva, 4200 Porto, Portugal

Accepted 10 April 2000

The proliferation–differentiation behaviour of human alveolar bone cell cultures grown for 32 days in conditions that allowed the complete expression of the osteoblastic phenotype was significantly affected by the continuous presence of parathyroid hormone, 1,25-dihydroxyvitamin D_3, or dexamethasone. Parathyroid hormone and, in particular, dexamethasone significantly induced the differentiation of osteoblastic cells. Moreover, cultures exposed to these hormones presented an earlier appearance and higher levels of alkaline phosphatase, and an increased ability to form calcium phosphate deposits in the extracellular matrix.

KEY WORDS: human alveolar bone cell cultures, parathyroid hormone, 1,25-dihydroxyvitamin D_3, dexamethasone, osteoblastic proliferation–differentiation.

INTRODUCTION

Studies performed in well-characterized osteoblast-like cell cultures have contributed significantly to the understanding of the effects of compounds that influence bone metabolism (hormones, drugs) [1–3], and also the interactions of the bone tissue with implant biomaterials and their degradation products [3–10]. These cultures should present a proliferation–differentiation behaviour representative of the *in vivo* osteoblastic cell population: in particular, the ability to produce a mineralized collagenous bone matrix, a key feature of the osteoblastic phenotype [1–3, 5, 9].

References in the literature concerning human bone cell cultures derived from alveolar bone are sparse and, most frequently, bone cell cultures are obtained from trabecular bone from other skeletal sites [11]. However, there is a considerable amount of information showing differences in the behaviour among osteoblastic cells from different origins concerning growth characteristics, expression of osteoblastic markers, and response to hormones and growth factors [12–14]. Also, several studies have shown that *in vitro* bone cell culture differences correlate well with *in vivo* bone cell activity [13–15], and, as alveolar bone has a very high turnover rate [16], it should be expected that bone cell cultures established from this biological material might be a useful tool to study bone physiology and pharmacology.

Previous work has shown that, as observed in other bone cell systems [1, 2, 17–19], human alveolar bone cells maintained in long-term culture in the presence of ascorbic acid and β-glycerophosphate (βGP) present osteoblastic features, namely a high alkaline phosphatase activity (ALP) and ability to form a mineralized matrix [9]. Ascorbic acid is an enzyme cofactor and antioxidant that stimulates the transcription, translation, and post-translational processing of collagen in connective tissue cells [20]. In cultures of bone-derived cells, ascorbic acid plays an important role in the production of the collagenous extracellular matrix [1, 2, 20, 21]. βGP is routinely added to bone cell cultures to induce osteogenesis and promote calcium phosphate deposition by a mechanism that appears to be closely linked to the high ALP activity of bone cell cultures. Hydrolysis of βGP by ALP produces high levels of local phosphate ions, providing the chemical conditions for mineral deposition [1, 2, 8–10, 18, 19, 22, 23].

Parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], and dexamethasone (Dex) are known to exert significant effects on bone metabolism. A wide variety of *in vitro* studies have been conducted to analyse the effects of these hormones in the proliferation and differentiation of osteoblastic cells. However, the results obtained are inconsistent due to a number of factors related to the experimental conditions used, such as the particular system investigated, hormone concentration, time and length of exposure, stage of the osteoblast

*Corresponding author.

1043–6618/00/100345–09/$35.00/0

differentiation, and the presence of other compounds that also influence osteoblast cell growth and function [1, 2, 24–35].

The aim of this work was to analyse the long-term effects of PTH, $1,25(OH)_2D_3$ and Dex, or a combination of these hormones, in the proliferation–differentiation behaviour of human alveolar bone cell cultures grown in experimental conditions that allowed the complete expression of the osteoblastic phenotype. Alveolar bone-derived cells, cultured in the presence of βGP and ascorbic acid, were exposed continuously to the hormones. The cultures were then analysed concerning cell growth, ALP activity, and ability to form calcium phosphate deposits in the extracellular matrix.

MATERIALS AND METHODS

Cell cultures

Alveolar bone fragments (obtained from oral surgery procedures) were washed extensively with α-minimal essential medium (α-MEM), minced into small pieces, and cultured in α-MEM containing 10% foetal bovine serum, 2.5 μg ml^{-1} fungizone, and 50 μg ml^{-1} gentamicin (standard medium). Cell growth from the bone fragments was observed after approximately 10–15 days.

Primary cultures were maintained until near confluence. At this stage, adherent cells were enzymatically released (0.04% trypsin and 0.025% collagenase), counted using a haemocytometer, and subcultured at a density of 10^4 cells cm^{-2}. Cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every 2–3 days, the monitoring of which was done using phase contrast inverted microscopy.

First-passage alveolar bone-derived cells were cultured in standard medium supplemented with 5×10^{-3} M βGP and 2.52×10^{-4} M ascorbic acid (50 μg ml^{-1}) in the absence (control cultures) and in the presence of (1) PTH, (2) $1,25(OH)_2D_3$, (3) Dex, (4) PTH + $1,25(OH)_2D_3$, (5) Dex + PTH, and (6) Dex + $1,25(OH)_2D_3$. Stock solutions of the hormones were then prepared in ethanol, with the final concentration of ethanol in the culture medium being 0.1% even when different hormones were combined; preliminary studies showed no effect of this amount of ethanol in the behaviour of the cell cultures. The hormones were used in the culture medium at a concentration of 10^{-8} M and were added on day 3, being present throughout the entire incubation time.

Cultures were maintained for 32 days. They were characterized by biochemical and histochemical assays, and observed by scanning electron microscopy (SEM) for the evaluation of DNA and protein content, ALP, acid phosphatase (ACP), tartaric acid resistant phosphatase (TRAP) activities, and identification of the presence of mineralized calcium phosphate deposits in the extracellular matrix. Cultures were evaluated at days 1, 4, 7, 11, 18, 25 and 32, i.e. at 3 to 7 day intervals. Under these conditions, it was not possible to determine accurately the peak response of the measured parameters. All the experiments were performed in the first subculture, as previous work showed a loss of osteoblastic features on serial passage [36–39].

Characterization of the cell cultures

Biochemical assays.

DNA and protein content. DNA concentration was determined fluorometrically using a technique based on the method described by West, Satter, and Kumar in 1985 [40]. The medium was aspired from the wells and the cell layer washed twice in phosphate-buffered saline (PBS). To solubilize the DNA, the cell layer was treated with 10^{-2} M EDTA adjusted to pH 12.3 with NaOH, and incubated at 37 °C for 20 min. The plates were cooled on ice and the pH was adjusted to 7.0 by the addition of KH_2PO_4. A solution of Hoescht 33258, 200 ng ml^{-1} (prepared in 0.1 M NaCl and 10^{-2} M Tris, pH 7), was added to each well and fluorescence was measured immediately at excitation and emission wavelengths of 350 nm and 455 nm, respectively. Salmon DNA (0-100 μg ml^{-1}) was used as a standard.

Protein content was determined in 0.1 M NaOH cell lysates according to the method of Lowry using bovine serum albumin as a standard.

ALP, ACP, and TRAP activities. Activities of ALP, ACP, and TRAP were determined in cell lysates (obtained by treatment of the cultures with 0.1% triton) and assayed by the hydrolysis of p-nitrophenyl phosphate in the appropriate buffer: alkaline buffer solution, pH 10.3 (Sigma) (ALP); citrate buffer solution, pH 4.8 (Sigma) (ACP); and tartaric acid buffer solution, pH 4.8 (Sigma) (TRAP). Hydrolysis was carried out for 30 min at 37 °C and the p-nitrophenol formed was measured at $\lambda = 405$. Results are expressed in nanomoles of p-nitrophenol produced per min per μg of DNA (nM min^{-1} μg DNA^{-1}).

Histochemical assays.

For histochemical staining, cultures were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer, and rinsed with distilled water.

ALP. Fixed cultures were incubated for 1 h in the dark with a mixture, prepared in Tris buffer pH 10, containing 2 mg ml^{-1} of Na-α-napthyl phosphate and 2 mg ml^{-1} of fast blue RR salt (Sigma); the incubation was stopped by rinsing the samples with water. The presence of ALP was identified by a brown-black staining.

Calcium and phosphate deposits. For calcium staining, the fixed cultures were covered with a 1.0% S alizarin sodium solution (0.028% in NH_4OH), pH = 6.4, for

2 min and then rinsed with water and acid ethanol (ethanol, 0.01% HCl). Calcium deposits stained red.

Phosphate deposits were assessed by the von Kossa technique [41]. The fixed cultures were covered with a 1.0% silver nitrate solution and kept for 1 h under UV light. After rinsing, a 5.0% sodium thiosulphate solution was added for 2 min and cultures were washed again. Phosphate deposits stained black.

SEM. For SEM observation, cell cultures were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.3), then dehydrated in graded alcohols, critical-point dried, sputter-coated with carbon, and analysed in a JeoL JSM 6301F scanning electron microscope equipped with an X-ray energy-dispersive spectroscopy (EDX) microanalysis capability (Voyager XRMA System, Noran Instruments).

Statistical analysis

For biochemical data (DNA and protein content, and ALP activity), each point represents the mean ± standard deviation of 6–8 replicates. Statistical analyses were done by one-way analysis of variance (ANOVA). The statistical differences between the different groups were determined by the Bonferroni method. *P*-values <0.05 were considered significant.

RESULTS

Human alveolar bone-derived cells, growing in a medium containing βGP and ascorbic acid, were cultured in the absence and in the presence of PTH, $1,25(OH)_2D_3$ and Dex, added alone or in combination. Cultures were exposed to 10^{-8} M (the concentration most frequently used to study the effects of such compounds in bone cell systems) of the various hormones. Cultures were characterized throughout the 32-day incubation period concerning the cell proliferation–differentiation behaviour. A preliminary account of the effects of Dex in alveolar bone cell cultures has been previously reported [38, 42].

Cells in culture

Alveolar bone cells in subculture proliferated gradually with incubation time. Cell proliferation was accompanied by the synthesis of an abundant extracellular matrix, clearly visible on observation of the cultures by phase contrast microscopy and SEM. Also, cultures showed the formation of randomly located multilayered cell tridimensional nodules that increased with incubation time and were visible macroscopically as clear white spots from 2–3 weeks onwards (depending on the experimental conditions). The presence of the hormones resulted in an increase in the number and in the size of the nodules. These structures were not quantified, but it was clearly evident that the presence of Dex resulted in a higher induction in the formation and growth of the nodules compared to the other hormones. This effect is presented in Figs 1a and 1b, which show the appearance of 25-day-old cultures stained for ALP and grown in the presence of βGP + ascorbic acid (Fig. 1a) and βGP + ascorbic acid + Dex (Fig. 1b).

DNA content

Results concerning DNA content measured in the cultures grown in the various experimental conditions are presented in Figs 2a and 2b.

Cultures grown in control conditions proliferated markedly from day 10 onwards, and maximal values for DNA content were observed by day 25, after which it decreased significantly. In the cultures exposed to PTH, $1,25(OH)_2D_3$ and PTH + $1,25(OH)_2D_3$, the variation of DNA content during the incubation period followed a similar pattern, i.e. maximal values were observed by day 25. These hormones did not significantly affect cell growth, although in the presence of $1,25(OH)_2D_3$ and PTH + $1,25(OH)_2D_3$ a small increase in the DNA content was observed, specifically at days 18 and 25 (Fig. 2a).

In contrast, cultures grown in the presence of Dex (Fig. 2b) presented increased DNA content from the beginning of the incubation period, and maximal values were attained one week earlier, by day 18, after which the concentration decreased. In these cultures, maximal values for DNA content, observed at day 18 (Fig. 2b), were similar to those found in the cultures grown in the presence of $1,25(OH)_2D_3$ and PTH + $1,25(OH)_2D_3$, observed at day 25 (Fig. 2a).

Alveolar bone cells were also cultured in the presence of a combination of Dex and the other hormones, namely Dex + PTH and Dex + $1,25(OH)_2D_3$. In these cultures, the pattern of variation of DNA content was similar to that observed in the cultures grown only in the presence of Dex (Fig. 2b). The additional presence of the other hormones resulted in small increases in the DNA content, particularly in the case of $1,25(OH)_2D_3$.

Measurement of the total protein content in the cultures grown in the various experimental conditions provided the same kind of information as the measured DNA content (results not shown).

ALP, ACP, and TRAP activities

Results concerning ALP activity are shown in Figs 2c and 2d. Cultures grown in the presence of βGP and ascorbic acid (control cultures) showed levels of ALP that increased with incubation time in a way similar to that observed for cell proliferation; maximal ALP activity was found by day 25, after which it slowly decreased.

Cultures treated with $1,25(OH)_2D_3$ showed a high increase in ALP activity during the few days after the addition of the hormone (*c*. 145%, at day 11). However, later levels were similar to those observed in cultures grown under controlled conditions. Cultures exposed to PTH presented increased ALP activity from day 7 on-

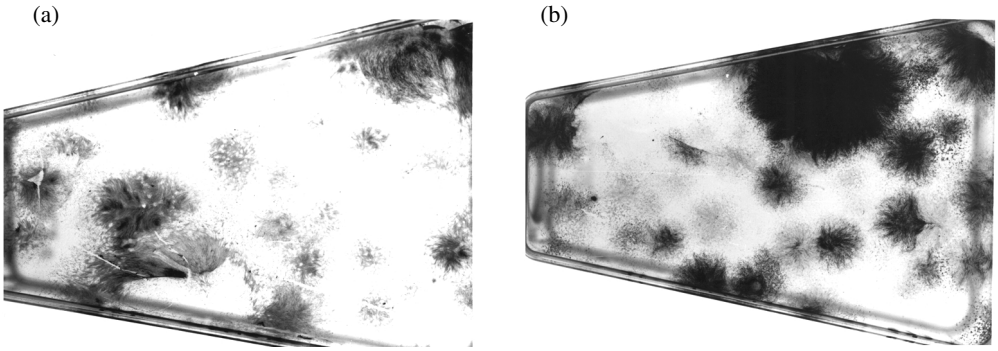

Fig. 1. Appearance of 25-day-old human alveolar bone cell cultures stained for ALP. First subculture cells were cultured in 50 cm^2 culture flasks in the presence of (a) βGP + ascorbic acid and (b) βGP + ascorbic acid + Dex (x 2).

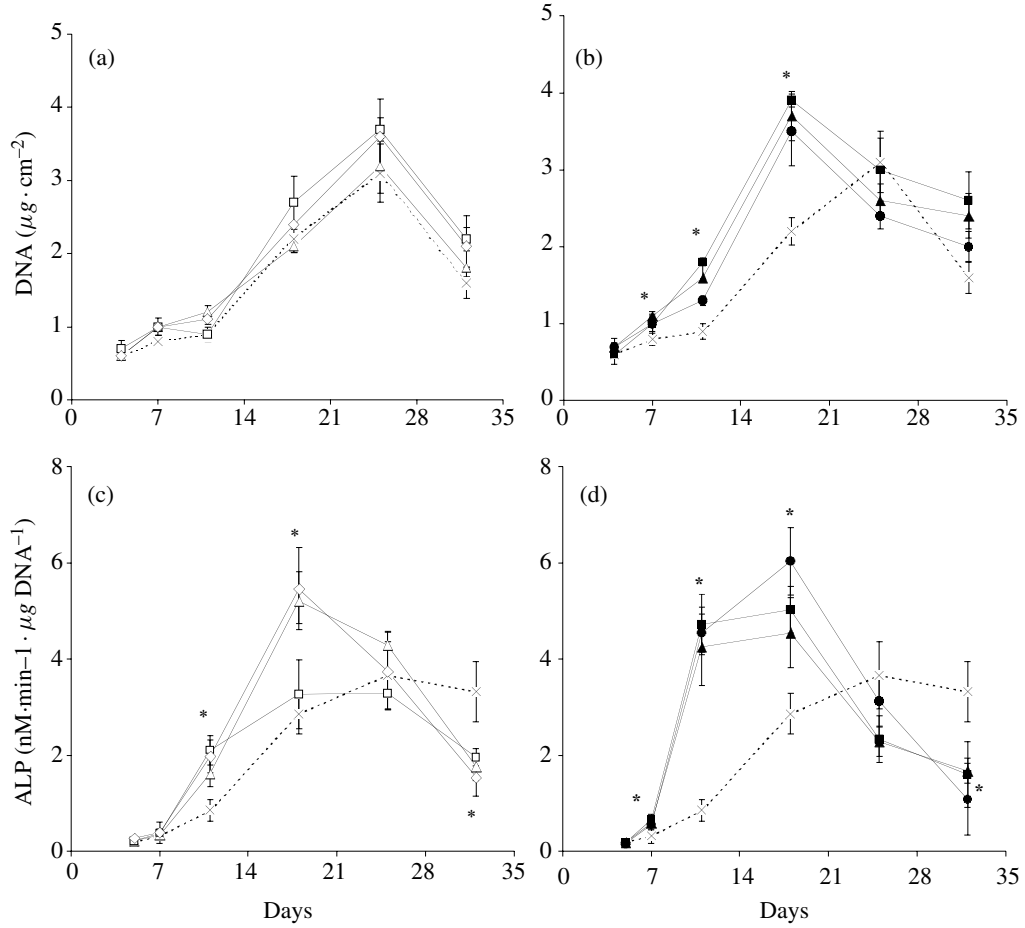

Fig. 2. (a, b) Total DNA content and (c, d) ALP activity measured in human alveolar bone cell cultures grown for 32 days in the absence (control cultures) and in the presence of hormones. (a, c) Cultures grown in the absence of Dex (open symbols): control cultures (dotted line, ×) and cultures treated with PTH (△), 1,25$(OH)_2D_3$ (□) and PTH + 1,25$(OH)_2D_3$ (△). (b, d) Cultures grown in the presence of Dex (filled symbols): control cultures (dotted line, ×) and cultures treated with Dex (●), Dex + PTH (▲) and Dex + 1,25$(OH)_2D_3$ (■). *Statistically different from control cultures.

wards (*c*. 88% and 80%, at days 11 and 18, respectively), and those treated with PTH + 1,25$(OH)_2D_3$ showed a similar behaviour.

The presence of Dex resulted in a high induction of ALP activity at the early stages of the culture, namely during the first 10 days (four-fold, at day 11), as compared to that observed in cultures treated with PTH or 1,25$(OH)_2D_3$. Cultures treated with a combination of Dex and the other hormones presented similar ALP activity until approximately day 11. By day 18, the stage at which ALP attained maximal levels, the levels of the enzyme had decreased significantly (Fig. 2d). This decrease was more significant in the presence of PTH, the experimental conditions that caused the more pronounced increase in the ALP activity in the cultures grown in the absence of Dex.

Levels of ACP and TRAP were insignificant in all cultures, suggesting a negligible contamination by fibrob-

Table I
Effect of 1,25$(OH_2)_2D_3$ (vitamin D_3), PTH, Dex, or their combination on the formation of calcium phosphate deposits (von Kossa reaction) in alveolar bone cell cultures grown for 18, 25 and 32 days

Culture	*No. of days*		
	18	*25*	*32*
Control cultures	−	+/−	+
Vitamin D_3	−	+	++
PTH	−	+	++
Vitamin D_3 + PTH	−	+	++
Dex	+	++	+++
Dex + Vitamin D_3	+	++	+++
Dex + PTH	+	++	+++

Intensity of staining was graded as follows: −, negative staining; +/−, week to undetectable staining; +, definite staining, but of low intensity; ++, moderate staining; + + +, intense staining.

lasts and/or osteoclasts (results not shown). These results are in agreement with previous work performed in human alveolar bone cell cultures treated with Dex in similar experimental conditions [38, 42].

Formation of calcium phosphate deposits

The formation of calcium phosphate deposits in the cell cultures growing in the various experimental conditions was estimated by histochemical assays (Table I), and observation of the cultures was carried out by SEM (Fig. 3).

Cell cultures grown in control conditions and in the presence of the hormones presented positive staining for the formation of calcium phosphate deposits (Table I). However, in the absence of hormones, cultures began to mineralize later, during the last week of culture. Cultures grown in the presence of Dex (added alone or in combination) mineralized earlier, and the intensity of the staining was higher than that observed in the cultures treated with PTH, 1,25$(OH)_2D_3$, or PTH + 1,25$(OH)_2D_3$ (Table I).

In agreement with the histochemical assays, all the cultures showed the presence of mineral deposits. In the absence of hormones, they were observed only in 32-day-old cultures, but in hormone-treated cultures, the presence of these structures was evident by day 25. However, the abundance of the mineral deposits was higher in Dex-treated cultures. Figure 3 shows the SEM appearance of 25-day-old cultures grown in the presence of PTH (Fig. 3a) and Dex (Figs 3b and 3c). In all the experimental situations, X-ray microanalysis of the mineral deposits showed the presence of calcium (Ca) and phosphorous (P) peaks at an atomic ratio of around 1.6, which is in agreement with the Ca/P ratio of the hydroxyapatite (Fig. 3d, for cultures grown in the presence of Dex).

DISCUSSION

The development of the osteoblastic phenotype from the osteoprogenitor proliferative cell to the osteocyte embedded in the extracellular matrix has been observed in many *in vivo* and *in vitro* studies. These studies suggest a temporal sequence of differentiation involving active cell proliferation and extracellular matrix biosynthesis, extracellular matrix maturation, and organization and matrix mineralization (the latter event reflecting the terminal expression of the osteoblastic phenotype [1, 2, 23]). Proliferation of osteoblastic cells is functionally related to the synthesis of an organized bone-specific extracellular matrix, and its accumulation, maturation, and organization, essential to the mineralization process, contribute to the shutdown of proliferation. With the initiation of the mineralization process, cell proliferation ceases, and the osteoblasts become trapped and embedded in the mineralizing matrix [1, 2, 23, 26, 43]. Results presented in this work concerning the proliferation–differentiation behaviour of human alveolar bone cell cultures grown only in the presence of βGP and ascorbic acid appear to be in agreement with this model. These cultures undergo progressive differentiation as they present a proliferative phase, accompanied by the synthesis of an abundant extracellular matrix that is further mineralized. The initiation and propagation of the matrix mineralization, which occurred during the last week of culture, was associated with a decrease in cell proliferation (Table I, Fig. 2a). This culture system appears to be a potential *in vitro* model for addressing the proliferation–differentiation behaviour relationship during the progressive development of the osteoblastic phenotype in selected experimental conditions, namely in the presence of compounds that affect bone regulatory mechanisms (hormones, drugs).

PTH, 1,25$(OH)_2D_3$, and Dex are known to exert significant effects on bone metabolism, i.e. to influence the proliferation and functional activity of the osteoblastic cells. Results presented in Figs 1–3 and Table I show that the presence of these compounds in the culture medium influences cell proliferation (Figs 2a, b), ALP activity (Figs 2c, d), and the mineralization process (Fig. 3, Table I) of alveolar bone cell cultures.

Results concerning cell proliferation show that there is a significant difference in behaviour between the cultures growing in the absence or in the presence of Dex, independently of the presence of other hormones. In the absence of the glucocorticoid, maximal values for DNA content were attained by day 25 (Fig. 2a), whereas in the presence of the glucocorticoid, cultures presented significantly increased values for DNA content in the exponential phase of cell growth, and maximal values were observed by day 18 (Fig. 2b). This effect is most probably related to the ability of glucocorticoids to stimulate the proliferation of osteoprogenitor cells [1, 2, 26, 44–48].

Osteoblastic cells present high ALP activity. It is generally accepted that as the specific activity of ALP in a population of bone cells increases, there is a corresponding shift to a more differentiated state, and the levels of the enzyme have been routinely used in *in vitro* experiments as a relative marker of osteoblastic

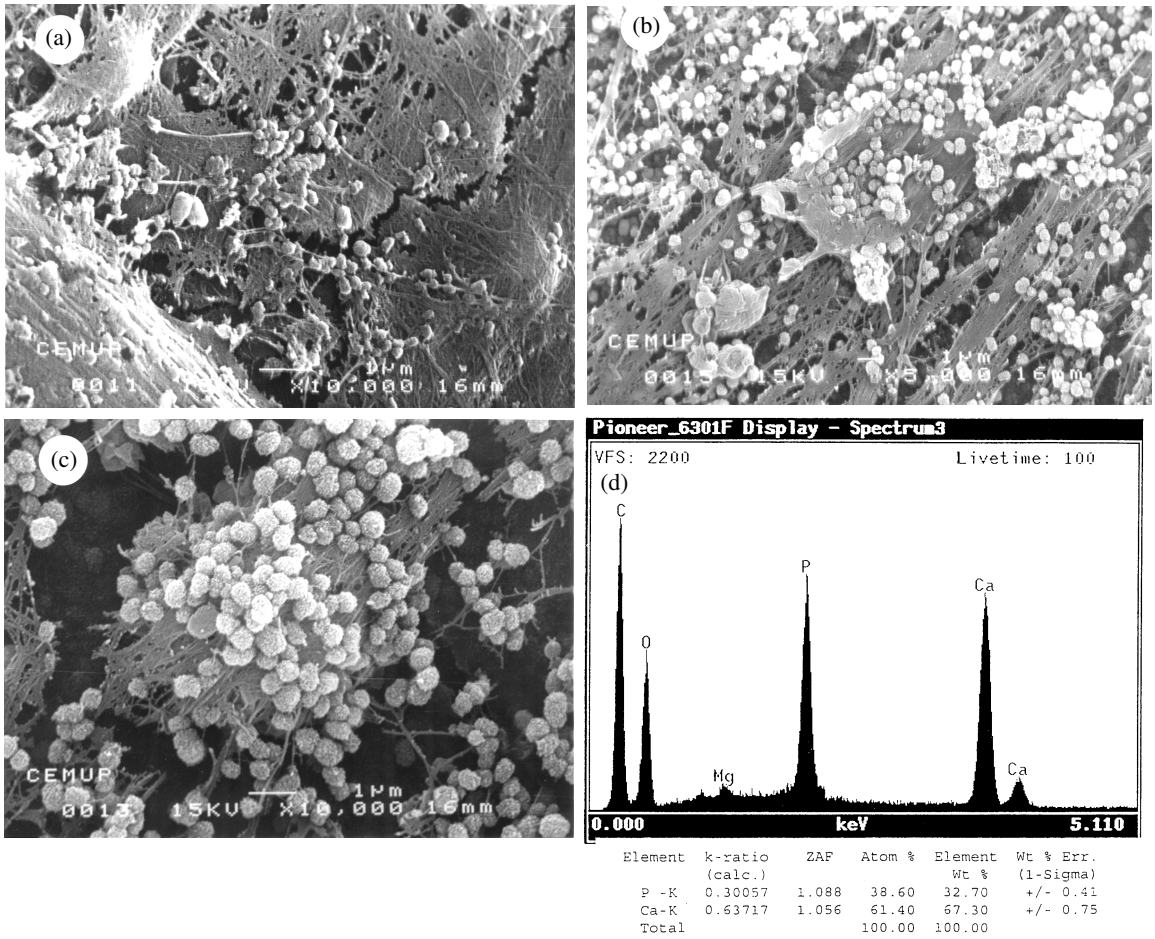

Fig. 3. SEM appearance of 25-day-old human alveolar bone cell cultures grown in the presence of (a) βGP + ascorbic acid + PTH and (b, c) βGP + ascorbic acid + Dex. (d) Energy-dispersive X-ray spectrum of the mineral deposits present in Dex-treated cultures (shown in Fig. 3b and 3c).

differentiation [1, 2]. The addition of PTH,1,25$(OH)_2D_3$, or Dex to alveolar bone cell cultures results in an increase in the ALP activity throughout the incubation time, with the expression of the enzyme and maximal levels also occurring earlier (Figs 2c, d). PTH and Dex cause the most significant increase in the activity of the enzyme.

Cell cultures grown in the presence of a combination of Dex and the other hormones present a decrease in the maximal values observed for ALP activity, as compared to those found in cultures exposed only to Dex. The high induction of the osteoblastic differentiation caused by the presence of Dex in the early stages of incubation should be taken into account when observing the decrease in the maximal values for ALP activity found in cultures treated with a combination of Dex and the other hormones, as the effect of PTH and 1,25$(OH)_2D_3$ on ALP activity appears to be dependent on the differentiation status of the culture [1, 2].

Results concerning the histochemical and SEM assays (Table I, Fig. 3) show that the cultures grown in the presence of Dex (added alone or in combination with other hormones) present a higher ability to form calcium phosphate deposits in the extracellular matrix. This is in agreement with a number of studies performed in rat and human osteoblast-like cell cultures showing that chronic treatment with glucocorticoids at near physiological concentrations generally stimulates parameters associated with bone formation [1, 2, 20, 26, 38, 42–50].

In all the experimental situations, similar to those observed in other bone cell systems [1, 2, 18, 19, 44, 45], the formation of calcium phosphate deposits in the cultures began to occur following the maximal ALP activity, as is evident when comparing the results presented in Fig. 2c and Table I. ALP has long been associated with biological mineralization [1, 2, 23], and the presence of high ALP activity at sites of mineralizable matrices increases the local phosphate concentration (in the conditions of this work, by hydrolysing βGP) and, together with the Ca^{2+}-binding properties of the enzyme, may regulate and provide appropriate localized concentrations of both ions to initiate mineralization [1, 2, 23]. Figure 2 shows that decreased values for DNA content and ALP activity were repeatedly observed later in the culture time, whereas the intensity of the von Kossa reaction increased (Table I). During this stage of the culture, i.e. after the initiation of the mineral deposition, the fully differentiated osteoblast trapped in the mineralizing matrix becomes an osteocyte that neither proliferates nor expresses ALP activity [1, 2, 51]. Also, it could be speculated that the induction of apoptosis occurred late in the culture, in some cases pos-

sibly aiding in the creation of the matrix vesicles necessary for the formation of the observed mineralized globules. This effect seems to be apparent under SEM observation of the cultures (Fig. 3). In addition, and probably related to this, displacement of the cell layer from the plastic culture surface began to be observed soon after the onset of mineralization, an event that is more significant in heavily mineralized cultures. These observations are in agreement with previous studies reporting that apoptosis has been observed during mineralization in bone nodule-forming cultures [2]. Also, there is evidence that cells are more importantly involved in the initiation of mineralization (through matrix vesicles containing high levels of ALP), while propagation remains primarily physicochemical and, in heavily mineralized cultures, ALP activity is low [1, 2, 23].

A large number of studies suggest that while glucocorticoid excess *in vivo* is associated with bone loss, *in vitro* glucocorticoids can either stimulate or inhibit bone formation [52]. Regarding this, it is important to distinguish between the developmental and regulatory effects of glucocorticoids on bone formation [52]. Generally, these compounds promote the growth and differentiation of osteoprogenitor cells, abbreviating the proliferation period of the developmental sequence in the expression of the osteoblastic phenotype (developmental effects) [1, 2, 20, 26, 38, 42–52]. However, glucocorticoids decrease bone formation, the expression of several osteoblastic marker genes, and cell proliferation in mineralized bone (regulatory effects) [52]. The presence of Dex in alveolar bone cell cultures enhances the differentiation of osteoblastic cells; cell proliferation ceased earlier, being followed by matrix mineralization, apparently as a result of the developmental effects of glucocorticoids on osteoblastic cells.

It is widely known that $1,25(OH)_2D_3$ anabolically and catabolically modulates bone cell function. Some reports show the inhibition of bone cell proliferation [37, 53–55], the decrease of ALP activity [56, 57] or increase on the levels of this enzyme [30, 37, 48, 54–60], and inhibition of the mineralization process [61]. Similarly, many studies have shown that PTH has either proliferative or antiproliferative effects on osteoblastic cells [27, 62–68], and the effect of this hormone on ALP and osteoblastic differentiation is either stimulatory [69–71] or inhibitory [24, 56, 57, 72, 73]. In alveolar bone cell cultures, the long-term effect of $1,25(OH)_2D_3$ results in a discrete increase in cell proliferation and ALP activity (in this case, only during the few days after the addition of the hormone), whereas the presence of PTH causes a significant increase in ALP activity.

A common issue in analysing the effects of compounds that affect osteoblastic cells is the heterogeneity of the responses observed in different studies. Reported data show that PTH, $1,25(OH)_2D_3$, and Dex can both positively and negatively regulate the expression of osteoblastic phenotypic markers, such as ALP, type I collagen, osteopontin and osteocalcin, depending on the cell system, culture time, concentration and duration of hormone treatment, stage of differentiation of the osteoblastic cells, and the presence of other compounds that influence osteoblastic properties [1, 2, 24–35]. Hormones may affect osteoblastic cell growth and differentiation by inducing direct effects on osteoblastic cells, but also by affecting the production and bioavailability of growth factors [1, 2, 25]. In addition, hormones and growth factors may influence osteoblastic cells by acting at several steps during the sequence of differentiation. A variety of local factors and hormones have a major influence on the commitment and recruitment of undifferentiated cells. Some affect the differentiation of osteoprogenitors, some of them are potent mitogens, whereas others influence the expression of phenotypic markers, suggesting that the interactions of the various hormones and growth factors are important in determining cell function [1, 2, 26, 30, 34, 35, 74, 75].

The results presented in this work show a similar tendency of effects as those observed in some, but not all, studies. It is worthwhile to note that this study was performed in cultures obtained from alveolar bone, a tissue that has a very high turnover rate due to its constant adaptation to the stresses of occlusal impact. In addition, alveolar bone cells appear to have some physiological differences compared to bone cells from other skeletal origins in the sense that they are related to the presence of teeth. Unlike most of the related studies, human alveolar bone cells were kept in culture for an extended period of time under experimental conditions that allowed the opportunity to study the full differentiation of these cells from osteoblast precursor through to matrix mineralization in the presence of PTH, $1,25(OH)_2D_3$, and Dex.

Alveolar bone cell cultures were exposed to PTH, $1,25(OH)_2D_3$, and Dex during the entire incubation time, and there is increasing evidence that some compounds may elicit different effects on osteoblastic cells as they mature from osteoprogenitor to more mature cells, whereas others may affect only particular subpopulations within the lineage, as referred to above. In this context, the results of this work do not allow for a discrimination of the differential effects of the various hormones on the osteoblastic cells. With this in mind, we are now studying the proliferation–differentiation behaviour of alveolar bone cell cultures exposed to the same hormones at different phases of the culture period, representative of cell populations at different stages of osteoblastic differentiation.

ACKNOWLEDGEMENTS

The authors would like to thank Ms Cláudia Marina Martins for technical assistance and Mr Emanuel Carvalho Monteiro for preparation of the samples for SEM analysis.

REFERENCES

1. Martin TJ, Findlay DM, Hath JK, Ng KW. Osteoblasts: Differentiation and function. In: *Handbook of Experimental Pharmacology*. Mundy JR, Martin TJ, eds. New York: Springer-Verlag, 1993: 149–83.
2. Stein GS, Lian JB, Stein JL, van Wijnen AJ, Frenkel B, Montecino M. Mechanisms regulating osteoblast proliferation and differentiation. In: *Principles of Bone Biology*. Bilizikian JP, Raisz LG, Rodan GA, eds. New York: Academic Press Inc, 1996: 69.
3. Beresford JN, Graves SE, Smoothy CA. Formation of mineralized nodules by bone derived cells *in vitro*: A model of bone formation? *Am J Med Genet* 1993; **45:** 163–78.
4. Bizios R. Mini review: Osteoblasts: An *in vitro* model of bone-implant interactions. *Biotechnol Bioeng* 1994; **43:** 582–85.
5. KirkPatrick CJ, Wagner M, Kohler H, Bittinger F, Otto M, Klein CL. The cell and molecular biological approach to biomaterial research: A perspective. *J Mater Sci: Mater Med* 1997; **8:** 131–41.
6. Tomás H, Carvalho GS, Fernandes MH, Freire AP, Abrantes LM. The use of rat, rabbit or human bone marrow derived cells for cytocompatibility evaluation of metallic elements. *J Mater Sci: Mater Med* 1997; **8:** 233–38.
7. Morais S, Sousa JP, Fernandes MH, Carvalho GS, de Bruijn JD, van Blitterswijk CA. Decreased consumption of Ca and P during *in vitro* biomineralization and biologically induced deposition of Ni and Cr in presence of stainless steel corrosion products. *J Biomed Mater Res* 1998; **42:** 199–212.
8. Morais S, Dias N, Fernandes MH, Carvalho GS. *In vitro* osteoblastic differentiation of human bone marrow cells in the presence of metal ions. *J Biomed Mater Res* 1999; **44:** 176–90.
9. Fernandes MH. Effect of stainless steel corrosion products on *in vitro* biomineralization. *J Biomat App* 1999; **14:** 113–68.
10. Ferraz MP, Fernandes MH, Trigo-Cabral A, Santos JD, Monteiro JF. *In vitro* growth and differentiation of osteoblast-like human bone marrow cells on a glass reinforced hydroxiapatite plasma-sprayed coating. *J Mat Sci: Mat Med* 1999; **10:** 567–76.
11. Gundle R, Beresford JN. The isolation and culture of cells from explants of human trabecular bone. *Calcif Tissue Int* 1995; **56:** S8–S10.
12. Ninomiya JT, Tracy RP, Calore JD. Heterogeneity of human bone. *J Bone Miner Res* 1990; **5:** 933–38.
13. Denkouski P, Tertinegg I, Heersche JNM. Heterogeneity between osteoblastic populations isolated from different adult rat skeletal sites. *Bone Miner* 1994; **25:** S8.
14. Kasperk C, Wergedal J, Strong D. Human bone cell phenotypes differ depending on their skeletal site of origin. *J Clin Endocrinol Metab* 1995; **80:** 2511–17.
15. Marie PJ, de Vernejoul MC. Proliferation of bone surface derived osteoblast cells and control of bone formation. *Bone* 1993; **14:** 463–68.
16. Avery JK. Histology of the periodontium: alveolar bone, cementum and periodontal ligament. In: *Oral Development and Histology*. Avery JK, ed. New York: Thieme Medical Publishers, 1994: 144–62.
17. Bellows CG, Aubin JE, Heersch JNM. Initiation and progression of mineralization of bone nodules formed *in vitro*: The role of alkaline phosphatase and organic phosphate. *Bone and Mineral* 1991; **14:** 27–40.
18. Bellows CG, Heersch JNM, Aubin JE. Inorganic phosphate added exogenously or released from β-glycerophosphate initiates mineralization of osteoid nodules *in vitro*. *Bone and Mineral* 1992; **17:** 15–29.
19. Coelho MJ, Fernandes MH. Human bone cell cultures in biocompatibility testing. Part II: Effect of ascorbic acid, β-glycerophosphate and dexamethasone in the osteoblastic differentiation. *Biomaterials* in press.
20. Franceschi RT. The role of ascorbic acid in mesenchymal differentiation. *Nutr Rev* 1992; **50:** 60–5.
21. Lynch MP, Stein JL, Stein GS, Lian JB. The influence of type I collagen on the development and maintenance of the osteoblastic phenotype in primary and passaged rat calvarial osteoblasts: modification of expression of genes supporting cell growth, adhesion and extracellular matrix mineralization. *Exp Cell Res* 1995; **216:** 35–45.
22. Chung CH, Golub EE, Forbes E, Tokuoka T, Shapiro IM. Mechanism of action of β-glycerophosphate on bone cell mineralization. *Calcif Tissue Int* 1992; **51:** 305–11.
23. Anderson HC, Morris DC. Mineralization. In: *Handbook of Experimental Pharmacology*. Mundy JR, Martin TJ, eds. New York: Springer-Verlag, 1993: 267–98.
24. Jongen JWJM, Bos MP, Meer JMVD, Herrmann-Erlee MPM. Parathyroid hormone-induced changes in alkaline phosphatase expression in fetal calvarial osteoblasts: differences between rat and mouse. *J Cell Physiol* 1993; **155:** 36–43.
25. Lian JB, Stein GS. The developmental stages of osteoblast growth and differentiation exhibit selective responses of genes to growth factors (TGFβ1) and hormones (vitamin D and glucocorticoids). *J Oral Implant* 1993; **19:** 95–105.
26. Stein GS, Lian JB. Molecular mechanisms mediated proliferation–differentiation interrelationships during progressive development of the osteoblastic phenotype: Update 1995. *Endocrinol Rev* 1995; **4:** 290–97.
27. Scutt A, Duvos C, Lauber J, Mayer H. Time-dependent effects of parathyroid hormone and prostaglandin E_2 on DNA synthesis by periosteal cells from embryonic chick calvaria. *Calcif Tissue Int* 1994; **55:** 208–15.
28. Kasperk C, Schneider U, Sommer U, Niethard F, Ziegler R. Differential effects of glucocorticoids on human osteoblastic cell metabolism *in vitro*. *Calcif Tissue Int* 1995; **57:** 120–26.
29. Isogai Y, Akatsu T, Ishizuya T, Yamaguchi A, Hari M, Takahashi N, Suda T. Parathyroid hormone regulates osteoblast differentiation positively or negatively depending on the differentiation stages. *J Bone Miner Res* 1996; **11:** 1384–93.
30. Rao LG, Wylie JN, Sutherland MS, Murray TM. 17β-estradiol enhances the stimulatory effect of 1,25-dihydroxyvitamin D_3 on alkaline phosphatase activity in human osteosarcoma cells in a differentiation-dependent manner. *J Endocrinol* 1996; **148:** 181–7.
31. Centrella M, Rosen V, Wozney JM, Casinghino SR, McCarthy TL. Opposing effects by glucocorticoid and bone morphogenetic protein-2 in fetal rat bone cell cultures. *J Cell Biochem* 1997; **67:** 528–40.
32. Fromigué O, Marie JP, Lomri A. Differential effects of transforming growth factor β2, dexamethasone and 1,25-dihydroxyvitamin D on human bone marrow stromal cells. *Cytokine* 1997; **9:** 613–23.
33. Ishizuya T, Yokose S, Hori M, Noda T, Suda T, Yoshiki S, Yamaguchi A. Parathyroid hormone exerts disparate effects on osteoblastdifferentiation depending on exposure time in rat osteoblasticcells. *J Clin Invest* 1997; **99:** 2961–70.
34. Marie PJ. Cellular and molecular alterations of osteoblasts in human disorders of bone formation. *Histol Histopathol* 1999; **14:** 525–38.
35. Civitelli R, Bacskai BJ, Mahaut-Smith MP, Adams SR, Avioli LV, Tsien RI. Single-cell analysis of cyclic AMP response to parathyroid hormone in osteoblastic cells. *J Bone Min Res* 1994; **9:** 1407–17.
36. Hayflick L. The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res* 1965; **37:** 614–36.
37. Auf'mkolk B, Hanschka PV, Schwartz ER. Charaterization of human bone cells in culture. *Calcif Tissue Int* 1985; **37:** 228–35.
38. Fernandes MH, Costa MA, Carvalho GS. Mineralisation in serially passaged human alveolar bone cells. *J Mater Sci: Mat Med* 1997; **8:** 61–5.
39. Coelho MJ, Trigo Cabral A, Fernandes MH. Human bone cell cultures in biocompatibility testing. Part I: Osteoblastic differentiation of serially passaged human bone marrow cells cultured in α-MEM and in DMEM. *Biomaterials* in press.
40. West DC, Sattar A, Kumar S. A simplified *in situ* solubilization procedure for the determination of DNA and cell number in tissue cultured mammalian cells. *Anal Biochem* 1985; **147:** 289–95.
41. McGee-Russel SM. Histochemical methods for calcium. *J Histochem Cytochem* 1958; **6:** 22–42.

42. Fernandes MH, Costa MA, Carvalho GS. *In vitro* osteogenesis by human alveolar bone cells in the presence of dexamethasone. *Pharmacol Res* 1995; **31(Suppl):** 88.
43. Owen TA, Aronow M, Shalhoub V. Progressive development of the rat osteoblastic phenotype *in vitro*: reciprocal relationships in expression of genes associated with osteoblast proliferation during formation of bone extracellular matrix. *J Cell Phys* 1990; **143:** 420–30.
44. Cheng SL, Yang JW, Riffins T. Differentiation of human bone marrow osteogenic stromal cells *in vitro*. Induction of the osteoblastic phenotype by dexamethasone. *Endocrinology* 1994; **134:** 277–86.
45. Gundle R, Joyner CJ, Triffit JT. Human bone tissue formation in diffusion chamber culture *in vivo* by bone derived cells and marrow stromal fibroblastic cells. *Bone* 1995; **16:** 597–601.
46. Aronow MA, Gerstenfeld LC, Owen TA. Factors that promote progressive development of the osteoblastic phenotype in cultured fetal rat calvaria cells. *J Cell Phys* 1990; **143:** 213–21.
47. Kamalia N, McCulloch CAG, Tenembaum HC, Limeback H. Dexamethasone recruitment of self-renewing osteoprogenitor cells in chick bone marrow stromal cell culture. *Blood* 1992; **79:** 320–6.
48. Beresford JN, Joyner CY, Devlin C, Triffitt JT. The effects of Dex and 1,25-dihydroxyvitamin D on osteogenic differentiation of human marrow stromal cells *in vitro*. *Archs Oral Biol* 1994; **39:** 941–7.
49. Maniatopoulos C, Sodek J, Melcher A. Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res* 1988; **254:** 317–30.
50. Cheng SL, Zhang SF, Avioli LV. Expression of bone matrix proteins during dex-induced mineralization of human bone marrow stromal cells. *J Cell Biochem* 1996; **61:** 182–93.
51. Aubin JE, Liu F. The osteoblast lineage. In: *Principles of Bone Biology*. Bilizikian JP, Raisz LG, Rodan GA, eds. New York: Academic Press Inc, 1996: 51–67.
52. Lukert BP, Kream BE. Clinical and basic aspects of glucocorticoid actions in bone. In: *Principles of Bone Biology*. Bilizikian JP, Raisz LG, Rodan GA, eds. New York: Academic Press Inc, 1996: 533–48.
53. Franceschi RT, James WN, Zerlauth G. 1α-25-dihydroxyvitamin D_3 specific regulation of growth, morphology, and fibronectin in a human osteosarcoma cell line. *J Cell Physiol* 1985; **123:** 401–09.
54. Beresford JN, Gallagher IA, Russell RGG. 1,25-Dihydroxyvitamin D_3 and human bone-derived cells *in vitro*: Effects on alkaline phosphatase, type I collagen, and proliferation. *Endocrinology* 1986; **119:** 1176–85.
55. Marie PJ, Lomri A, Sabbagh A, Basle M. Culture and behaviour of osteoblastic cells isolated from normal trabecular bone surfaces. *In vitro*. *Cell Develop Biol* 1989; **25:** 373–80.
56. Wong GL, Luben RA, Cohn DV. 1,25-dihydroxycholecalciferol and parathormone: Effects on isolated osteoclast-like and osteoblast-like cells. *Science* 1977; **197:** 663–5.
57. Canalis E. Effect of hormones and growth factors on alkaline phosphatase activity and collagen synthesis in cultured rat calvariae. *Metabolism* 1983; **32:** 14–20.
58. Manolagas SC, Spiess YH, Burton DW, Deftos LJ. Mechanism of action of 1,25-dihydroxyvitamin D_3-induced stimulation of alkaline phosphatase in cultured osteoblast-like cells. *Mol Cell Endocrinol* 1983; **333:** 27–36.
59. Fritsch J, Grosse B, Lieberherr M, Balsan S. 1,25-dihydroxyvitamin D_3 is required for growth-independent expression of alkaline phosphatase in cultured rat osteoblasts. *Calcif Tissue Int* 1985; **37:** 639–45.
60. Wergedal JE, Matsuyama T, Strong DO. Differentiation of normal human bone cells by transforming growth factor-β and $1,25(OH)_2$vitamin D_3. *Metabolism* 1992; **41:** 42–8.
61. Ishida H, Bellows CG, Aubin JE, Heersch JNM. Characterization of the $1,25\text{-}(OH)_2D_3$-induced inhibition of bone nodule formation in long term cultures of fetal rat calvaria cells. *Endocrinology* 1993; **132:** 61–6.
62. Partridge NC, Opie AL, Opie RT, Martin TJ. Inhibitory effects of parathyroid hormone on growth of osteogenic sarcoma cells. *Calcif Tissue Int* 1985; **37:** 519–25.
63. Van der Plas A, Feyen JHM, Nijweide PJ. Direct effect of parathyroid hormone on the proliferation of osteoblast-like cells; a possible involvement of cAMP. *Biochem Biophys Res Commun* 1985; **129:** 918–25.
64. MacDonald BR, Gallagher JA, Russell RGG. Parathyroid hormone stimulated the proliferation of cells derived from human bone. *Endocrinology* 1986; **118:** 2445–9.
65. Reid IR, Civitelli R, Avioli LV, Hruska KA. Parathyroid hormone depresses cytosolic pH and DNA synthesis in osteoblast-like cells. *Am J Physiol* 1988; **255:** E9–E15.
66. Kano J, Sugimoto T, Fukase M, Fujita T. The activation of cAMP-dependent protein kinase is directly linked to the regulation of osteoblasts proliferation (UMR-106) by parathyroid hormone. *Biochem Biophys Res Commun* 1991; **177:** 365–9.
67. Fang MA, Kujubu DA, Hahn TJ. The effects of prostaglandin E_2, parathyroid hormone, and epidermal growth factor on mitogenesis, signaling, and primary response genes in UMR 106-01 osteoblast-like cells. *Endocrinology* 1992; **131:** 2113–9.
68. Finkelman RD, Mohan S, Linkhart TA, Abraham SM, Boussy JP, Baylink DY. PTH stimulates the proliferation of TE-85 human osteosarcoma cells by a mechanism not involving either increased cAMP or increased secretion of IGF-I, IGF-II or TGF-β. *Bone Miner* 1992; **16:** 89–100.
69. Nakatani Y, Tsunoi M, Hakeda Y, Kurihara N, Fugita K, Kumegawa M. Effects of parathyroid hormone on cAMP production and alkaline phosphatase activity in osteoblastic clone MC3T3-E1 cells. *Biochem Biophys Res Commun* 1984; **123:** 894–8.
70. Yee JA, Sutton JK, Shew RL, Olansky L. Parathyroid hormone stimulation of alkaline phosphatase activity in cultured neonatal mouse calvarial bone cells: involvement of cyclic AMP and calcium. *J Cell Physiol* 1986; **128:** 246–50.
71. Onishi T, Hruska K. Expression of $p27^{kip1}$ in osteoblast-like cells during differentiation with parathyroid hormone. *Endocrinology* 1997; **138:** 1995–2004.
72. Majeska RJ, Rodan GA. Alkaline phosphatase inhibition by parathyroid hormone and isoproterenol in a clonal rat osteosarcoma cell line. Possible mediation by cyclic AMP. *Calcif Tissue Int* 1982; **34:** 59–66.
73. Bellows CG, Ishida H, Aubin JE, Heerche JNM. Parathyroid hormone reversibly suppresses the differentiation of osteoprogenitor cells into functional osteoblasts. *Endocrinology* 1990; **127:** 3111–6.
74. Chaudhary LR, Avioli LV. Dexamethasone regulates 1L-1β and TNF-α-induced interleukin-8 production in human bone marrow stromal and osteoblast-like cells. *Calcif Tissue Int* 1994; **55:** 16–20.
75. Ishibe M, Nojima T, Ishibashi T, Koda T, Kaneda K, Rosier RN, Puzas JE. 17β-estradiol increases the receptor number and modulates the action of 1,25-dihydroxyvitamin D_3 in human osteosarcoma-derived osteoblast-like cells. *Calcif Tissue Int* 1995; **57:** 430–5.