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Human bone cell cultures in biocompatibility testing. Part II: effect of ascorbic acid, β-glycerophosphate and dexamethasone on osteoblastic differentiation

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Abstract

This work analyses the proliferation/differentiation behaviour of human bone marrow cells cultured in α-minimum essential medium supplemented with 10% foetal bovine serum (standard medium) and in the presence of ascorbic acid (AA, 50 μg ml^{-1}), β-glycerophosphate (βGP, 10 mmol) and dexamethasone (Dex, 10 nmol) under selected experimental conditions. Cultures were compared concerning cell morphology, cell growth, ALP activity and ability to form calcium phosphate deposits. Cells growing in the various experimental conditions proliferated gradually with the incubation time and presented high ALP activity. Cultures grown in standard medium and in the presence of either AA or Dex failed to form calcium phosphate deposits. Cultures grown in the presence of βGP, βGP + AA and βGP + AA + Dex, i.e. in the presence of a source of phosphate ions, showed the formation of a mineralised extracellular matrix. The presence of Dex resulted in a significant induction in the ALP activity and ability to form mineral deposits. The behaviour of the various cell cultures is in agreement with previous studies stating a reciprocal and functionally coupled relationship between proliferation and differentiation, i.e. cultures grown in a medium containing βGP presented a less proliferative but more differentiated osteoblastic cell population, as compared to cultures lacking the mineralisation process.

Keywords: Osteoblastic proliferation/differentiation; Ascorbic acid; β-Glycerophosphate; Dexamethasone

1. Introduction

Human bone marrow osteoblast-like cell cultures have been used as suitable in vitro models to study biocompatibility and bioactivity aspects of bone replacement materials [1,2]. These cultures should present a proliferation/differentiation behaviour representative of the in vivo osteoblastic cell population, namely, a high alkaline phosphatase (ALP) activity and ability to produce a mineralised collagenous bone matrix. Despite of the several advantages in using normal bone cell cultures, it is not possible to use the same cell line for several experiments, as diploid cells have a limited in vitro lifetime [3] and serial subculture results in a loss of the osteoblastic phenotype [3–5]. There are many parameters that influence the expression of the osteoblastic phenotype in culture, i.e. culture medium, culture time and the presence of compounds that influence cell proliferation and differentiation [5–8], therefore, it is convenient to select suitable experimental conditions in order to obtain bone cell cultures with defined and reproducible osteoblastic behaviour. In a previous work [5], we analysed the influence of the culture medium on the proliferation and functional activity of serially passaged bone marrow cells. Bone marrow was cultured in two widely used culture media (α-Minimum Essential Medium Eagle, alpha modification, α-MEM; Dulbecco's Modified Eagle's Medium, DMEM) in the presence of ascorbic acid (AA), β-glycerophosphate (βGP) and dexamethasone (Dex), compounds known to favour the expression of the osteoblastic phenotype in several bone cell systems [9,10]. The results of this study showed that different nutrient-containing media affect the expression of the osteoblastic parameters and, also, the importance in the definition of the experimental conditions in studies involving bone cell cultures. To further characterise this bone cell culture system, the present work aims to analyse the effect of AA, βGP and Dex on the proliferation/differentiation behaviour of human bone marrow

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cells cultured in α-MEM. Cultures growing under selected experimental conditions were maintained for periods of up to 42 days and compared with regard to cell growth parameters and functional activity.

2. Materials and methods

2.1. Cell culture

Human bone marrow (obtained from surgery procedures) was cultured in α-MEM containing 10% foetal bovine serum (FBS), 2.5 μg ml^{-1} fungizone and 50 μg ml^{-1} gentamicine (standard medium) until near confluence (approximately, 10–15 days). At this stage, adherent cells were enzymatically released (0.04% trypsin and 0.025% collagenase), counted using a hemocytometer and cultured at a density of 10^4 cells cm^{-2}, in 96-well culture plates, for periods of up to 42 days, under the following experimental conditions: standard medium; standard medium + AA (50 μg ml^{-1}); standard medium + βGP (10 mmol); standard medium + Dex (10 nmol); standard medium + AA + βGP; standard medium + AA + βGP + Dex.

Cultures were characterised at days 3, 7, 14, 21, 28, 35 and 42 for cell morphology appearance, cell growth parameters (cell viability/proliferation and total protein content) and functional activity (ALP activity and ability to form calcium phosphate deposits).

Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and culture medium was changed twice a week; monitoring of the cultures was done daily using phase contrast inverted microscopy.

2.2. Culture characterisation

2.2.1. Cell morphology

Bone marrow cells cultured in the six experimental conditions described were observed during the initial phase of the culture, i.e. at 30 min, 1, 4 and 24 h after being plated and also throughout the culture period. Cells were observed by phase contrast microscopy and scanning electron microscopy (SEM).

2.2.2. Cell viability/proliferation and total protein content

MTT assay (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple Formazan product) was used to estimate cell viability/proliferation [11]. Cells were incubated with 0.5 mg ml^{-1} of MTT in the last 4 h of the culture period tested; the medium was then decanted, Formazan salts were dissolved with dimethylsulphoxide and the absorbance was determined at 600 nm.

Protein content was determined in 0.1 M NaOH cell lysates according to the method of Lowry using bovine serum albumin as a standard. Results are expressed as micrograms per square centimetre (μg cm^{-2}).

2.2.3. ALP activity

ALP activity was determined in cell lysates (obtained by treatment of the cultures with 0.1% triton) and assayed by the hydrolysis of *p*-nitrophenyl phosphate in alkaline buffer solution, pH 10.3, and colorimetric determination of the product (*p*-nitrophenol) at $\lambda = 405$ nm (hydrolysis was carried out for 30 min at 37°C). Results are expressed in nanomoles of *p*-nitrophenol produced per min per μg of protein (nmol min^{-1} μg protein^{-1}).

2.2.4. Formation of mineralised calcium phosphate deposits

Formation of calcium phosphate deposits was estimated by histochemical assays (Alizarin red assay and the von Kossa method, respectively, for calcium and phosphate deposits) and observation of the cultures by SEM.

2.2.5. Histochemical assays

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

Phosphate deposits were assessed by the von Kossa technique [12], i.e. 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.

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 distilled water and acid ethanol (ethanol, 0.01% HCl) [12]. Calcium deposits stained red.

2.2.6. 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 a X-ray energy dispersive spectroscopy (EDX) microanalysis capability (Voyager XRMA System, Noran Instruments).

2.2.7. Statistical analysis

Data presented in this work are the result of three separate experiments performed in cell cultures established from three different bone marrow donors (male patients, aged 20–40 years old). For biochemical data (MTT reduction, total protein content and ALP activity) each point represents the mean ± standard deviation of 6–8 measurements of each sample. Statistical analysis was done by one-way analysis of variance (ANOVA). The statistical differences between the different groups were determined by the Bonferroni method. P values $\leqslant 0.05$ were considered significant.

3. Results

Human bone marrow cells were cultured in standard medium and in the presence of AA, βGP and Dex for periods of up to 42 days under selected experimental conditions. All the experiments were performed in the first subculture as previous results showed that serial passage of bone marrow cells results in a progressive loss of the osteoblastic phenotype [3,5].

Results concerning the cell proliferation/differentiation behaviour of the cultures growing in the different experimental conditions are shown in Figs. 1–4. Bone marrow cells proliferated gradually with the incubation time and presented high ALP activity, an osteoblastic marker. Cultures grown in standard medium and in the presence of either AA or Dex failed to form calcium phosphate deposits (biochemical data plotted as dotted lines). However, cultures grown in the presence of βGP, βGP + AA and βGP + AA + Dex showed the formation of a mineralised extracellular matrix (biochemical data plotted as continuous lines).

3.1. Cells in culture. SEM and histochemical assays

Bone marrow cells were cultured in standard medium until near confluency (primary culture); adherent cells were enzymatically released and cultured in the experimental conditions described in the previous section. Observation of the cultures by phase contrast microscopy and SEM during the first hours after plating showed that cell attachment to the standard plastic surface culture was similar in all the situations tested. Expansion of the cytoplasm was already visible after 30 min of culture time and, 4 h later, it was almost completely spread with the bulge of the nucleus apparent (although, coexistence of cells in different stages of spreading could be observed); 24 h later, cells presented a fibroblastic morphology but, in 7-day cultures, cells showed a polygonal appearance. Results concerning the SEM observation of the cultures growing in the presence of AA + βGP + Dex (30 min, 4 h, 24 h and 7 days) are shown in Fig. 1.

Fig. 2 shows the results concerning the histochemical assays for the identification of calcium

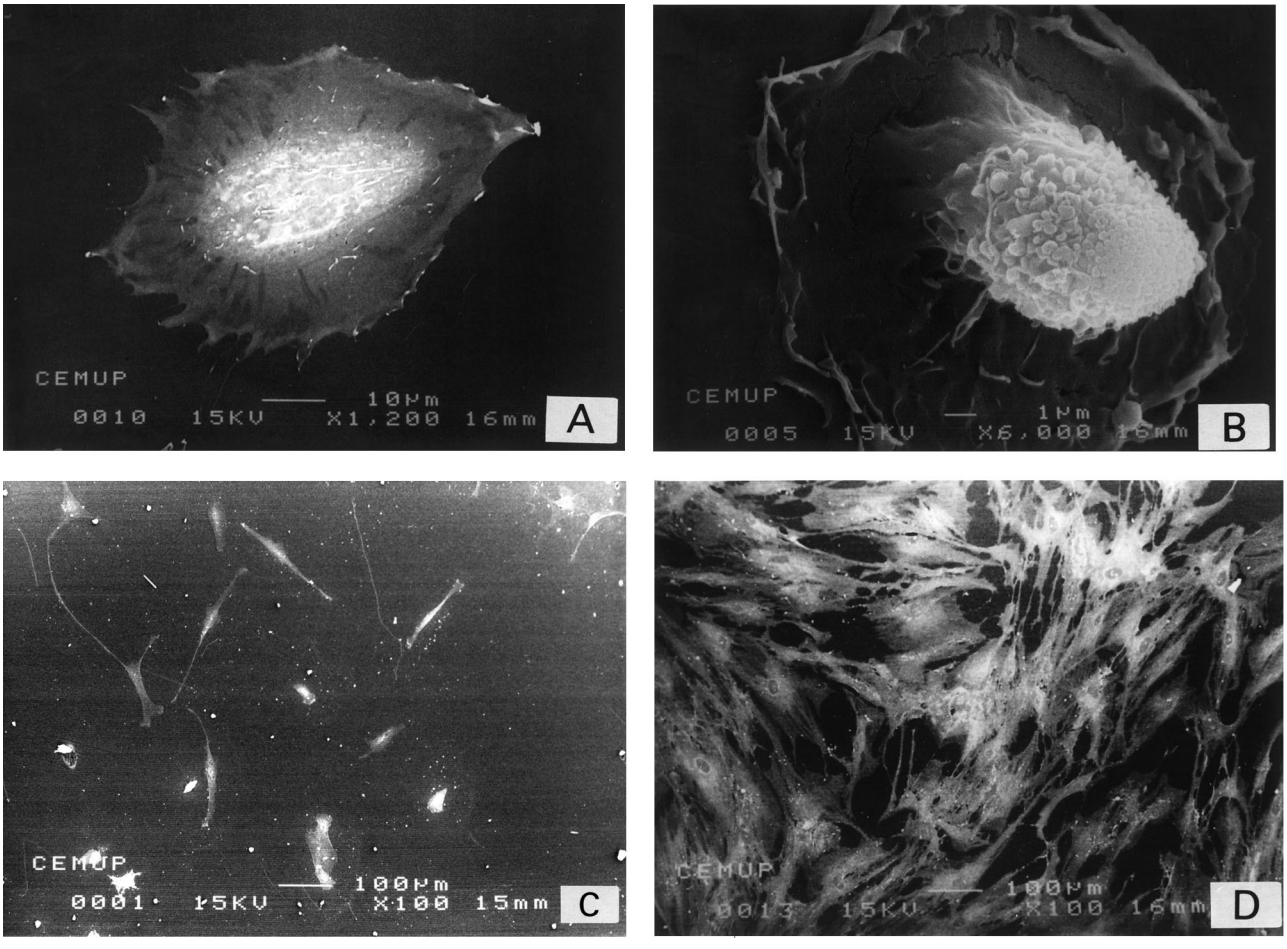


Fig. 1. SEM appearance of the cell morphology of human bone marrow cultures grown in the presence of βGP + AA + Dex, at 30 min (A), 4 h (B), 24 h (C) and 7 days (D).

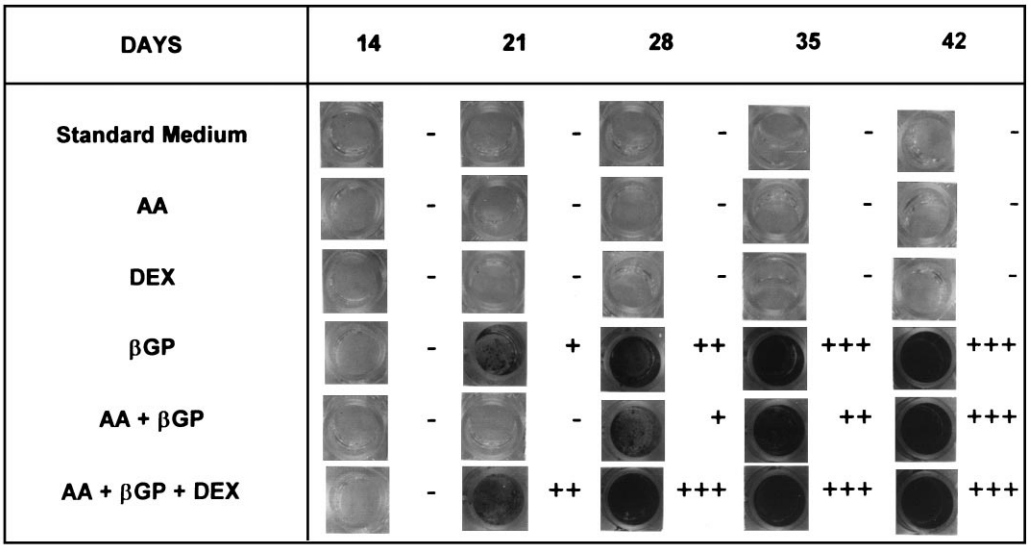


Fig. 2. Von Kossa staining of 14- to 42-day human bone marrow cultures grown under various experimental conditions. Intensity of staining was graded as follows: (−), negative staining; (+), definite staining but of low intensity; (+ +), moderate staining; (+ + +), intense staining.

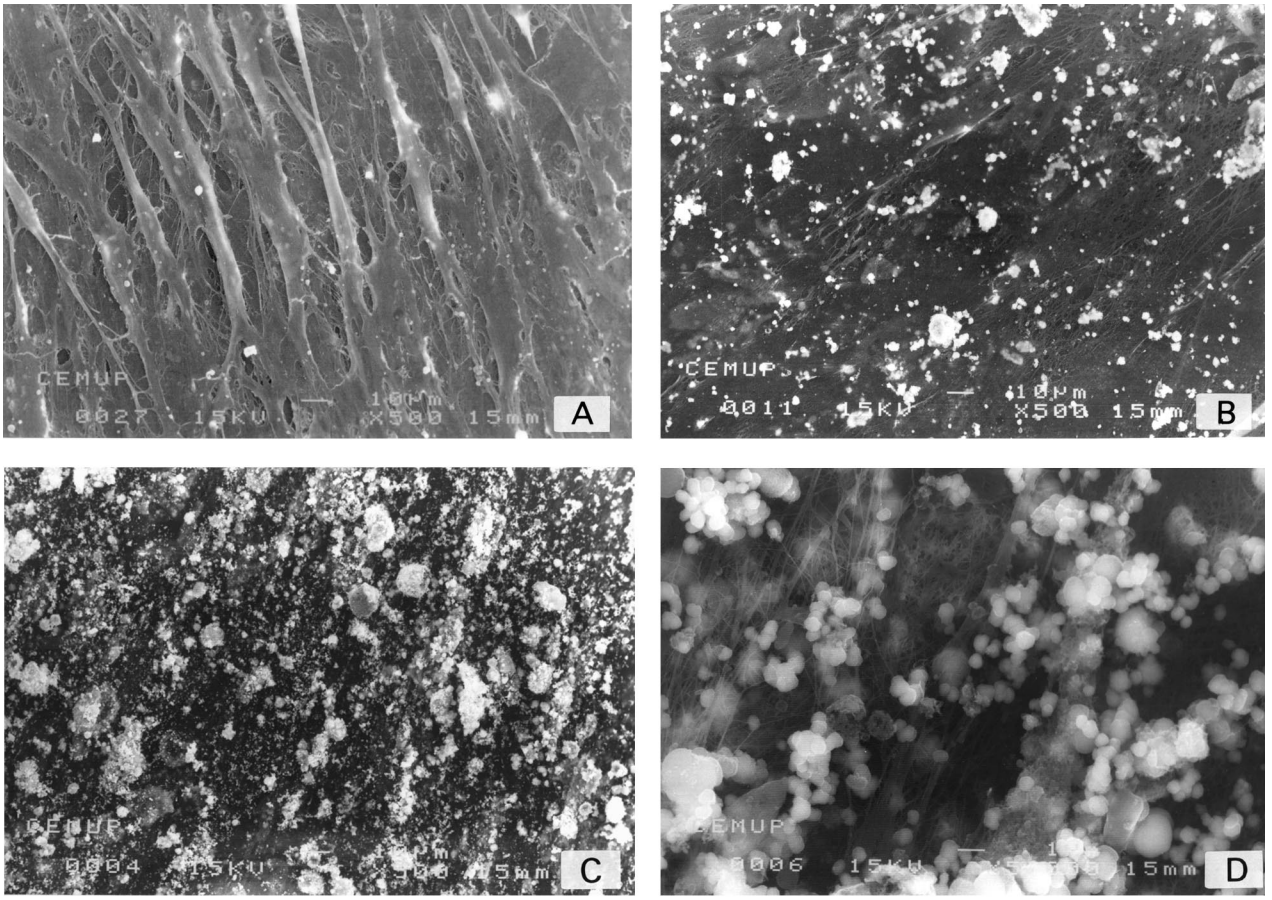
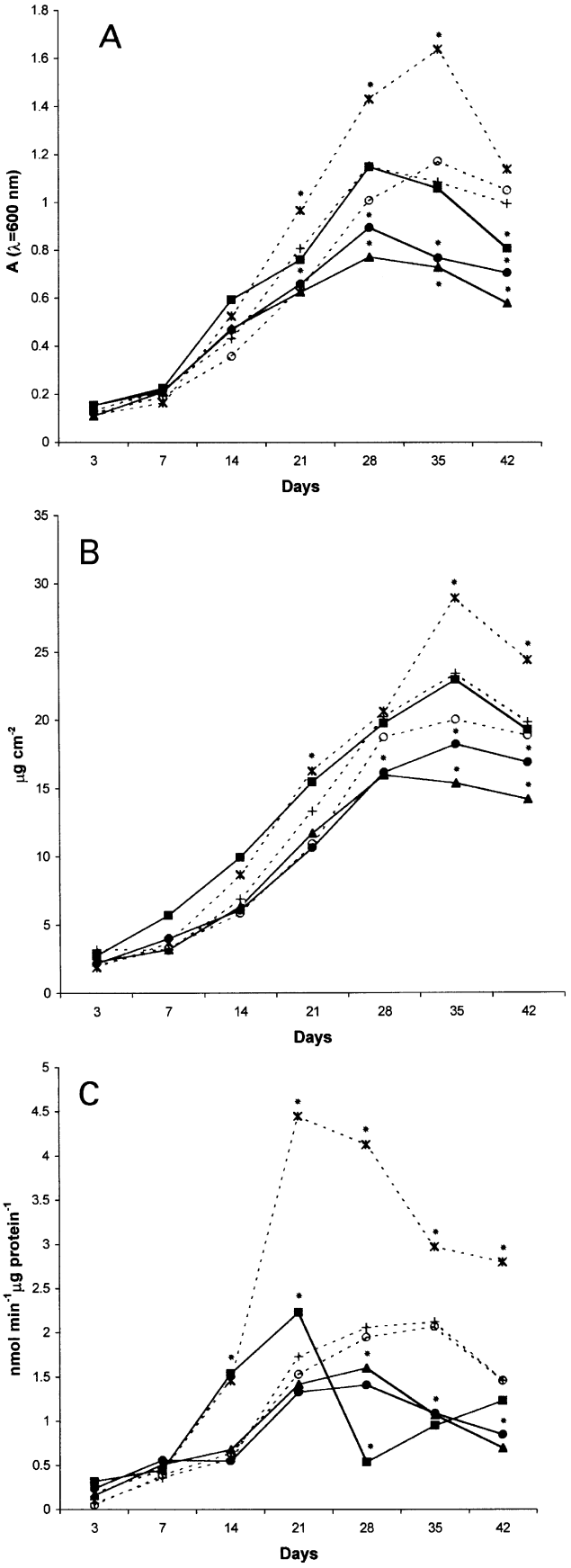


Fig. 3. SEM appearance of 35-day cultures grown in standard medium (A), βGP + AA (B) and βGP + AA + Dex (C, D: higher magnification).

phosphate deposits (von Kossa reaction) observed in 14 to 42-day cultures; similar information was provided by the Alizarin red assay (results not shown).

Fig. 3 shows the SEM appearance of 35-day cultures grown in the various experimental conditions. Cultures grown in standard medium and in the presence of either AA or Dex presented negative histochemical reactions



throughout the incubation period (Fig. 2). SEM observation of these cultures showed the presence of continuous cell multilayers and fibrillar extracellular matrix without any evidence of the presence of mineralised deposits, as exemplified in Fig. 3A for 35-day cultures grown in standard medium.

Cultures grown in the presence of βGP, alone or in combination with other supplements, presented ability to form mineralised calcium phosphate deposits. By day 21, von Kossa reaction was slightly positive in cultures grown in the presence of βGP and positive in cultures grown in the presence of AA + βGP + Dex; cultures growing in a medium containing AA + βGP began to mineralise later (around day 28). In the three experimental situations, intensity of the staining increased with the incubation time (Fig. 2). SEM observation of 35-day cultures grown in the presence of AA + βGP (Fig. 3B) and AA + βGP + Dex (Fig. 3C) showed the presence of numerous globular mineral deposits. As observed, abundance of mineralised deposits was significantly higher in the cultures grown in the presence of the three supplements. Fig. 3D shows a more detailed micrograph of the SEM appearance of these cultures, revealing that the mineral globules are incorporated in a network of fibres and, in regions of high density, they tend to fuse, forming big mineral deposits.

3.2. Cell viability/proliferation and total protein content

Results concerning cell viability/proliferation are shown in Fig. 4A. After a lag phase of approximately one week, bone marrow cells entered a period of active proliferation until days 28–35 and, after that, decrease in cell growth was observed.

Cultures grown in standard medium and in the presence of AA presented similar cell growth, although maximal values for the MTT reduction were attained, respectively, at days 28 and 35. The addition of Dex to the standard medium resulted in a significant increase in cell proliferation from the second week onwards.

Cultures grown under experimental conditions that allowed the formation of mineralised calcium phosphate deposits attained maximal values for cell proliferation by day 28. Cultures growing in the presence of βGP presented the lowest values for the MTT reduction and the additional presence of AA resulted in a slight increase in cell proliferation, especially from day 28 onwards, although, differences were not statistically significant.

Fig. 4. Cell viability/proliferation (A), total protein content (B) and ALP activity (C) of human bone marrow cell cultures grown under the following experimental conditions: (+) standard medium; (○) AA; (*) Dex; (▲) βGP; (●) βGP + AA; (■) βGP + AA + Dex; (dotted lines) cultures lacking the mineralisation process; (continuous lines) cultures that showed the formation of mineral calcium phosphate deposits; (*) significantly different from cultures grown in standard medium; the standard deviations were <20%.

Cultures grown in the presence of the three supplements presented a higher proliferation rate, the effect most probably related to the presence of Dex.

Results observed for total protein content in the various cultures followed a pattern similar to that observed for the MTT reduction, providing comparable information (Fig. 4B).

Results presented in Fig. 4A and B show that, as compared to cultures grown in standard medium, the presence of AA did not significantly influence cell growth but the presence of either Dex or βGP resulted in, respectively, an increase and a decrease in cell proliferation.

3.3. ALP activity

ALP activity is routinely used in in vitro experiments as a relative marker of osteoblastic differentiation [13,14]; the levels of this enzyme in the various cultures are shown in Fig. 4C.

Cultures grown in standard medium presented low levels of ALP during the first two weeks but, after that, activity of the enzyme increased until approximately day 35, decreasing afterwards. Cultures grown in the presence of AA presented similar levels of this enzyme throughout the incubation time.

Also, cultures grown in the presence of either βGP or AA + βGP presented a similar behaviour concerning ALP activity. As compared to cultures grown in standard medium, ALP presented the same pattern of variation, although, maximal values were lower and attained earlier (by day 28).

Results showed that cultures grown in the presence of Dex, i.e. cultures grown either in standard medium + Dex or in standard medium + AA + βGP + Dex, presented a significant induction in ALP activity, as compared to those growing in a similar medium lacking only the glucocorticoid (respectively, standard medium and standard medium + AA + βGP). ALP activity increased from the first week onwards and maximal values were higher and attained earlier (by day 21), decreasing significantly after that.

4. Discussion

Bone marrow tissue is known to contain osteogenic precursor cells [15,16]. Studies concerning the development of the osteoblast phenotype, from the osteoprogenitor proliferative cell to the osteocyte embedded in the extracellular matrix, suggest a temporal sequence of differentiation involving active cell proliferation, expression of osteoblastic markers, synthesis, deposition and maturation of a collagenous extracellular matrix and, matrix mineralisation [13,14,17]. Expression of the osteoblastic phenotype in culture depends on the composition of the culture medium, namely, the presence of compounds that influence cell proliferation and differentiation [5–8]. In this work, several in vitro culturing conditions were compared in order to maximise the induction and expression of osteoblastic markers in human bone marrow cell cultures. Cells were cultured in standard medium and in the presence of: (i) AA, (ii) βGP and (iii) Dex; cells were also cultured in the presence of two combinations of these compounds, namely, (iv) AA + βGP and (v) AA + βGP + Dex. Cultures growing in the various experimental situations were compared concerning cell growth (MTT reduction and total protein content), ALP activity and ability to form mineralised calcium phosphate deposits.

Cell growth and differentiation are known to depend in part on initial cell attachment [18]. Morphological changes occurring during the attachment and spreading of the cells correspond to the reorganisation of the cytoskeleton, the structure that plays a role in the control of the cell shape and behaviour [19]. Under the given experimental conditions, cell morphology changed from a fibroblastic (in the first days of culture) to a polygonal appearance (Fig. 1), suggesting a development in the stage of the osteoblastic differentiation [13,14].

Results reported in Figs. 2 and 3 showed that only the cultures grown in the presence of βGP, alone or in combination with other supplements, presented ability to form mineral deposits. βGP is routinely added to bone cell cultures to induce osteogenesis and promote calcium phosphate deposition [9,20–23]. The mechanism by which βGP induces mineralisation is closely linked to the high ALP activity of bone cell cultures; this compound is rapidly hydrolysed by ALP to produce high levels of local phosphate ions providing the chemical conditions for mineral deposition [20–23]. Previous studies performed with human bone cell cultures under similar experimental conditions to those reported in this work also showed that βGP is almost completely degraded to inorganic phosphate during the first two-three weeks of culture [24]. Several studies show that the addition of βGP to bone cell cultures induces osteogenesis but also affects cell growth parameters and/or ALP activity but data are not always consistent, probably due to differences in the cell systems and culture conditions [23].

The presence of dexamethasone, either alone or in combination, resulted in an evident increase in cell growth (MTT reduction and total protein content) (Fig. 4A and B). This compound also induced ALP activity: levels of the enzyme began to increase from the first week onwards and maximal values were significantly higher and attained earlier, as compared to cultures grown under similar experimental conditions but lacking glucocorticoid (Fig. 4C). In mineralising cultures, Dex increased the ability to form calcium phosphate deposits, as shown by the von Kossa reaction (Fig. 2) and observation of the cultures by SEM (Fig. 3C and D). These effects are most probably related to the ability of

glucocorticoids, near physiological concentrations, to stimulate the proliferation and/or the differentiation of osteoprogenitor cells resulting in an induction of the osteoblastic markers in bone cell cultures [4,9,10,17,25,26].

It is well known that AA plays an important role in the production of the collagenous bone extracellular matrix; some studies showed that the presence of various concentrations of this compound results in a dose-dependent synthesis of collagen and suggested that the resulting increase in the accumulation of the extracellular matrix is associated with a higher ALP activity and ability to form a mineralised matrix [13,17,27,28]. In the present work, bone marrow cells were cultured in α-MEM that already contains AA ($50\ \mu g\ ml^{-1}$) and, in this culture system, a further amount of AA in the medium apparently does not result in a significant effect on cell proliferation and ALP activity (Fig. 4A–C). However, as compared to cultures grown in the presence of βGP, the additional presence of AA resulted in a delay in the initiation of the mineralisation process (Fig. 2). This is probably related to differences in the balance accumulation/maturation of the collagenous matrix in the two experimental situations, as the amount, composition and maturation of the extracellular matrix affect bone cell differentiation [27,28].

Proliferation of osteoblastic cells is functionally related to the synthesis of a bone extracellular matrix and its accumulation and maturation, essential to the mineralisation process, contributes to the shutdown of proliferation [13,17,28,29]. In the presence of a source of phosphate ions, mineralisation does occur when an appropriate relationship between the amount of the extracellular matrix and its maturation is achieved; as a result, cell proliferation ceases with the osteoblasts being trapped and embedded in the mineralising matrix [23]. According to this model, there is a reciprocal and functionally coupled relationship between proliferation and differentiation [28,29]. The behaviour of human bone cell cultures (results presented in Figs. 2–4) appears to be in agreement with this model of osteoblastic differentiation. Cultures grown in the absence of a source of phosphate ions (βGP) showed a higher proliferation rate; these cultures presented osteoblastic features, namely, a high ALP activity, but complete expression of the osteoblast phenotype was not observed. However, in the presence of βGP further osteoblastic differentiation was achieved and the formation of a mineralised matrix contributed to a decrease in cell proliferation. As a result, these cultures presented a less proliferative but more differentiated osteoblastic cell population, as compared to those lacking the mineralisation process.

5. Conclusion

This work analysed the effect of AA, βGP and Dex on the proliferation/differentiation behaviour of human bone marrow cells cultured in α-MEM supplemented with 10% FBS. Cultures growing under all the experimental conditions tested presented high ALP activity, although, only the cultures grown in the presence of βGP showed the formation of a mineralised extracellular matrix, a key feature of the osteoblastic phenotype; the additional presence of Dex resulted in a significant induction of the two osteoblastic markers.

Human osteoblastic cultures with osteogenic ability may be regarded as a potential in vitro model to study biomaterial/bone tissue interactions, as the utilisation of bone replacement materials aims to induce the deposition of a mineralised collagenous matrix on the implanted material.

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