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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

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Abstract

Well-characterised human osteoblastic bone marrow cell cultures are a useful in vitro tool to analyse bone tissue/biomaterials interactions. In this work, human bone marrow was cultured in experimental conditions described to favour osteoblastic differentiation and, serially passaged cells were cultured in two widely used culture media, minimum essential medium Eagle, alpha modification (α-MEM) and Dulbecco's modified Eagle's medium (DMEM). Cultures were grown for 35 d and compared concerning morphologic appearance on scanning electron microscopy (SEM), cell viability/proliferation, total protein content, activity of alkaline phosphatase (ALP) and ability to form calcium phosphate deposits. Results showed that cell proliferation was similar in cultures grown in the two media but ALP activity and ability to form mineralised deposits were lower in DMEM cultures. In both experimental situations, osteoblastic parameters were strongly reduced on cell passage, particularly from the first to the second subculture. In the experimental conditions used (presence of ascorbic acid, sodium β-glycerophosphate and dexamethasone in the primary and secondary cultures), osteoblastic differentiation was observed in the first and second subcultures grown in α-MEM and in the first subculture grown in DMEM. These results underline the importance of the definition of the experimental conditions in studies involving bone cell cultures.

Keywords: Osteoblastic differentiation; Serial passage; Culture medium

1. Introduction

In the last years, research in dental and orthopaedic implants has focused on bone/biomaterials interactions, as long-term performance of materials requires direct and intimate contact between the biomaterial and the active biological environment [1].

Studies involving human and rodent bone cell cultures have contributed significantly to an understanding of the biological events occurring at the bone tissue/material interface [2–5]. These studies require a cell population with the relevant phenotype and, normally, large numbers of cells. Because of the small amount of biological material available to obtain the primary culture (particularly, of human origin), serial subculture is often performed in order to obtain a sufficient number of cells. As loss of the phenotype may be observed when diploid cells are cultured for a long time [6], phenotype evaluation during long-term culture (serial subculture) is essential to the selection of the appropriate cell population (subculture) to be used in in vitro studies [7,8].

The discovery that cells could be cultured and propagated in vitro led to attempts to provide more defined media to sustain continuous cell growth. Even after many years of research into matching particular media to specific cell types and culture conditions, the choice of the medium is not obvious and is often empirical [9]. Expression of a particular cell phenotype in culture depends on the biological material used (and its manipulation) and the culture conditions, namely, culture medium, culture time and the presence of compounds that influence cell proliferation and differentiation [10]. This is a matter of particular concern considering the diversity and multiplicity of methodologies described in the literature, which leads to

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difficulty in interpretation and comparison of results obtained in similar studies.

In this work, human bone marrow was cultured in experimental conditions described to favour osteoblastic differentiation and serially passaged cells were cultured in two widely used culture media, minimum essential medium Eagle, alpha modification (α-MEM) and Dulbecco's modified Eagle's medium (DMEM). These media have different characteristics, namely, DMEM is a less nutrient-rich medium with respect to aminoacids and vitamins, although, nutrient concentrations are, on the whole, higher than those found in α-MEM. Cultures growing in the two media were compared concerning morphologic appearance on scanning electron microscopy (SEM), cell viability/proliferation, total protein content, activity of alkaline phosphatase (ALP) and ability to form calcium phosphate deposits.

2. Materials and methods

2.1. Cell culture

Human bone marrow (obtained from surgery procedures, patients aged 30–60 yr) was cultured in α-MEM (SIGMA M 0894) and also in DMEM (SIGMA D 5523), both containing 10% foetal bovine serum (FBS), 2.5 μg/ml fungizone and 50 μg/ml gentamicine and supplemented with 50 μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate and 10 nM dexamethasone. Primary cultures were maintained until near confluence (approximately, 10–15 d).

Starting on primary culture, cells were serially passaged on the exponential cell growth phase. Adherent cells were enzymatically released (0.04% trypsin and 0.025% collagenase), counted using a haemocytometer, seeded at a density of 10^4 cells/cm^2 and cultured under the same experimental conditions as those used in the primary cultures. First to third passage cells were cultured for periods up to 35 d in both culture media (α-MEM and DMEM) and monitoring of the cultures was done daily using phase contrast inverted microscopy.

Cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 and culture medium was changed twice a week.

Subcultures were characterised at days 1, 7, 14, 21, 28 and 35 for cell viability/proliferation, total protein content and ALP activity (biochemical assays), ability to form calcium phosphate deposits (histochemical assays) and were observed by SEM.

2.2. Culture characterisation

2.2.1. Biochemical assays

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 of MTT in the last 4 h of the culture period tested; the medium was then decanted, Formozan salts were dissolved with dimethylsulphoxide and the absorbance was determined at 600 nm in an ELISA reader.

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 cm^2 (nmol/min cm^2).

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 μg/cm^2.

2.2.2. Histochemical assays

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

ALP staining. Fixed cultures were incubated during 1 h in the dark with a mixture, prepared in Tris buffer pH = 10, containing 2 mg/ml of Na-α-naphtyl phosphate and 2 mg/ml of fast blue RR salt; the incubation was stopped by rinsing the samples with water. The presence of ALP was identified by a brown to black stain.

Phosphate staining. 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.

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) [12]. Calcium deposits stained red.

2.2.3. Scanning electron microscopy

For SEM observation, cell cultures were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate (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 microanalysis capability, Voyager XRMA System, Noran Instruments.

2.2.4. 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 yr). In each experiment, the comparison of cultures grown in the two culture media was

established from the same donor. Student t-test was applied to determine the statistical significance of the differences observed between groups: P values $\leqslant 0.05$ were considered significant.

Results showed that, among donors, the behaviour of the cultures grown in the two culture media followed a similar pattern.

3. Results

Serially passaged human bone marrow cells were cultured in α-MEM and in DMEM in the presence of ascorbic acid, sodium β-glycerophosphate and dexamethasone, experimental conditions that favour the development of osteoblastic cells in several bone cell systems [13–20].

3.1. Cell viability/proliferation and total protein content

Subcultured cells growing in the two culture media presented a similar pattern of cell proliferation (Fig. 1). First passage cells proliferated gradually with incubation time and during the exponential cell growth, values for the MTT reduction were higher in cultures grown in DMEM, but maximal values were similar in the two experimental situations and were observed by the end of the third week, decreasing significantly after that (Fig. 1A).

As compared to the first subculture, a different pattern on cell growth was observed in the second and third subcultures, however, maximal values for MTT reduction were similar in the three subcultures (Fig. 1B, C). In DMEM cultures, maximal values for MTT reduction were observed later, by the end of the fourth week, decreasing during the fifth week (only a slight decrease was observed in the third subculture). In α-MEM cultures, cells from the second and third passages proliferated throughout the incubation period.

Total protein content (Fig. 2) of cultures grown in the two culture media followed a pattern similar to that observed for the MTT reduction, providing comparable information.

3.2. ALP activity

Results concerning ALP activity are shown in Fig. 3.

First passage cells (Fig. 3A) presented low ALP activity during the first week in both culture media. In α-MEM cultures, levels of the enzyme increased significantly during the second week, attaining maximal values around day 14 and decreased after that; also, in DMEM cultures, ALP activity increased during the second week, reached a plateau during the third week, decreasing afterwards. ALP activity was significantly lower in cultures growing in DMEM, particularly around day 14.

For cells growing in the two culture media, serial passage resulted in a decrease in ALP activity, especially from the first to the second subculture (Fig. 3B); in addition, variation of ALP activity with the incubation time followed a different pattern. In α-MEM cultures, second passage cells reached maximal levels of the enzyme later (15.01 ± 2.66 nmol/min cm^2, around day 21, compared with 49.73 ± 3.93 nmol/min cm^2 at day 14, in the first subculture) and remained approximately constant until the end of the incubation period; the third subculture presented even lower ALP activity (Fig. 3C). In DMEM cultures, very low levels of ALP were detected in the second and third subcultures and, as observed in the first subculture, levels of the enzyme were significantly lower than those observed in α-MEM cultures (Fig. 3B, C).

3.3. Histochemical assays

Subcultured cells were stained for the presence of ALP and calcium phosphate deposition.

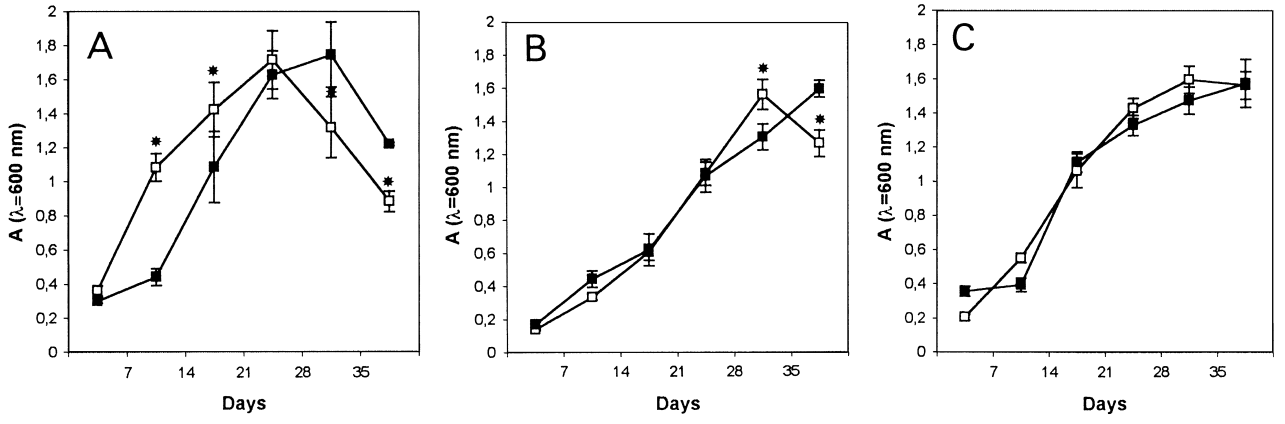

Fig. 1. Cell viability/proliferation (MTT assay) of serially passaged human bone marrow cells cultured in α-MEM (■) and in DMEM (□). First (A), second (B) and third (C) subcultures. * Significantly different from cultures grown in α-MEM.

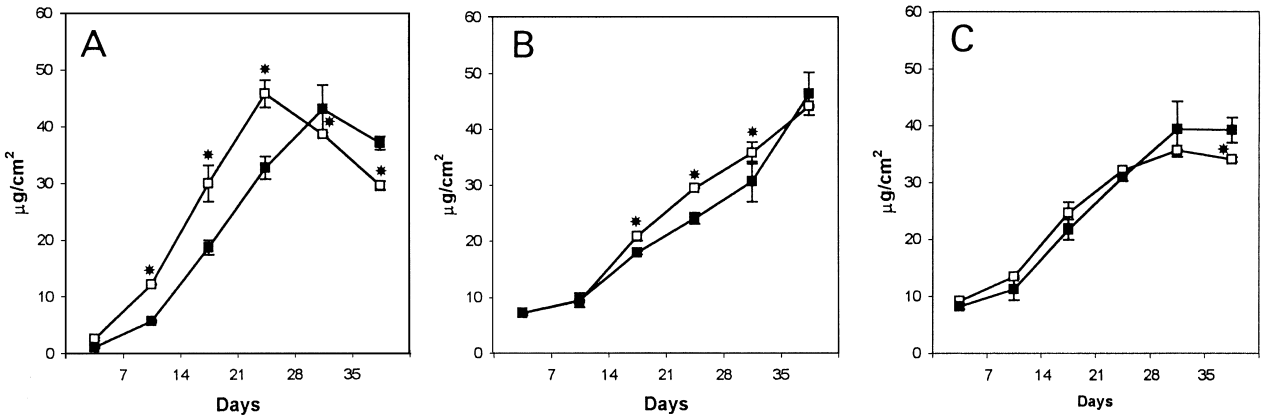

Fig. 2. Total protein content of serially passaged human bone marrow cell cultures grown in α-MEM (■) and in DMEM (□). First (A), second (B) and third (C) subcultures. * Significantly different from cultures grown in α-MEM.

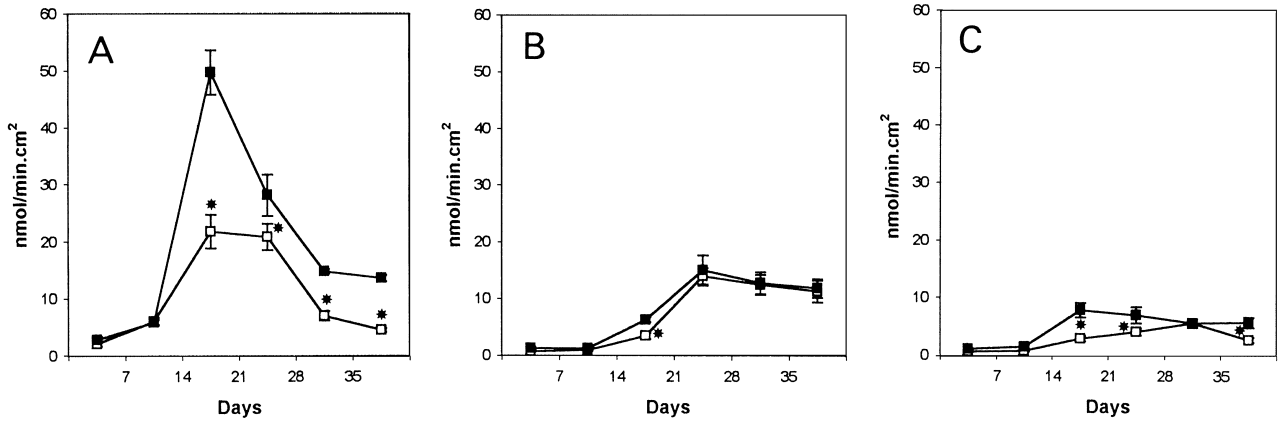

Fig. 3. ALP activity of serially passaged human bone marrow cell cultures grown in α-MEM (■) and in DMEM (□). First (A), second (B) and third (C) subcultures. * Significantly different from cultures grown in α-MEM.

Cultures stained for ALP in the tested days showed results that were in agreement with those observed in the biochemical determination of the enzyme and are not shown. Histochemical staining of the cultures by the Alizarin red assay and von Kossa method gave the same kind of information and the results concerning 21, 28 and 35 days cultures are summarised in Table 1. Cultures grown in α-MEM appeared to have more favourable conditions for calcium phosphate deposition; in the first subculture staining was already positive at day 21 and 28 and 35 days cultures presented strong positive reactions, whereas, in the first subculture grown in DMEM, mineral deposits were detected later, only around day 28, and the intensity of the staining was lower than that observed in α-MEM cultures (even in 35 days cultures).

Serial passage resulted in a decreased formation of mineral deposition. A positive histochemical reaction was observed only in the second subculture grown in α-MEM and staining was weaker and detected later than in the first subculture.

Table 1
Histochemical reaction for calcium phosphate deposits (von Kossa assay) in 21, 28 and 35 days serially passaged human bone marrow cell cultures grown in α-MEM and in DMEM[a]

Subculture	21 days		28 days		35 days	
	α-MEM	DMEM	α-MEM	DMEM	α-MEM	DMEM
1st	+	−	++	+	+++	++
2nd	−	−	+	−	++	−
3rd	−	−	−	−	−	−

[a]Intensity of staining was graded as follows: −, negative staining; +, definite staining but of low intensity; ++, moderate staining; +++, intense staining.

Fig. 4 compares the appearance of the first and the second subcultures grown for 28 days in the two culture media and stained for calcium phosphate deposits (von Kossa assay).

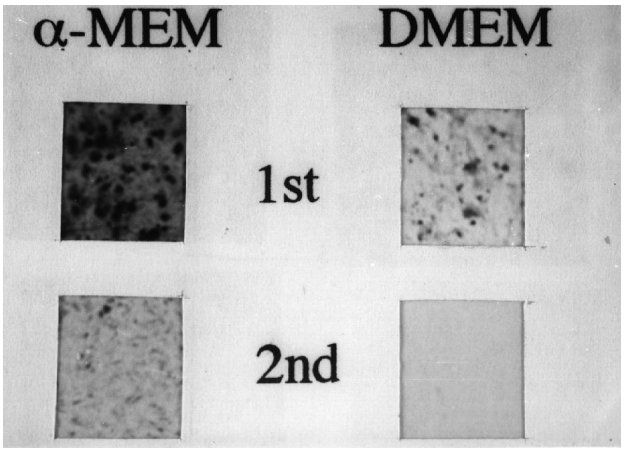

Fig. 4. Microscopic appearance of the first and the second subcultures grown for 28 days in α-MEM and in DMEM and stained for calcium phosphate deposits (von Kossa assay) (20×).

3.4. Scanning electron microscopy

SEM observation of the cultured cells showed that proliferation of bone marrow cells was accompanied by the production of a fibrillar extracellular matrix, in both culture media. In α-MEM cultures, the presence of mineralised deposits was evident in 21 day cultures and 28 and 35 day cultures presented numerous globular structures, shown by X-ray microanalysis to contain Ca and P; this aspect is in accordance with other previously reported studies in this culture system [21].

In agreement with the histochemical results, the presence of mineral deposits was observed later in cultures grown in DMEM, only around day 28; in addition, mineral deposition was significantly lower than that observed in the α-MEM first subculture. Fig. 5 compares the appearance of 28 days first subcultures grown in the two culture media and also shows the X-ray spectrum of mineralised areas present in α-MEM first subculture.

SEM observation of the cultures also showed that the abundance of the mineral structures decreased with culture passage; the presence of mineral deposits was still observed in the second subculture grown in α-MEM, but other subcultures showed a structure formed by cells and a fibrillar extracellular matrix with a continuous

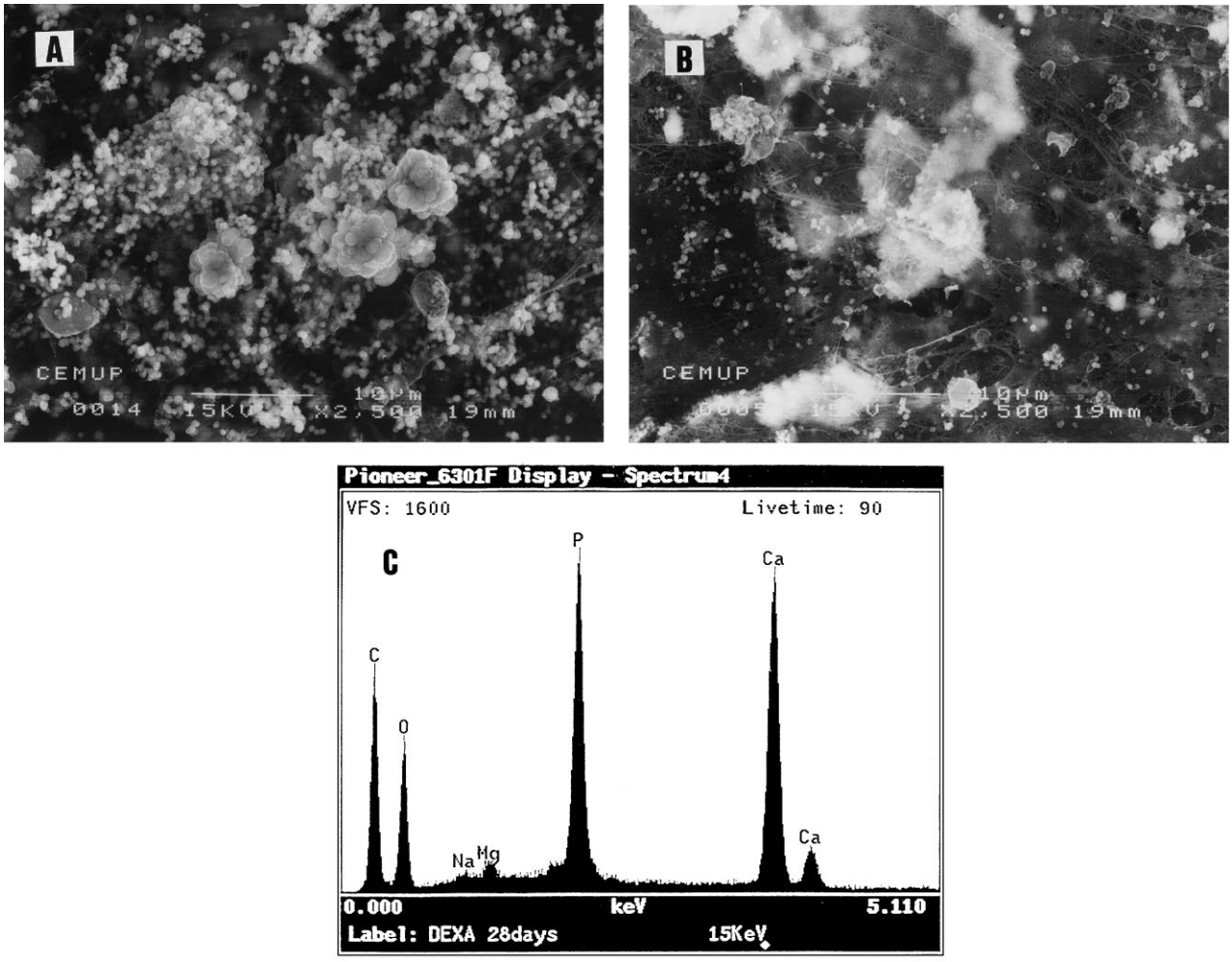

Fig. 5. SEM appearance of 28 days first subcultures grown in (A) α-MEM and (B) DMEM (C) Energy-dispersive X-ray spectrum of mineral spherules present in α-MEM cultures.

Fig. 6. SEM appearance of 28 days third subculture grown in α-MEM.

appearance, as can be observed in Fig. 6, showing a 28 days third subculture grown in α-MEM.

4. Discussion

In this work, serially passaged osteoblastic human bone marrow cells were cultured in α-MEM and in DMEM and compared concerning cell growth and differentiation.

Cell proliferation of subcultured cells was similar in cultures grown in the two culture media, as shown by the results obtained for the MTT reduction and total protein content (Figs. 1 and 2). In the first subculture, active proliferation was observed during approximately three weeks, a process that is functionally related to the synthesis of a bone-specific extracellular matrix. Afterwards, cell growth decreased significantly, most probably as a result of the accumulation, maturation and mineralisation of the extracellular matrix [22–26]. In this stage of the culture, osteoblasts became embedded in the mineralising matrix and were unable to proliferate. It is worthwhile to mention that during the first two weeks, cell growth was significantly higher in cultures growing in DMEM (especially during the first week) (Fig. 1A). The second and the third subcultures presented a different pattern on cell proliferation with the incubation time, i.e, a tendency for a continuous cell growth was observed in α-MEM cultures and, in DMEM cultures, only a small decrease was observed in the last week of culture. However, maximal values for MTT reduction (and also total protein content) were similar in the three subcultures growing in the two culture media.

Results concerning ALP activity showed that, both the culture medium and also serial cell passage affected the levels measured in the cell cultures. In the first subculture, levels of the enzyme were low in the first week but increased significantly during the second week, reaching maximal levels around day 14 in α-MEM cultures and at days 14–21 in DMEM cultures, decreasing after that (Fig. 3A). The significant increase in the ALP levels during the second week suggested that the cells were shifting to a more differentiated stage, as the level of this enzyme has been routinely used in in vitro experiments as a relative marker of osteoblastic differentiation [23,24,26,27]. Formation of mineral deposits occurred following the maximal ALP activity, earlier in α-MEM cultures and approximately one week later in DMEM cultures, as shown by the histochemical results (Table 1) and also SEM observation of the cell cultures. ALP has long been associated with biological calcification and an enhanced expression of this enzyme is apparently needed just before the onset of matrix mineralisation, providing localised enrichment of inorganic phosphate for hydroxyapatite crystal nucleation and proliferation [23,24,28,29]. Comparison of the results presented in Fig. 2 and in Table 1 showed that ALP activity decreased significantly in mineralised cultures, similar to that reported in other bone cell culture systems [23,24,28]. Previous work has shown that rat and human bone marrow cells grown in these experimental conditions and in α-MEM presented a high ability to hydrolyse the β-glycerophosphate present in the culture medium, reflected by the high levels of inorganic phosphate measured in the medium during the second week of incubation [21,30,31], most probably by ALP known to have a high efficacy in hydrolysing this substrate [28,29,32].

Serial subculture resulted in a decrease in the ALP activity and also in the ability to form mineralised deposits, both in cultures grown in α-MEM and in DMEM. Considering that cell proliferation was not significantly affected by subcultivation and that ALP activity has been regarded as an in vitro marker of the osteoblastic differentiation, this observation suggested that serial passage results in the presence of a cell population progressively less differentiated concerning the osteoblastic phenotype. This is probably related with a progressive selection of a more proliferative cell population on serial culture passage [33], therefore in an earlier state of differentiation [23,24,26]; hence, the effective density of osteoblast competent cells may have decreased upon subcultivation [15]. This hypothesis is suggested by the results observed for cell proliferation (Fig. 1), i.e., a tendency for a continuous cell growth on serial passage and also that the maximal ALP activity tends to be observed later (around day 21) and remained constant after that. The ability to form mineralised deposits also decreased significantly on serial passage. The second subculture grown in α-MEM presented a weak positive staining for calcium phosphate deposits and a more dispersive mineralisation pattern of the extracellular matrix was observed; these cultures were slightly mineralised and, accordingly, ALP activity did

not decrease significantly after the onset of mineralisation (Figs. 3 and 4). Formation of mineral deposits could not be demonstrated in the third subculture grown in α-MEM and also in the second and third subcultures grown in DMEM.

Results presented in Figs. 1 and 2 showed that similar cell proliferation was observed in the cultures growing in the two culture media. ALP activity was significantly lower in DMEM first subculture (Fig. 3A). Also, in the cultures grown in DMEM, formation of mineral deposits occurred later and were observed only in the first subculture (Table 1). In both culture media, ALP activity was very low in the second and third subcultures (Fig. 3B and C). Differences in the behaviour of the cell cultures grown in the two culture media were specially evident in the first subculture. By contrast, the second and third subcultures presented a similar behaviour in the two culture media. As serial passage results in the selection of a less differentiated cell population [33], this would contribute to a decreased heterogeneity of cells in different stages of differentiation, which most probably results in an attenuation of the differences in the behaviour of the cell cultures grown in different experimental conditions, namely the culture medium.

The results of this work showed that different nutrient-containing media were found to affect expression of the osteoblastic parameters. DMEM is a less nutrient-rich medium, particularly with respect to aminoacids and vitamins, although, nutrient concentrations are, on the whole, higher than those found in α-MEM and this probably explains the higher values for cell proliferation found in DMEM first subculture during the exponential cell growth, as compared with those observed in α-MEM first subculture. As cell differentiation appears to be affected, an important difference between the two media is, probably, that α-MEM contains ascorbic acid (0.05 g/l), therefore, α-MEM and DMEM cultures were grown in the presence of, respectively, 100 and 50 μg/ml of this compound. Ascorbic acid (ascorbate) is an enzyme cofactor and antioxidant that stimulates the transcription, translation and post-translational processing of collagen in connective tissue cells [34]. In cultures of bone-derived cells, ascorbate stimulates osteoblastic differentiation, synthesis and deposition of collagen as well as mineralisation [22,24,26,35]; a series of studies in which cells were cultured at various concentrations of ascorbic acid showed a dose-dependent synthesis of collagen and that the resulting increase in the accumulation of the extracellular matrix was associated with an higher ALP activity and ability to form a mineralised matrix [22]. These and several others studies suggest that the development of an appropriate extracellular matrix is related to the osteoblastic differentiation [22–26]. As serial passage appears to contribute to the selection of a less differentiated cell population, the decrease in the osteoblastic parameters observed in the second and third subcultures, namely, a lower ALP activity and a decreased ability to form mineralised deposits, may be related with the formation of an inadequate extracellular matrix (in quantity and quality). This process is more significant in DMEM cultures which appears to correlate with the fact that, in this situation, cultures were grown in the presence of a lower concentration of ascorbic acid.

5. Conclusions

The present study showed that subcultivated human bone marrow cells cultured in α-MEM and in DMEM were able to proliferate and differentiate, although, ALP activity and ability to form mineral deposits were lower in DMEM cultures. In addition, osteoblastic parameters were strongly reduced on cell passage, particularly from the first to the second subculture. In the experimental conditions used (presence of ascorbic acid, sodium β-glycerophosphate and dexamethasone in the primary and secondary cultures), osteoblastic differentiation was observed in the first and second subcultures grown in α-MEM and in the first subculture grown in DMEM. Considering that human osteoblastic bone marrow cell cultures are used as an in vitro model to study biological events occurring at the bone cells/biomaterial interface, these results underline the importance of the definition of the experimental conditions in these studies.

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