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Osteoblast Differentiation of Human Bone Marrow Cells Under Continuous and Discontinuous Treatment with Dexamethasone

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Dexamethasone (Dex) has been shown to induce osteoblast differentiation in several cell culture systems. This study investigated the effect of continuous and discontinuous treatment with Dex on osteoblast differentiation of human bone marrow stromal cells (BMSC). Primary culture and first passage were cultured in media with or without Dex 10^{-7} M. During the culture period, cells were incubated at 37ºC in humidified atmosphere of 5% CO_2 and 95% air. At 7, 14, and 21 days, cell proliferation, cell viability, total protein content, alkaline phosphatase (ALP) activity and bone-like formation were evaluated. Data were compared by two-way analysis of variance. Dex did not affect cell viability and total protein content, but reduced cell number. ALP activity and bone-like formation increased when only first passage or both primary culture and first passage were treated with Dex, in comparison to the groups that did not have contact with Dex after first passage. The results of this study indicate that, for human BMSC, continuous presence of Dex did not appear to be required for development of the osteoblast phenotype, but Dex must be present after first passage to allow osteoblast differentiation expressed by reduced cell proliferation and increased ALP activity and bone-like formation.

Key Words: bone-like formation, cell culture, cell differentiation, glucocorticoid, osteoblast phenotype.

INTRODUCTION

Bone formation requires differentiated and active osteoblasts to synthesize the extracellular matrix that will support the mineralizing process. Bone marrow stromal cells (BMSC) have been used as suitable *in vitro* model to study some aspects of bone formation and the interactions between bone cells and biomaterials used in orthopedic and maxillofacial surgeries (1,2). Such cultures should present proliferative and differentiation patterns representative of the *in vivo* osteoblast population, namely, a high alkaline phosphatase (ALP) activity and ability to produce mineralized bone matrix (3).

To obtain bone cell cultures with defined and reproducible osteoblast characteristics, appropriate experimental procedures should be selected. There are many factors that influence the expression of the osteoblast phenotype in culture, i.e. cell source, culture medium, culture time and the presence of compounds that influence cell proliferation and differentiation (4). Several systemic and local hormones, growth factors and cytokines have been shown to be involved in osteoblast differentiation process (5).

Previous studies have suggested that glucocorticoids can cause *in vivo* bone loss by two mechanisms: suppression of bone formation and stimulation of bone resorption (6). Data regarding the effects of glucocorticoids on osteoblast activities *in vitro,* however, are conflicting. They appear to depend upon whether physiological or pharmacological concentrations of the hormone are used, time and duration of exposure to the drug, and the specific system investigated (7). In osteoblast-like cell cultures, glucocorticoids decrease cell proliferation but increase ALP activity (8). The effects of glucocorticoids on

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collagen expression are controversial, as both stimulation and inhibition have been reported depending on the cell source and culture conditions (9,10). Despite this, it has been well documented that BMSC, including actively proliferating osteoprogenitors, can be induced to differentiate into cells exhibiting osteoblast phenotype by dexamethasone (Dex), a synthetic glucocorticoid, at both 10^{-7} M and 10^{-8} M concentrations (11,12).

Although the range of Dex concentration is well established, little is known about how long the treatment with glucocorticoids should be to induce osteoblast differentiation from BMSC. Moreover, up to date, the stage of cell development at which exposure to Dex is required in the process of osteoblast differentiation has not yet been investigated. In view of this, we hypothesized that both duration of treatment and stage of cell development would influence the effect of Dex on osteoblast differentiation. To test this hypothesis, human BMSC were cultured in continuous and discontinuous presence of Dex and the following parameters were evaluated: cell proliferation, cell viability, total protein content, ALP activity and bone-like formation.

MATERIAL AND METHODS

Cells were obtained according to research protocols approved by the Brazilian National Commission of Ethics in Research for human tissue specimens, by iliac aspiration of healthy male bone marrow donors aged 24 and 33 years. The cells were cultured in α-MEM (Gibco - Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco), 50 µg/mL gentamicin (Gibco), 0.3 µg/mL fungizone (Gibco), 5 µg/L ascorbic acid (Gibco) and 7 mM â-glycerophosphate (Sigma, St. Louis, MO, USA). Two experimental tracks were followed. In track 1, cells were seeded in two culture flasks of 75 cm^2 (Nunc, Copenhagen, Denmark) and the added medium was supplemented with dexamethasone (+Dex) at 10^{-7} M (Sigma). In track 2, a similar procedure was followed. However, the cells were provided with medium that did not contain dexamethasone (-Dex). Subconfluent cells in the primary culture were harvested after treatment with 1 mM EDTA (Gibco) and 0.25% trypsin (Gibco) and the first passage were subcultured in 24-well culture plates (Falcon, Franklin Lakes, NJ, USA) at a cell density of 2 x 10^4 cells per well. Cells in the first passage were also cultured in media either supplemented with (+Dex) or not containing (-Dex) dexamethasone at 10^{-7} M concentration. Therefore, cells were cultured in four groups, according to Dex supplementation in both the primary culture and the first passage: +/+Dex; +/-Dex; -/+Dex; -/-Dex. Protocol for cell culture is shown in Figure 1. During the entire culture period, cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2 and 95% air, and the medium were changed every 3 or 4 days.

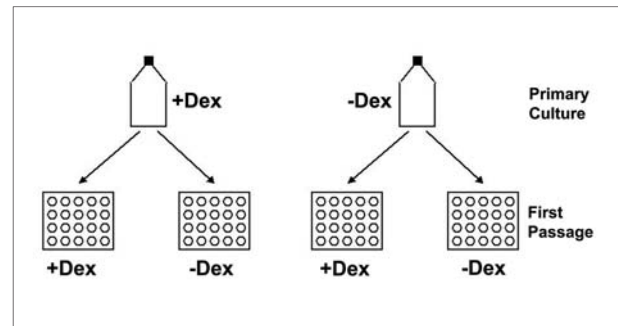

Figure 1. Schematic representation of the cell culture protocol. Human BMSC were isolated and cultured in absence or presence of Dexamethasone (Dex) during primary culture and first passage. Four groups were evaluated: +/+Dex; +/-Dex; -/+Dex; -/-Dex.

Cell Proliferation. For proliferation assessment, cells were cultured for 7, 14 and 21 days, enzymatically released (1 mM EDTA, 1.3 mg/mL collagenase and 0.25% trypsin; Gibco) and counted using a hemacytometer.

Cell Viability. For cell viability, aliquots of the same solutions used for calculating cell number were assayed. These aliquots were incubated for 5 min with 1% trypan blue (v/v) (Sigma) and non-viable cells were counted using a hemacytometer. Cell viability was expressed as percentage of viable cells counted at 7, 14, and 21 days, respectively.

Total Protein Content. Total protein content was calculated at 7, 14, and 21 days in culture, according to modified Lowry method (13). The wells were filled with 2 mL of 0.1% sodium lauryl sulfate (Sigma) and, after 30 min, 1 mL of this solution from each well was mixed with 1 mL of Lowry solution (Sigma) and left for 20 min at room temperature. Thereafter, it was added to 0.5 mL of the phenol reagent of Folin and Ciocalteau (Sigma) and left at room temperature for 30 min. Absorbance was then measured (CE3021; Cecil, Cambridge, UK) at 680 nm and total protein content was calculated from a

standard curve. Data were normalized by the number of cells counted at 7, 14, and 21 days, respectively and expressed as µg protein/10^4 cells.

ALP Activity. ALP activity was evaluated as the release of tymolphtaleine from tymolphtaleine monophosphate using a commercial kit (Labtest Diagnostica, Belo Horizonte, MG, Brazil). Aliquots of the same solutions used for calculating total protein content were assayed and specific activity was calculated. Absorbance was measured at 590 nm and ALP activity was calculated from a standard value. Data were normalized by the number of cells counted at 7, 14, and 21 days, respectively, and expressed as µmol tymophtaleine/h/10^4 cells.

Bone-like Formation. At 7, 14, and 21 days, attached cells were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h at room temperature and rinsed once in the same buffer. After fixation, the specimens were dehydrated through a graded series of alcohol and processed for staining with Alizarin red S (Sigma). The specimens were evaluated using an image analyzer (Image Tool; University of Texas Health Science Center, San Antonio, TX, USA) and the amount of bone-like formation was calculated as a percentage of well area.

Data from this study are the representative results of two separate experiments in cell cultures established from two different bone marrow donors. For each parameter, evaluations were carried out in quintuplicate (n=5). All data were submitted to two-way analysis of variance (ANOVA) and Duncan's multiple range test, when applicable. Differences at $p \leq 0.05$ were considered statistically significant.

RESULTS

Cell number was affected by Dex (ANOVA: df=3; F=5.68; p=0.002) in the following order: +/+Dex = -/+Dex < -/-Dex < +/-Dex. Moreover, cell growth was a time-dependent process (ANOVA: df=2; F=54.95; p=0.00001) being greater after 21 days (Fig. 2). Cell viability was not affected by either Dex (ANOVA: df=3; F=0.98; p=0.41) or period of culture (ANOVA: df=2; F=0.01; p=0.92). Total protein content was not affected

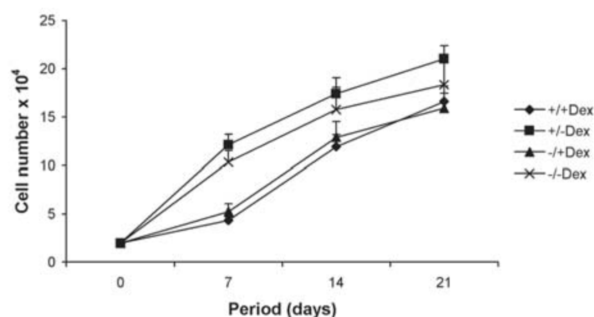

Figure 2. Proliferation of human BMSC at 7, 14, and 21 days. Data are reported as mean ± standard deviation (n=5).

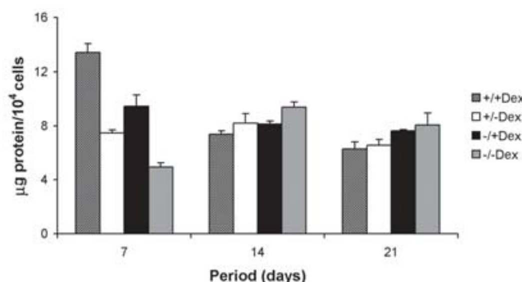

Figure 3. Total protein content normalized by the number of cells (µg protein/10^4 cells) counted at 7, 14 and 21 days. Data are reported as means ± standard deviation (n=5).

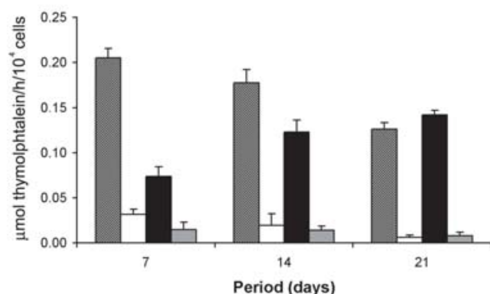

Figure 4. Alkaline phosphatase (ALP) activity normalized by the cell number (µmol tymolphtaleine/h/10^4 cells) at 7, 14 and 21 days. Data are reported as means ± standard deviation (n=5).

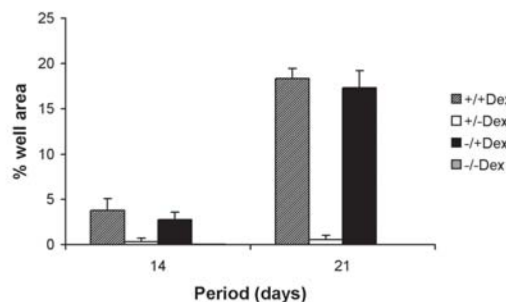

Figure 5. Bone-like formation expressed as percentage of total well area at 14 and 21 days. Data are reported as mean ± standard deviation (n=5).

by Dex (ANOVA: df=3; F=2.23; p=0.094), but it was instead affected by period of culture (ANOVA: df=2; F=3.63; p=0.032), as follows: 21 days = 14 days < 7 days (Fig. 3). ALP activity was affected by Dex (ANOVA: df=3; F=137.68; p=0.00001) in the following order: -/-Dex = +/-Dex < -/+Dex < +/+Dex, but it was not affected by period of culture (ANOVA: df=2; F=0.17; p=0.84) (Fig. 4). After the 7th day in culture, there was no evident bone-like formation in any of the study group, thus only data from the 14th and 21st days of evaluation were included in statistical analysis. Bone-like formation was affected by Dex (ANOVA: df=3; F=11.70; p=0.00001) in the following order: -/-Dex = +/-Dex < -/+Dex = +/+Dex. Bone-like formation process increased in a time-dependent way (ANOVA: df=1; F=457.97; p=0.00001) being greater after 21 days (Fig. 5).

DISCUSSION

Cells isolated from bone marrow provide an excellent source of proliferative osteoprogenitor cells that can be induced to differentiate into osteoblasts. Studies concerning the development of osteoblast phenotype, from osteoprogenitor proliferative cells to osteocytes embedded in the extracellular matrix, suggest a temporal sequence of events involving active cell proliferation, expression of osteoblast markers, synthesis, deposition and maturation of a collagenous extracellular matrix, and matrix mineralization (14). The present study was designed to investigate how continuous and discontinuous treatment with Dex could affect the process of osteoblast differentiation. It has been shown that when human BMSC are treated with Dex either continuously (+/+Dex) or only after first passage (-/+Dex) they differentiate into osteoblasts. This was demonstrated by the expression of markers characteristic of the osteoblast phenotype, such as ALP activity and bone-like formation. Moreover, osteoblast differentiation did not occur in absence of Dex, mainly after first passage.

Regarding the relationship between cell growth and phenotypic expression during osteoblast differentiation, Owen et al. (15) observed that there is a reciprocal relationship between the decrease in proliferation and the subsequent induction of cell differentiation in rat calvarial cell cultures. Therefore, the decrease in cell number caused by Dex in both groups +/+Dex and -/+Dex, as noted in this study, seems to be related to the progression of differentiation of BMSC into mature osteoblasts. In this regard, our results differ from those using rat BMSC, in which the presence of Dex stimulated cell proliferation and differentiation (16). It is not known whether these differences indicate that BMSC derived from rats and humans behave differently or simply reflect differences in isolation procedures and culture conditions, i.e. the initial cell number, fetal serum used or Dex concentration (either 10^{-8} M or 10^{-7} M, for example). The methods used for assessing cell proliferation should also be considered. While Atmani et al. (16) used an indirect method, 6-h [methyl-^{3}H] thymidine incorporation, a direct cell counting method was used in this study. In spite of reducing cell proliferation, Dex did not affect cell viability, as observed by trypan blue staining.

Dex exposure did not result in an increase in the cell synthesis activity. Considering that the amount of protein was normalized by the number of cells, these results evaluated the cell secretory activities that were not stimulated by Dex. However, the lack of bone-like formation in the absence of Dex suggests that the composition of protein produced when cells were treated with Dex was different and such proteins were more related to the process of matrix mineralization.

Human BMSC show an increase of ALP activity in presence of Dex, with maximal effects at concentrations ranging from 10^{-7} M to 10^{-6} M (3). In the present study, when only first passage (-/+Dex) or both primary culture and first passage (+/+Dex) were treated with Dex, the ALP activity was enhanced to values far greater than those observed in the other groups that did not have contact with Dex after the first passage. The findings of this experiment also showed that Dex exposure of the primary culture exerts a crucial role in the early development of osteoblast phenotype, as revealed by greater ALP activity in the earlier period (7 days) of evaluation. In addition, the absence of Dex in the primary culture caused a delay in osteoblast differentiation, as the treatment with Dex only after first passage (-/+Dex) produced a significant ALP activity in the later periods, 14 days and 21 days.

In a comparable fashion of ALP activity, our results indicate that Dex plays a critical role in the process of matrix mineralization. No bone-like formation occurred in absence of dexamethasone (-/-Dex), in agreement with Ter Brugge and Jansen (17), who also

reported lack of matrix mineralization in rat BMSC untreated with Dex.

In this investigation, cells presenting significant ALP activity also produced mineralized matrix, which indicates a relationship between both markers of osteoblast differentiation. These results are consistent with those by Sugawara et al. (18), who showed that ALP enzymatic activity is essential for mineralization process of osteoblastic cells because bone-like formation did not occur in cells cultured in presence of tetramisole, an inhibitor of ALP activity. Nevertheless, the positive correlation between ALP activity and bone-like formation, which has been supported by other authors (19) and is suggestive that greater ALP activity would imply in more bone-like formation, was not observed here. It may be speculated that there would be a specific level of ALP activity that is required to produce matrix mineralization. Once this level is reached, an increase in ALP activity does not result in a corresponding increase in bone-like formation.

Dex bone stimulator effect has been documented *in vitro* in different cell culture systems. Results on the length of Dex exposure necessary to promote osteoblast differentiation, however, are conflicting. Together, the findings of this study indicated that, for human BMSC, continuous presence of Dex did not appear to be required for development of osteoblast phenotype. However, Dex must be present at least after first passage to allow osteoblast differentiation, expressed by reduced cell proliferation and increased ALP activity and bone-like formation. Such culture conditions should be considered in studies on both bone biology and bone biomaterials interactions.

RESUMO

A dexametasona (Dex) induz diferenciação osteoblástica em diversos modelos de cultura de células. Este estudo investigou o efeito do tratamento contínuo e descontínuo com Dex sobre a diferenciação de células de medula óssea humana (BMSC). Células da cultura primária e da primeira passagem foram cultivadas em meio de cultura com e sem Dex 10^{-7} M (37ºC e 5% CO_2 / 95% ar atmosférico). Aos 7, 14 e 21 dias, os seguintes parâmetros foram avaliados: proliferação e viabilidade celulares, conteúdo de proteína total, atividade de fosfatase alcalina (ALP) e formação de matriz mineralizada. Os dados foram comparados por análise de variância a dois critérios. A Dex não afetou a viabilidade celular e o conteúdo de proteína total, mas reduziu o número de células. A atividade de ALP e a formação de matriz mineralizada foram aumentadas quando apenas a primeira passagem ou cultura primária e primeira passagem foram tratadas com Dex, em comparação aos grupos que não tiveram contato com Dex após a primeira passagem. Estes resultados indicam que, para BMSC humanas, a presença contínua de Dex não parece ser necessária para o desenvolvimento do fenótipo osteoblástico. Contudo, a Dex deve estar presente após a primeira passagem para permitir a diferenciação osteoblástica expressa por proliferação celular reduzida e aumento da atividade de ALP e da formação de matriz mineralizada.

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