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ARTICLES

Effect of Dexamethasone Withdrawal on Osteoblastic Differentiation of Bone Marrow Stromal Cells

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Abstract Dexamethasone is capable of directing osteoblastic differentiation of bone marrow stromal cells (BMSCs) in vitro, but its effects are not lineage-specific, and sustained exposure has been shown to down-regulate collagen synthesis and induce maturation of an adipocyte subpopulation within BMSC cultures. Such side effects might be reduced if dexamethasone is applied in a regimented manner, but the discrete steps in osteoblastic maturation that are stimulated by dexamethasone are not known. To examine this, dexamethasone was added to medium to initiate differentiation of rat BMSCs cultures and then removed after a varying number of days. Cell layers were analyzed for cell number, rate of collagen synthesis, expression of osteocalcin (OC), bone sialoprotein (BSP) and lipoprotein lipase (LpL), and matrix mineralization. Withdrawal of dexamethasone at 3 and 10 days was found to enhance cell number relative to continuous exposure, but did not affect to decrease collagen synthesis slightly. Late markers of osteoblastic differentiation, BSP expression and matrix mineralization, were also sensitive to dexamethasone and increased systematically with exposure while LpL systematically decreased. These results indicate that dexamethasone acts at both early and late stages to direct proliferative osteoprogenitor cells toward terminal maturation. J. Cell. Biochem. 90: 13–22, 2003.

Key words: dexamethasone; bone marrow stromal cells; bone sialoprotein; osteocalcin; osteoblast; differentiation; lipoprotein lipase

The bone marrow stroma is a heterogeneous tissue consisting of fibroblasts, endothelial cells, reticulocytes, and adipocytes, which provide structural support for hematopoiesis. This connective tissue is also a repository for mesenchymal stem cells [Caplan, 1991], which have the capacity to differentiate into osteoblasts [Maniatopoulos et al., 1988; Haynesworth et al., 1992; Jaiswal et al., 1997], adipocytes [Beresford et al., 1992; Caplan and Dennis, 1996], and chondrocytes [Johnstone et al., 1998]. Consequently, bone marrow stroma has become an attractive tissue for the clinical repair of skeletal defects. However, to direct differentiation into a specific lineage an optimal treatment regimen, involving inductive factors applied in the appropriate concentration and sequence [Caplan, 1994], must be identified.

Osteoblastic differentiation in vitro is marked by three distinct stages of cellular activity: proliferation, extracellular matrix maturation, and matrix mineralization [Lian and Stein, 1992]. Initially, osteoprogenitor cells are highly mitotic, as demonstrated by their expression of the cell-growth associated genes H4 histone and *c*-fos [Pockwinse et al., 1992]. During this proliferative stage, genes associated with extracellular matrix formation (type I collagen, fibronectin, and transforming growth factor-β) are expressed at peak levels [Owen et al., 1990]. As cells form multilayered clusters the rate of proliferation decreases and the expression of the bone/liver/kidney isoform of alkaline phosphatase (AP) increases [Owen et al., 1990; Turksen and Aubin, 1991; Malaval et al., 1994]. Following the period of matrix maturation,

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nodule cells begin to mineralize the extracellular matrix. The expression of osteocalcin (OC) and bone sialoprotein (BSP) increases with mineral deposition [Owen et al., 1990; Pockwinse et al., 1992], while AP levels decline [Turksen and Aubin, 1991; Malaval et al., 1994].

Dexamethasone—a synthetic glucocorticoid —induces osteoblastic differentiation of bone marrow stromal cells (BMSCs) in vitro [Maniatopoulos et al., 1988; Cheng et al., 1994; Peter et al., 1998; Atmani et al., 2002]. Continuous treatment with dexamethasone has been shown to increase AP activity [Maniatopoulos et al., 1988; Cheng et al., 1994; Rickard et al., 1994], expression of OC and BSP [Kasugai et al., 1991; Leboy et al., 1991; Rickard et al., 1994; Aubin, 1999], and matrix mineralization [Maniatopoulos et al., 1988; Cheng et al., 1994; Aubin, 1999; Dieudonne et al., 1999]. However, this glucocorticoid also stimulates adipocytic differentiation of BMSCs [Bennett et al., 1991; Beresford et al., 1992] and down-regulates expression of type I collagen [Beresford et al., 1992]. The effects of dexamethasone on osteoprogenitor cell proliferation and phenotypic development also depend on both the dosage and timing of treatment [Beresford et al., 1992; Aubin, 1999; Atmani et al., 2002]. In particular, dexamethasone treatment in both primary and secondary culture further enhances osteoblastic differentiation when compared with treatment in either passage alone [Beresford et al., 1992; Aubin, 1999; Atmani et al., 2002; Ter Brugge and Jansen, 2002]. However, no studies have specifically looked at the effects of removing dexamethasone during a single passage, and such experiments would indicate the necessity of this glucocorticoid for particular stages of osteoblastic differentiation.

In this study, secondary cultures of rat BMSCs were induced to differentiate in the presence of 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbate-2-phosphate. To assess the effects of dexamethasone on the progression of osteogenesis, the glucocorticoid was removed from the culture medium after 3, 7, 10, 17, 24, or 31 days. Cell number and collagen synthesis were measured at days 10 and 14. Expression of OC, BSP and the adipocytic gene lipoprotein lipase (LpL) was analyzed at day 24, and areas of bone-like mineral deposition were quantified at day 31.

MATERIALS AND METHODS

Primary Cell Isolation and Culture

Bone marrow stroma was extracted from the femurs and tibias of male Sprague-Dawley rats (125–150 g; Harlan, Dublin, VA) as described previously [Maniatopoulos et al., 1988; Dennis et al., 1992; Goldstein et al., 2001]. Dispersed marrow plugs were centrifuged at 1000 rpm (206*g*) for 5 min, buoyant adipocytes were removed by vacuum aspiration, and the pellet resuspended and seeded into two 100-mm culture dishes per animal (approximately 10^8 nucleated cells/dish). The cells were maintained in 7 ml of growth medium (Dulbecco's modified Eagle's medium (DMEM), Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA) and 1% antibiotic/antimycotic (penicillin, streptomycin, neomycin, and fungizone; Gibco BRL) in a 37°C, 5% CO_2 incubator for 14 days. Growth medium was replaced every 3 or 4 days.

Following 14 days in primary culture, cells were lifted with trypsin/EDTA (Gibco BRL), seeded into 12-well culture plates (Corning, Corning, NY) at 10^5 cells/well (2.6×10^4 cells/cm^2) with 2 ml of growth medium, and allowed to attach overnight. The following day (designated as day 0) growth medium was replaced with osteogenic medium (growth medium supplemented with 10 nM dexamethasone (Sigma, St. Louis, MO), 10 mM β-glycerophosphate (Sigma) [Peter et al., 1998], and 50 μg/ml ascorbate-2-phosphate (Sigma) [Johnstone et al., 1998]). Medium was replaced every 3 or 4 days. To evaluate the effect of dexamethasone on the progression of osteoblastic differentiation medium was replaced with dexamethasone-free osteogenic medium at specific time points (days 3, 7, 10, 17, 24, or 31).

Measurement of Cell Number

Total cell number within each well was determined by flourometric analysis of DNA content using Hoechst 33258 (Sigma) [West et al., 1985; Ishaug et al., 1997; Goldstein et al., 2001]. Wells were rinsed twice with PBS and cells were lifted with a cell scraper. The cells were collected with two 0.7 ml aliquots of 10 mM EDTA (pH 12.3) into 1.5 ml microcentrifuge caps. Caps were stored at −70°C until analysis. For analysis, samples were thawed, sonicated on ice for 10 min., and 0.2 ml of 1 M KH2PO4 was added to neutralize pH. DNA standards were prepared

by adding known volumes (0 to 160 μl) of a 50 ng/ml DNA solution (50 ng/ml corresponds to an absorbance of 1.00 through a path of 1 cm at 260 nm) to 1.4 ml EDTA (pH 12.3), sonicating on ice for 10 min., and adding 0.2 ml of 1 M KH2PO4. Fluorescence measurements were performed by combining 0.5 ml of the homogenized supernatant or DNA standard with 1.5 ml of a 100 ng/ml solution of Hoechst 33258 dye (Sigma) in 100 mM NaCl and 10 mM Tris-buffer. Fluorescence measurements were made with a DyNAQuant 200 (Hoefer, San Francisco, CA). Measurements were made in duplicate. A linear relationship was used to correlate fluorescence with total mass of DNA, and a conversion factor of 10.4 pg DNA/cell [Goldstein et al., 2001] was used to calculate cell number.

Collagen Synthesis

Total collagen synthesis was estimated by the incorporation of [^{3}H]-proline [Ishaug et al., 1994; Goldstein et al., 2001]. Briefly, 24 h prior to sample collection, L[5-^{3}H]-proline (ICN, Irvine, CA) was added to the culture medium to a final concentration of 3 μCi/ml. After a 24 h incubation samples were rinsed twice with PBS and incubated in 1 ml of pepsin (1 mg/ml pepsin in 1 M acetic acid) for 4 h at room temperature. Following this incubation, wells were scraped and two 0.2 ml aliquots were collected. Samples were combined with 0.1 ml 0.6 M HEPES (N-2-hydroxyethylpiperazine N′-2-ethane sulfonic acid, pH 7.2) and 0.2 ml of collagenase (125 μg/ml collagenase (Sigma C0773), with 6.25 mM n-ethyl-maleimide, and 1.25 mg/ml CaCl2 in 0.02 N HCl) and incubated for 90 min at 37°C. At the end of the incubation the digestion was stopped by the addition of 0.5 ml of TCA/TA (10% trichloroacetic acid, 0.5% tannic acid). Samples were centrifuged for 5 min at 2,000*g* and the resultant supernatants were transferred to scintillation vials labeled "CP" (for collagen protein fraction). Pellets were resuspended in 0.25 ml TCA/TA and 0.25 ml deionized water, and centrifuged 5 min at 2,000*g*. Supernatants were added to "CP" scintillation vials. Pellets then were resuspended in 0.75 ml TCA/TA and 0.75 ml deionized water and transferred to scintillation vials labeled "NCP" (for non-collagen protein fraction). A volume of 4 ml of emulsion type liquid scintillant was added to each vial and counts were measured using a Tricarb 2100TR (Packard) beta-counter. Counts were then normalized by mean cell density as determined by DNA analysis. The percent collagenous protein (%CP) for each sample was calculated using the following formula [Ishaug et al., 1994]:

$$\%\mathrm{CP} = \frac{\mathrm{CP}}{(\mathrm{CP} + 5.2 \times \mathrm{NCP})} \times 100\%$$

Here, the number 5.2 accounts for the relative abundance of proline in collagen.

Northern Blot Analysis

Expression of mRNAs for OC, BSP, and LpL at day 24 were measured by northern blot analysis. Briefly, cell layers were rinsed twice with sterile PBS and total cellular RNA was isolated using a RNAqueous-4PCR kit (Ambion, Austin, TX) according to kit instructions. RNA concentrations were determined by measuring the absorbance at 260 nm and the samples were stored at −70°C until analysis. Unless otherwise noted, reagents for RNA electrophoresis and hybridization were provided in a NorthernMax kit (Ambion) and used according to kit instructions. Briefly, total RNAs (12 μg per sample) were lyophilized and reconstituted with 18 μl formaldehyde load dye and 2 μl of 100 μg/ml ethidium bromide (Gibco BRL). The reconstituted RNA samples were heated at 65°C for 15 min and immediately loaded onto a denaturing 1% agarose gel for fractionation at 100 V. RNA from the gel was transferred to a Zeta-Probe GT nylon membrane (BioRad, Hercules, CA) by capillary transport in 20× saline sodium citrate (SSC). The RNA was crosslinked to the membrane using a Stratalinker 1800 UV Crosslinker (Stratagene, La Jolla, CA). The wet membrane was then stored at −20°C until further analysis.

Complimentary DNAs (cDNAs) to the messenger RNAs for OC, LpL, and 18S ribosomal RNA were prepared by PCR amplification using Taq DNA polymerase (Qiagen) with the primers listed in Table I and rat placental cDNA template. PCR products were gel-purified, subcloned into pDrive (Qiagen), and sequenced to confirm identity. The BSP probe was provided by Dr. Marion Young (National Institutes of Health). Probes were excised from pDrive by digestion with *Eco*RI, gel purified, and labeled with [α-^{32}P]-dATP (50 μCi, 3,000 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, England) using a Prime-a-Gene kit (Promega, Madison, WI). Membranes were

TABLE I. List of Primer Sequences for cDNA Probe Synthesis

Target	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size (bp)	GenBank no.
OC	AGACTCCGGCGCTACCTCAA	CAGCTGTGCCGTCCATACTTTC	273	NM_013414
LpL	GTATCGGGCCCAGCAACATTATCC	GCCTTGCTGGGGTTTTCTTCATTC	522	L03294
18S rRNA	CGGCGCCCCCTCGATGCTCTTA	TCCTGGTGGTGCCCTTCCGTCAAT	474	V01270

prehybridized with ULTRAhyb buffer (preheated to 68°C) at 42°C for 30 min in a roller oven (VMR International, West Chester, PA). Radiolabeled probe solution was diluted 10-fold with 10 mM EDTA, denatured at 90°C for 10 min, added to the prehybridization buffer within the roller tube, and allowed to hybridize with the RNA overnight at 42°C. Following hybridization, the membranes were washed twice with a low stringency solution (equivalent to 2× SSC, 0.1% lauryl sulfate (SDS)) at room temperature and twice with a high stringency solution (equivalent to 0.1× SSC, 0.1% SDS) at 42°C. The membranes were placed in cassettes with BIOMAX X-ray film (Kodak) and the film was exposed for 1 h to overnight at −80°C. To probe for additional RNAs, the membranes were stripped using two 15 min washes with 0.1× SSC, 0.1% SDS solution at 95°C and reprobed as described above. Membranes were probed for RNAs in the sequence: OC, BSP, LpL, 18S rRNA. Processed autoradiographs were scanned into 8-bit, grayscale TIFF images using a VXR-12 Film Digitizer (Vidar Systems Corp., Herndon, VA). Bands were analyzed by densitometry using ImageJ v1.27 (National Institutes of Health, available at http://rsb.info.nih.gov/ij/). The optical density of each mRNA band was corrected for background noise and expressed as a ratio to the optical density of the 18S rRNA band from the same sample.

Histochemical Staining

Culture wells were assayed for deposition of mineral using the von Kossa silver staining technique [Goldstein et al., 2001]. Briefly, cell layers were rinsed with PBS, fixed in 4% glutaraldehyde (Sigma), rinsed again, and then incubated with 5% $AgNO_3$ (Sigma) under incandescent illumination for 30 min. Safrinin-O (Fisher) was then added to counter-stain the cells. Grayscale images of the stained cell layers were acquired at 4× using an Olympus model IX50 inverted microscope (Opelco, Sterling, VA) equipped with a Prior motorized stage and a Hamamatsu cooled CCD camera (I-cube, Crofton, MD). Individual images were tiled with ImagePro software (Media Cybernetics, Silver Spring, MD) to produce a mosaic image of an entire well. For each image, a triangle algorithm was used to determine the optimal threshold for distinguishing black mineralized regions from gray unmineralized regions. Image Pro was then used to identify objects that satisfied the threshold criterion. Artifacts and debris (objects less than 300 μm^2) were eliminated. The total area of mineral coverage within each well was determined by summing the areas of the remaining objects.

Statistics

Unless noted elsewhere, measurements are presented as the mean ± standard error of the mean (SEM) for $n = 8$ samples. A one-way analysis of variance in combination with Scheffe's multiple-comparison procedure was used to perform discrete pairwise comparisons of the means between groups. Statistical significance denotes a confidence of greater than 95% ($P \leq 0.05$).

RESULTS

Cell Morphology

BMSCs subcultured in the presence of dexamethasone initially exhibited a fibroblast-like spindle shape. As cultures reached confluence, cell merged into morphologically heterogeneous multilayered clusters with tightly packed polygonal cells in the center, and spindle shaped cells with membrane extensions at the periphery. Withdrawal of dexamethasone altered this morphology (Fig. 1). At day 14, when dexamethasone had been removed at day 3 cells within the middle of colonies appeared smaller and rounder (Fig. 1A) than those continuously treated (Fig. 1C). Likewise, at the periphery of these colonies cells exhibited fewer membrane extensions when dexamethasone was removed at day 3 (Fig. 1D) relative to cells continuously treated with dexamethasone (Fig. 1F). Similar numbers of adipocytes were observed in all

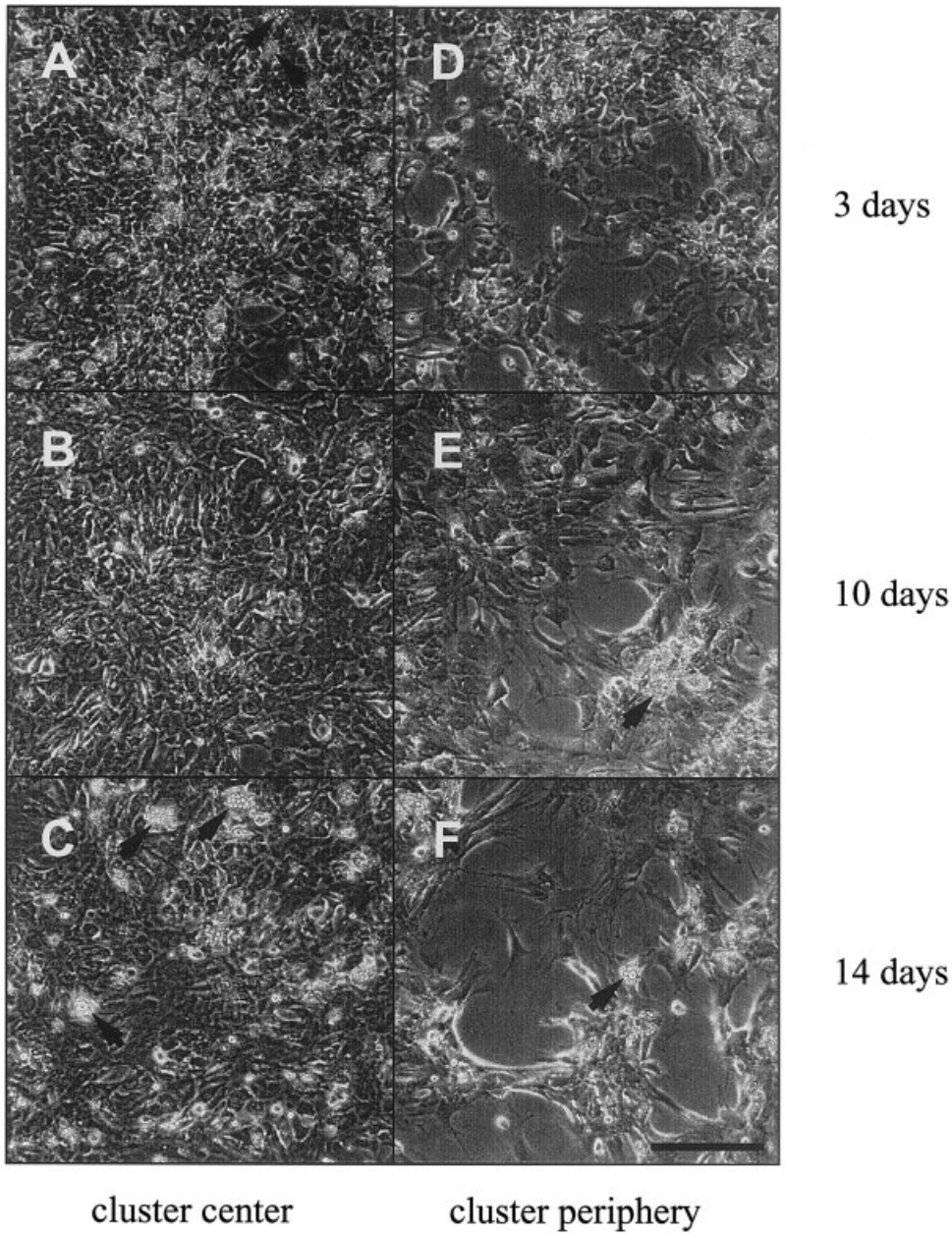

Fig. 1. Cell morphology of rat marrow stromal cells after 14 days in culture. Dexamethasone was removed after 3 (**A**,**D**), 10 (**B**,**E**), or 14 (**C**,**F**) days. Phase contrast images were taken for areas with relatively high (A–C) and low (D–F) degrees of confluence. Arrowheads indicate lipid vesicles (i.e., adipocytes). Scalebar corresponds to 200 μm.

cultures, as indicated by the presence of lipid vesicles in the cell colonies (arrowheads, Fig. 1).

Cell Number

Quantification of DNA indicated that cell number decreased with continued dexamethasone treatment (Fig. 2). On day 10, continuous exposure (gray bar) resulted in significantly fewer cells per well ($P < 0.05$) compared to discontinuation at day 3 (white bar). After 14 days, a similar systematic decrease in cell number ($P < 0.05$) was associated with the duration of dexamethasone treatment: discontinuation at day 3 (white bar) > discontinuation at day 10 (gray bar) > continuous treatment for 14 days (black bar).

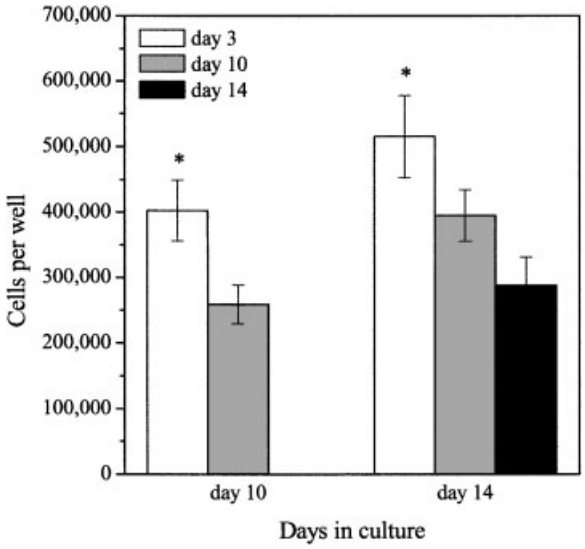

Fig. 2. Cell density determined by fluorometric quantification of DNA at 10 and 14 days in culture. Dexamethasone was removed after 3 (white), 10 (gray), or 14 (black) days. Each bar represents the mean ± SEM for 8 wells (over two separate experiments). A single asterisk (*) denotes statistically significant difference relative to the longest exposure time ($P < 0.05$).

Collagen Synthesis

^{3}H-proline incorporation into the collagenase-digestible protein fraction revealed a small but statistically insignificant increase in collagen synthesis rate per cell at days 10 and 14 with increasing duration of dexamethasone treatment (Fig. 3a). Similarly, a small but insignificant increase in the rate of synthesis of non-collagenase protein with increasing duration of treatment was also measured (data not shown). Together they indicated that collagen synthesis, as a percent of total protein synthesis, did not vary among treatments (Fig. 3b).

Messenger RNA Expression

Cells were assayed on day 24 for the expression of osteoblastic markers OC and BSP, and the adipocytic marker LpL. Groups included cultures exposed to dexamethasone for the 24-day period and cultures in which dexamethasone had been withdrawn after 10 or 17 days. Northern blots indicated an increase in BSP expression and a decrease in LpL expression with increasing duration of treatment (Fig. 4). Although a slight increase in OC expression with increasing duration of exposure was observed among samples from one 12-well culture plate (lanes 2, 4, and 6), a replicate plate revealed only trace levels of the mRNA (lanes 1, 3, and 5).

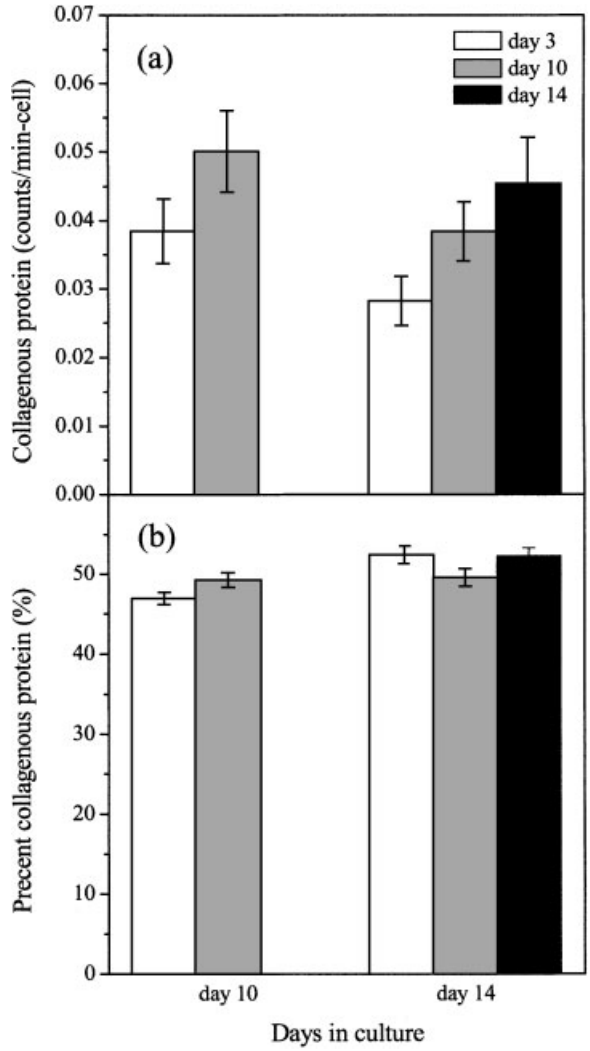

Fig. 3. Collagen synthesis determined by ^{3}H-proline incorporation at 10 and 14 days in culture. **a**: Counts per minute from collagen protein fractions normalized by mean cell number and (**b**) the collagen protein as a percentage of total protein synthesized are plotted versus subculture duration. Dexamethasone was removed after 3 (white), 10 (gray), or 14 (black) days. Each bar represents the mean ± SEM for 8 wells (over two separate experiments).

Matrix Mineralization

After 31 days in culture, histochemical staining revealed that cells formed multilayered colonies with varying degrees of mineral deposition (Fig. 5a). The continuous presence of dexamethasone (day 31) resulted in many mineralized nodules, but the early withdrawal of dexamethasone (days 7 and 10) resulted in the appearance of only a few nodules per well.

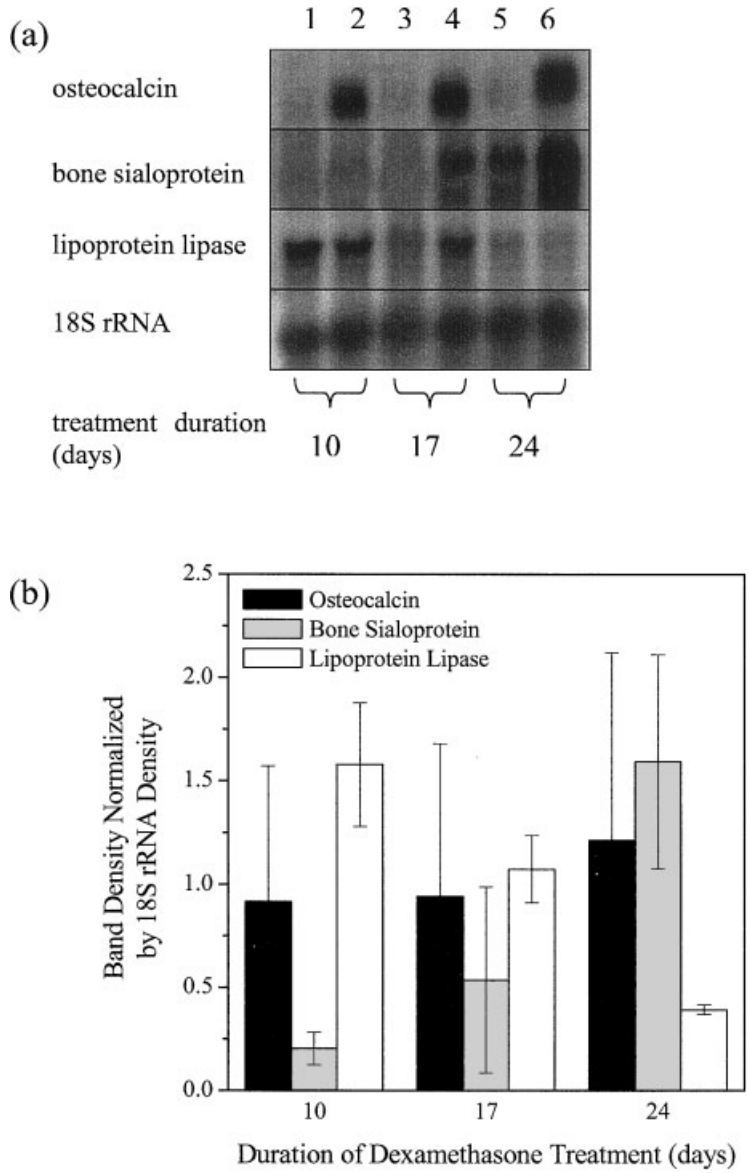

Fig. 4. Protein expression as determined by Northern blot analysis. **a**: Bands for OC, BSP, and LpL mRNAs, and 18S rRNA are presented for cells cultured for 24 days in which dexamethasone was removed after 10, 17, or 24 days. Each lane represents the RNA collected from 4 wells for two replicate studies (**odd lanes** and **even lanes**), which were prepared from the same primary cells but cultured in seperate well plates. **b**: Band densities (relative to 18S rRNA band densities) for OC (black), BSP (gray), and LpL (white) mRNAs are plotted versus dexamethasone treatment duration. Each bar represents the mean of two replications, and each error bar denotes the range between replicates.

Measurements of the total area of mineralized nodules demonstrated that wells with at least 17 days of exposure to dexamethasone yielded significantly higher areas of mineral deposition ($P < 0.01$) compared to shorter treatment durations (Fig. 5b). Black specks, which were thought to be mineral deposits on clusters of dead cells, were observed when dexamethasone was withdrawn after 7 and 10 days but were not included in measurements of total area.

DISCUSSION

This study examined the effects of dexamethasone treatment duration on the proliferation and differentiation of rat BMSCs. At early time points, continuous exposure to dexamethasone decreased cell number relative to discontinuation but did not affect collagen synthesis. At late time points, mineralization and expression of BSP increased systematically with the

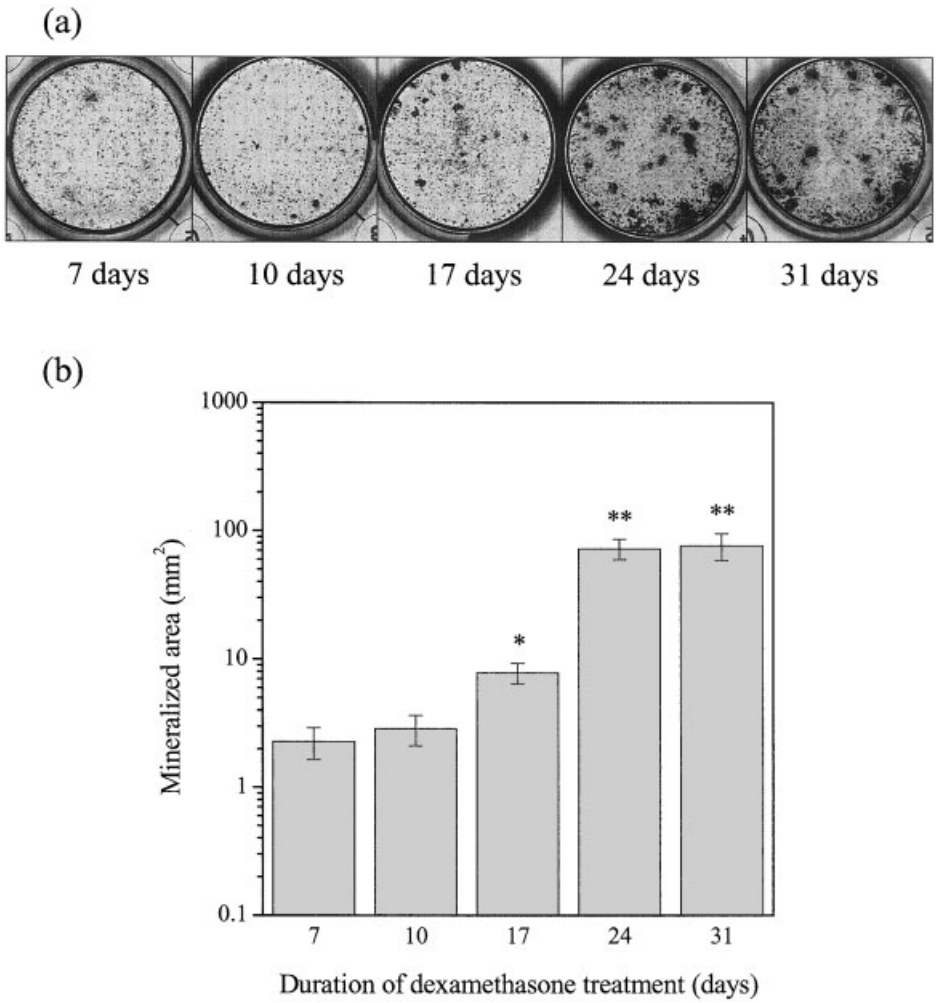

Fig. 5. Areas of mineralization as determined at day 31 by von Kossa silver stain. **a**: Representative wells obtained when dexamethasone was withdrawn from medium after 7, 10, 17, 24, or 31 days. Areas of mineral deposit appear black from the von Kossa stain. Small cell clusters appear gray. **b**: Area of mineral deposit per well is plotted versus the dexamethasone treatment duration. Each bar represents the mean and standard error of the mean (n = 8). A single asterisk (*) denotes statistically significant difference from day 3 removal ($P < 0.001$). A double asterisk (**) denotes statistically significant difference from day 17 removal ($P < 0.001$).

duration of exposure to dexamethasone, while LpL systematically decreased with increasing exposure. Together, these results indicate that dexamethasone acts at multiple points in the differentiation process to stimulate osteoblastic maturation.

In this study the adherent BMSCs formed multilayer cell clusters in subculture that were similar to those observed in previous studies [Maniatopoulos et al., 1988]. However, for cultures where dexamethasone had been removed the cells—particularly those at the cluster periphery—appeared less spread and more rounded. This might reflect alterations in adhesion receptor display [Lauffenburger and Griffith, 2001]. Previous studies have reported that dexamethasone regulates expression of various integrins [Cheng et al., 2000; Walsh et al., 2001] and cadherins [Lecanda et al., 2000]. The morphological observations could also indicate transdifferentiation into adipocytes, which is consistent with phenotypic plasticity between osteoblastic and adipocytic lineages [Aubin, 1998; Bianco et al., 2001]. However, no evidence of increased adipocyte density was found: rounded cells with lipid vesicles appeared with the same frequency under all conditions.

The effect of dexamethasone on BMSC proliferation has been shown to depend on glucocorticoid concentration and osteoprogenitor maturity. When added to primary explants,

dexamethasone can decrease proliferation [Leboy et al., 1991], have no effect, or increase proliferation [Rickard et al., 1994; Peter et al., 1998; Atmani et al., 2002]. Further, increasing concentration to supraphysiological levels has been shown to inhibit proliferation [Walsh et al., 2001]. In contrast, when it is introduced in secondary culture [Cheng et al., 1994; Kim et al., 1999] or its addition is delayed [Peter et al., 1998] it is inhibitory. This study—in which the withdrawal of dexamethasone enhanced proliferation—provides evidence that the presence of dexamethasone is inhibitory to cell proliferation. This suggests that dexamethasone acts to direct osteoprogenitor cells from a state of proliferation to that of matrix maturation. This transition is marked by increased secretion of AP and collagen type I [Lian and Stein, 1992]. Previous studies support this transition, and have shown that when dexamethasone is inhibitory to cell proliferation it stimulates AP activity [Leboy et al., 1991; Cheng et al., 1994; Peter et al., 1998; Kim et al., 1999; Walsh et al., 2001]. However, previous studies have shown that dexamethasone is inhibitory to collagen type I synthesis in BMSC cultures [Beresford et al., 1992; Kim et al., 1999]. In contrast, this study showed that continuous exposure to dexamethasone had no effect or stimulated collagenous protein synthesis.

The final stage of osteoblastic maturation is marked by the expression of several matrix proteins, including OC and BSP, and the deposition of a calcium phosphate layer [Lian and Stein, 1992]. In this study, a systematic increase in BSP expression with continued exposure to dexamethasone was measured, but no difference in OC expression was detected. This suggests that dexamethasone induces expression of BSP, but that BSP and OC expression are not stimulated through the same pathways. In contrast when dexamethasone was removed from culture medium, expression of the adipocytic marker LpL was enhanced. A possible explanation is that in the absence of the osteoinductive agent the inducible subpopulation of osteoprogenitor cells [Aubin, 1998] transdifferentiated into the adipocytic lineage. However, this would need to be examined more closely. Mineralization results were consistent with previous studies, which have shown very little mineralization in the absence of dexamethasone compared to continuous exposure [Herbertson and Aubin, 1995] and an intermediate level when dexamethasone is withdrawn after 11 days [Cheng et al., 1994]. In particular the significant increase in mineralized nodule formation when dexamethasone exposure is extended from 10 to 17 days may correspond to a transition from matrix maturation to mineralization [Owen et al., 1990]. Finally, it has been proposed that mineralization is regulated by deposition of negatively charged matrix proteins [Cheng et al., 1996]. The concurrent increases in BSP and mineralization in this study when dexamethasone exposure was extended from 10 to 24 days is consistent with this hypothesis.

The results of this study demonstrate that dexamethasone is required to direct osteoprogenitor cells from a proliferative but undifferentiated state toward terminal maturation. When dexamethasone is removed during culture the progression of BMSCs into subsequent stages of osteoblastic differentiation is undermined, and a population of cells may regress toward a more undifferentiated state or differentiate along alternative pathways (i.e., adipogenesis). Thus, although dexamethasone is a non-cell-specific agent, its continued presence is required to achieve maximal osteoblastic differentiation of BMSC cultures.

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