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Osteoblastic Phenotype of Rat Marrow Stromal Cells Cultured in the Presence of Dexamethasone, β-Glycerolphosphate, and L-Ascorbic Acid

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Abstract We investigated the effects of the time course of addition of osteogenic supplements dexamethasone, β-glycerolphosphate, and L-ascorbic acid to rat marrow stromal cells, and the exposure time on the proliferation and differentiation of the cells. It was the goal of these experiments to determine the time point for supplement addition to optimize marrow stromal cell proliferation and osteoblastic differentiation. To determine this, two studies were performed; one study was based on the age of the cells from harvest, and the other study was based on the duration of exposure to supplemented medium. Cells were seen to proliferate rapidly at early time points in the presence and absence of osteogenic supplements as determined by ^{3}H-thymidine incorporation into the DNA of replicating cells. These results were supported by cell counts ascertained through total DNA analysis. Alkaline phosphatase (ALP) activity and osteocalcin production at 21 days were highest for both experimental designs when the cells were exposed to supplemented medium immediately upon harvest. The ALP levels at 21 days were six times greater for cells maintained in supplements throughout than for control cells cultured in the absence of supplements for both studies, reaching an absolute value of 75×10^{-7} μmole/min/cell. Osteocalcin production reached 20×10^{-6} ng/cell at 21 days in both studies for cells maintained in supplemented medium throughout the study, whereas the control cells produced an insignificant amount of osteocalcin. These results suggest that the addition of osteogenic supplements to marrow-derived cells early in the culture period did not inhibit proliferation and greatly enhanced the osteoblastic phenotype of cells in a rat model. J. Cell. Biochem. 71:55–62, 1998.

Key words: osteoblast; marrow stromal cell; osteoblastic differentiation; dexamethasone; bone tissue engineering

Osteoblasts have been described as polyhedral mesenchymal cells responsible for synthesizing new bone matrix [Wlodarski, 1990; Caplan, 1991]. The differentiation of osteoblasts has been modeled as a three-step process consisting of a proliferation phase, a matrix maturation phase, and a mineralization phase [Lian and Stein, 1992]. Type I collagen, osteopontin (OP), and osteocalcin (OC) production in addition to increased alkaline phosphatase activity (ALP) are all markers of osteoblastic phenotype [Lian and Stein, 1993; Shi et al., 1996]. Additional evidence of differentiation is the expression of bone sialoprotein and the formation of bone nodules during the matrix mineralization phase [Li et al., 1996].

Marrow-derived cells can be encouraged to follow one of many lineages by the addition of various induction factors to their growth medium. Previous studies have shown that dexamethasone, a synthetic glucocorticoid, is capable of inducing human bone marrow stromal cells to express an osteoblastic phenotype [Cheng et al., 1994, 1996; Gronthos et al., 1994]. Similar results have been observed with rat marrow cells [Rickard et al., 1994]. Furthermore, formation of bone-like tissue was observed in vitro by stromal cells derived from rat bone marrow [Maniatopoulos et al., 1988]. Cells isolated from the bone marrow population and induced along the osteoblastic lineage with dexamethasone have been transplanted subcu-

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taneously while retaining their phenotype in vivo within porous ceramic matrices [Dennis et al., 1992]. Finally, marrow-derived mesenchymal stem cells have been used to regenerate bone in a critical-sized segmental defect [Kadiyala et al., 1997].

In addition to dexamethasone, other agents have been shown to have an effect on preosteoblast differentiation in culture. L-ascorbic acid (AA) is necessary for osteoblastic production of collagen and enhances ALP activity [Choong et al., 1993]. AA has also been shown to decrease cellular proliferation, possibly acting as one of the signals theorized as necessary for the progression from the proliferation to the matrix maturation phase [Lian and Stein, 1993]. Beta-glycerolphosphate and AA are requisite for the formation and mineralization of the extracellular matrix in culture [Maniatopoulos et al., 1988]. Vitamin D_3 added for a short duration of time can increase OC production but can also inhibit differentiation when added continuously to proliferating osteoprogenitor cells [Lian and Stein, 1993].

The growth medium affects the differentiation of marrow cells towards the osteoblastic phenotype. Jaiswal et al. [1997] described the variation of medium components and their concentration in an effort to induce the osteogenic differentiation of mesenchymal stem cells derived from human bone marrow. Dexamethasone, ascorbic acid or L-ascorbic acid-2-phosphate, and β-glycerolphosphate concentrations were found to have an effect on mesenchymal cell morphology, expression of ALP activity, OC production, and matrix mineralization. Further experimentation has shown that transient exposure of stem cells to dexamethasone may be effective in inducing and maintaining the osteoblastic phenotype [Jaiswal and Bruder, 1996].

Not only the medium components and their concentration affect cellular behavior, but the time frame in which osteogenic supplements are added can affect both the proliferative potential of the cells and their differentiated function. In the present study, marrow stromal cells were harvested from rats and expanded in medium either absent in or containing osteogenic supplements (dexamethasone, β-glycerolphosphate, and L-ascorbic acid). The time frame in which the supplements were added differed, and the effect on cellular proliferation and differentiation was investigated. We asked the following questions: (1) Does the cell age have a significant effect on differentiation when cells are exposed to osteogenic supplements at different time points, and (2) Does the duration of exposure to osteogenic supplements have an effect on the cellular differentiation and expression of phenotypic markers?

MATERIALS AND METHODS

Materials

Cell culture. Dulbecco's Modified Eagle Medium (DMEM), fungizone, trypsin, phosphate buffered saline (PBS), and penicillin/streptomycin/neomycin (PSN) were purchased from Gibco (Gaithersburg, MD). Dihydroxyvitamin D_3 and dexamethasone were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). All remaining chemicals for buffer solutions were purchased as cell culture grade from Sigma.

Assays. The ALP-10 alkaline phosphatase assay kit was purchased from Sigma. The Hoechst 33258 dye for the DNA assay was purchased from Polysciences (Warrington, PA). Tritiated thymidine was purchased from Sigma (catalog no. 32,222–9). The scintillation cocktail Ecolume was purchased from ICN (Irvine, CA). All osteocalcin assay materials were purchased from Biomedical Technologies (Stoughton, MA). Additional chemicals needed to make solutions or buffers for the assays were purchased as cell culture grade from Sigma.

Marrow Stromal Cell Isolation

Marrow stromal cells were harvested from the femurs and tibias of 6-week-old male Sprague-Dawley rats as previously described [Maniatopoulos et al., 1988; Ishaug et al., 1997]. Briefly, bones were aseptically excised from the hindlimbs of the rats following ether euthanasia. The soft tissue was removed, and the femurs and tibias were placed in 50 ml DMEM supplemented with 200 μg/ml of a PSN cocktail for approximately 10 min. This concentration of antibiotics is 10 times the normal concentration used for cell culture and is used to avoid contamination during the harvesting process. The proximal end of the femur and distal end of the tibia were clipped off with sterile scissors, a hole was created in the knee joint end of each bone with an 18-gauge needle, and the marrow was flushed from the shaft with primary media (DMEM supplemented with 20 μg/ml PSN, 20

μg/ml fungizone, and 10% FBS). This solution was resuspended, and the cell suspensions from all bones were combined in a centrifuge tube. The suspension was spun down at 400*g* for 5 min. The supernatant was aspirated, and the pellet was resuspended in fresh primary media and seeded into six-well plates at 84,000 cells/cm^2. This high seeding density was chosen because a large portion of the cells harvested from marrow are nonadherent. The wells were rinsed three times with PBS on the third day of expansion to remove the nonadherent cells. The medium was then exchanged every 3 days throughout the studies.

Media Addition I Study

The goal of this study was to determine whether the age of the cells in culture would have an effect on the proliferation and osteoblastic differentiation of primary marrow stromal cells. Upon harvest, one sample set received medium containing osteogenic supplements (DMEM supplemented with 20 μg/ml PSN, 20 μg/ml fungizone, 10% FBS, 1×10^{-8} M dexamethasone, 10 mM Na β-glycerol phosphate, and 50 μg/ml L-ascorbic acid); the remainder of the wells was maintained in primary medium. A second sample set was exposed to supplemented medium at 4 days, with the remaining samples sets exposed at 7 and 10 days. A control sample set was maintained in primary medium throughout the experiment. Samples were assayed for total DNA and ^{3}H-thymidine incorporation at 1, 4, 7, 14, and 21 days, with additional samples analyzed for ALP activity and osteocalcin production at the latter three time points. The time points were counted from the initial day of harvest.

Media Addition II Study

The goal of this study was to determine whether the duration of exposure of the cells to osteogenic supplements would have an effect on the proliferation and osteoblastic differentiation of primary marrow stromal cells. The addition of medium was the same as that described in Media Addition I Study. The sampling times, however, were taken at 1, 4, 7, 14, and 21 days for all sample sets, with the time point referring to the number of days the sample set was exposed to osteogenic supplements in the medium.

DNA Assay

A fluorimetric assay was performed to ascertain the total amount of DNA and subsequently determine the cell number [West et al., 1985]. At the appropriate time point, the medium was removed, the wells were rinsed with PBS three times to remove any nonadherent cells, and the samples were frozen (−80°C). At the end of all time points, the samples were removed from the freezer and allowed to come to room temperature. The cells were removed from the wells with a cell scraper under 1.4 ml of 10 mM EDTA (pH 12.3) and sonicated in 5-ml polypropylene tubes. The tubes were incubated in a water bath at 37°C for 20 min and then transferred to an ice chest. The pH was adjusted to approximately 7.0 by adding 0.2 ml of 1 M KH_2PO_4 to each sample. Immediately prior to analysis, 1.5 ml of a 200 ng/ml Hoechst solution were added to each sample tube. The fluorescence was then read on an Amino-Bowman Series 2 Luminescence Spectrometer (SLM-Aminco, Urbana, IL) with the emission wavelength adjusted to 455 nm and the excitation wavelength adjusted to 350 nm.

^{3}H-Thymidine Assay

Cellular proliferation was monitored by quantifying DNA synthesis through the incorporation of ^{3}H-thymidine [Wu et al., 1996]. Twenty-four hours prior to assaying, 10 μl of ^{3}H-thymidine were added to each well. At the time point, the medium was removed, the wells were rinsed with PBS three times to remove any nonadherent cells, and 0.5 ml of a trypsin solution was added to each well. Ten minutes after being returned to the incubator, the well plates were returned to the hood, and 1 ml of PBS was added to each well. The solutions in the individual wells were then resuspended, and each suspension was added to 10 ml of a scintillation cocktail. The beta-emission was counted on a Minaxi β Tricarb 4000 Series liquid scintillation counter (Parkard, Laguna Hills, CA) for a period of 2 min per sample. The results are presented as counts per cell by normalization with the cell numbers determined from the DNA assay.

Alkaline Phosphatase Assay

The method provided by Sigma for their ALP-10 assay kit was followed as described in Ishaug et al. [1997]. At the appropriate time

point, the medium was removed, and the wells were rinsed with PBS and frozen at −20°C. At the end of all time points, the samples were removed from the freezer and allowed to come to room temperature. The samples were removed from the wells with a cell scraper under 0.5 ml 1 M Tris buffer (pH 8.0). All samples were then sonicated for 10 min in an ice bath and stored on ice until analysis. A water blank was recorded on a Hewlett Packard 8452A Diode Array Spectrophotometer (Waldbronn, Germany). Immediately prior to insertion into the spectrophotometer, 20 µl of the sample solution were added to 1 ml of the ALP-10 reagent (30°C) in a cuvette, and the mixture was lightly inverted to induce mixing. The absorbance was recorded at 405 nm at time zero and every 1 min throughout 3 min, with the sample being maintained at 30°C between each reading. The slope of the absorbance versus time plot was used to calculate the ALP activity.

Osteocalcin Assay

Two days prior to assaying, the medium on the osteocalcin samples was exchanged for osteocalcin-specific medium (DMEM supplemented with 20 µg/ml PSN, 20 µg/ml fungizone, 1×10^{-8} M dexamethasone, 10 mM β-glycerol phosphate, 50 µg/ml L-ascorbic acid, 1×10^{-9} M dihydroxyvitamin D_3). On the day of the time point, the media were removed from each well, placed in individual polypropylene tubes, and frozen (−80°C). All samples were assayed at the end of the study according to the following procedure [Wake et al., 1998]. On day 1 of the assay, osteocalcin standards were prepared in concentrations of 0.03–2.0 ng/tube. Reserved media samples were added to RIA tubes in 100-µl aliquots. Samples and standards were incubated with goat anti-rat osteocalcin (100 µl, 1:50 dilution) and normal goat nonimmune serum (300 µl, 1:40 dilution) overnight at 4°C on an orbital shaker (80 rpm). On day 2 of the assay, ^{125}I-labeled osteocalcin (100 µl, 1:10 dilution) was added to all tubes, and they were incubated a second night under the same conditions. On the third day of the assay, samples were incubated for 2 h with donkey anti-goat IgG (1 ml, 1:50 dilution). All samples were centrifuged at 1,500*g* and 4°C for 20 min. Each sample was then rinsed with ddH_2O and centrifuged again. The supernatant was decanted, and the pellets were counted in a gamma counter for 2 min. A standard curve was constructed to evaluate the OC content of the samples from the gamma count. Results were reported as nanograms of OC per cell.

Statistical Analysis

All assays were performed in triplicate and expressed as means ± standard deviations. Single-factor analysis of variance was used to determine statistical significance of the results. A two-tailed unpaired t-test between sample sets with a significance level of $P < 0.05$ was used for multiple comparison tests.

RESULTS AND DISCUSSION

Cells isolated from bone marrow provide an excellent source of proliferative osteoprogenitor cells that can be induced to differentiate into osteoblasts. These experiments were designed to characterize marrow stromal cells further and determine the optimal environment for their expansion and differentiation for use as an in vivo bone formation model. We wanted to determine whether the cell age at the time of exposure to osteogenic supplements and the duration of exposure to osteogenic supplements would have an effect on cellular differentiation and expression of phenotypic markers.

Through examination of the results from the first media addition study, it was determined that the age of the cells prior to their exposure to osteogenic supplements had a significant effect on their ability to express markers of osteoblastic phenotype. Cellular proliferation was monitored by measuring the level of incorporation of ^{3}H-thymidine into the DNA of replicating cells. By normalizing this level with the total cell number found from the DNA assay, the fraction of cells that were replicating could be determined. For the first 24 h after harvest, cells were seen to replicate rapidly (Fig. 1a). After this time point, the wells were nearly confluent and the rate of cell division dropped significantly. This rapid proliferation followed by a more moderate increase in cell number was paralleled by the leveling off of the total cell number found from the DNA assay at day 7 (Fig. 1b). The cell number plateaued for all wells at day 7.

The alkaline phosphatase activity was seen to increase rapidly from day 7 to day 14 for cells exposed to supplements immediately upon harvest. A further increase in ALP was seen from day 14 to day 21 for media I sample sets receiving osteogenic supplements at 0, 4, and 7 days after harvest (Fig. 1c). The absolute values of ALP activity were similar to those found in

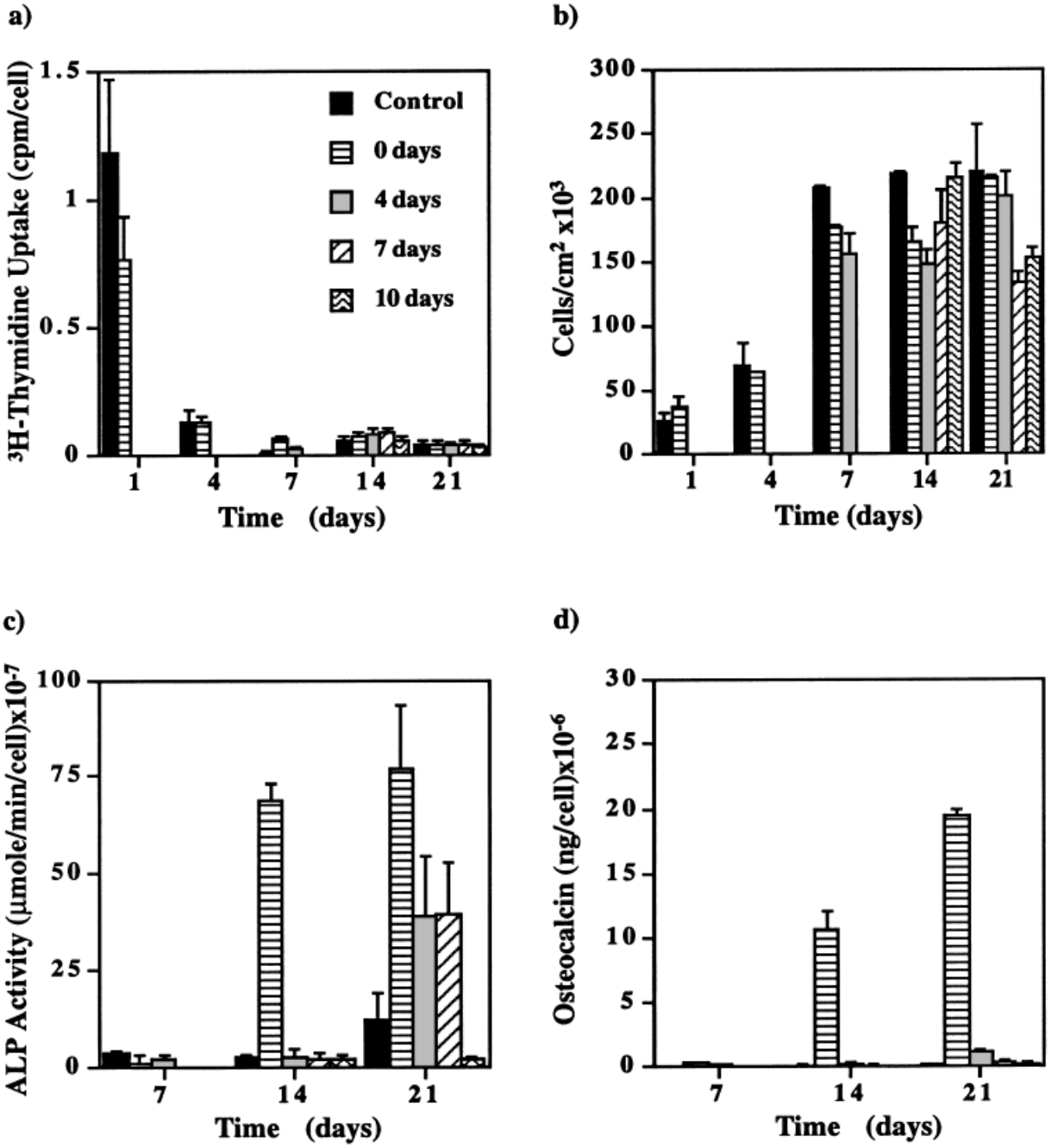

Fig. 1. The media I study evaluated the effects of adding osteogenic supplements to cells of different age. **a:** ^{3}H-thymidine was at a maximum at 1 day and dropped to a low level for the remainder of the 21 days. **b:** Total cellular DNA indicates cell number. This value leveled off for all sample sets at around 7 days. **c:** The alkaline phosphatase activity increased most significantly at 21 days after harvest. **d:** The osteocalcin level in the medium increased at both 14 and 21 days after harvest for sample sets receiving osteogenic supplements immediately upon harvest. The legend indicates the time at which osteogenic supplements were added: 0, 4, 7, and 10 days after harvest. The control cells were maintained in the absence of supplements throughout the 21 days of the assay. The x axis indicates the age of the cells. The error bars indicate means ± standard deviation for n = 3.

previous studies [Ishaug et al., 1997]. Other researchers have determined that dexamethasone plays a significant role in inducing the osteoblastic phenotype of human bone marrow stromal cells [Cheng et al., 1994, 1996; Gronthos et al., 1994]. The cell age before exposure to dexamethasone, L-ascorbic acid, and β-glycerolphosphate was found to have a great effect on cellular differentiation, similar to the results found in the present study.

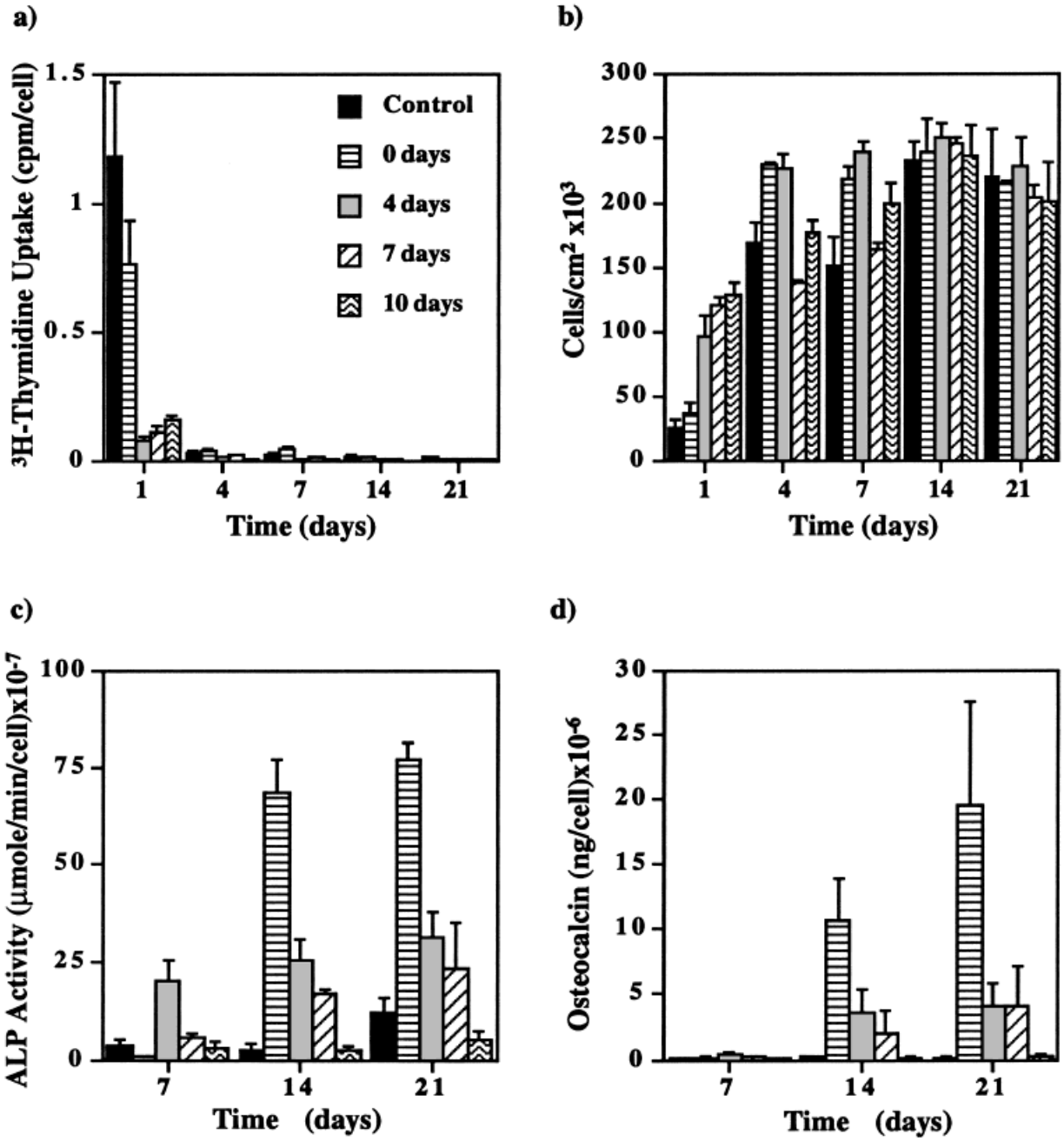

Fig. 2. The media II study evaluated the effects of the duration of exposure of osteogenic supplements on marrow stromal cell proliferation and differentiation. **a:** ^{3}H-thymidine was at a maximum at 1 day and dropped to a low level for the remainder of the 21 days. This plot is similar to the ^{3}H-thymidine incorporation for media I, indicating that proliferation is dependent on cell age and not the duration of exposure to supplemented medium. **b:** Total cellular DNA indicates cell number. The total cell number was consistent between sample sets, indicating that the duration of exposure to supplemented media does not have an effect on cell number. **c:** The alkaline phosphatase activity increased significantly at 14 and 21 days after harvest. **d:** The osteocalcin level in the medium increased at both 14 and 21 days after harvest for sample sets receiving osteogenic supplements immediately upon harvest and after 4 and 7 days. The absolute value of OC is higher for the 4-day and 7-day sample sets for media II than for media I. This indicates that prolonged exposure to osteogenic supplements may increase OC production. The legend indicates the time at which osteogenic supplements were added: 0, 4, 7, and 10 days after harvest. The control cells were maintained in the absence of supplements throughout the 21 days of the assay. The x axis indicates the number of days the cells were maintained in supplemented medium prior to assaying. The error bars indicate means $\pm$ standard deviation for $n = 3$.

Osteocalcin production increased significantly from day 7 to day 14 for the cells exposed to supplemented medium initially and increased again from day 14 to day 21 for these sample sets and those exposed after 4 days (Fig. 1d). At 21 days, the cells exposed to supplements for the entire time frame had an absolute value of 20×10^{-6} ng OC/cell. These levels of osteocalcin production coincided with those found by Wake et al. [1998] and demonstrated the commitment of these cells to follow the osteoblastic lineage [Beresford et al., 1984; Price, 1985].

Results from the second media addition study based on the duration of exposure of the cells to osteogenic medium were similar to those based on cell age. For ^{3}H-thymidine incorporation, the first 24 h showed rapid cellular replication (Fig. 2a). After this time point, the rate of cell division dropped significantly. The decrease in cellular proliferation was mirrored by the leveling off of the total cell number found from the DNA assay (Fig. 2b). The final cell number was approximately 200,000 cells/cm^2, which was similar to that of the media I results. After the cells reached confluency, they began their differentiation process and little replication occurred [Lian and Stein, 1992]. This is a possible explanation of how a longer duration of exposure to osteogenic supplements had no effect on cellular proliferation or total cell number.

The alkaline phosphatase activity was seen to increase from day 7 to day 14 for sample sets receiving osteogenic supplements at 0 and 7 days after harvest (Fig. 2c). The increase continued for the day-14 to day-21 time points for these sample sets but was only significant for those samples exposed to supplemented medium immediately upon harvest. The ALP level for those cells exposed to supplemented medium after 4 and 7 days was lower in the media II results than in the media I results at the 21-day time point. This decrease in ALP activity has been described as a characteristic of late stage osteoblastic differentiation [Lian and Stein, 1992; Li et al., 1996]. Therefore, a longer exposure to osteogenic supplements may enhance osteoblastic differentiation of marrow stromal cells. This hypothesis is supported by our osteocalcin data. The osteocalcin production increased significantly from day 7 to day 14 for the cells exposed to supplemented medium initially and after 4 and 7 days (Fig. 2d). For samples receiving supplements immediately upon harvest, the final OC production level rose to 20×10^{-6} ng OC/cell. These results match those found in media I, as expected. However, cells receiving supplements 4 and 7 days after harvest had final OC levels much higher for media II than those found for media I. The second media addition study allowed us to draw the conclusion that the duration of continuous exposure to osteogenic supplements does have a significant effect on the commitment of cells to pursue the osteoblastic phenotype.

The goal in expanding primary bone marrow cells for bone tissue engineering is to have both rapid proliferation of cells and differentiation along the osteoblastic lineage. The balance of proliferation versus differentiation may be optimized by adding osteogenic supplements early in the culture period because the presence of these factors did not inhibit proliferation and greatly enhanced the osteoblastic phenotype of stromal cells in a rat model.

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