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Importance of 1,25-Dihydroxyvitamin D_3 and the Nonadherent Cells of Marrow for Osteoblast Differentiation from Rat Marrow Stromal Cells

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Although steroid hormones regulate mature osteoblast function, much less is known about their actions on osteoprogenitor cells. The possibility of steroid hormone regulation of early stages in osteoblast differentiation was investigated by measuring the growth and induction of the osteoblast marker enzyme alkaline phosphatase (AP) in rat bone marrow stromal cell cultures. Experiments were performed in charcoal-stripped serum; conditions which markedly impaired stromal cell growth. However, growth could be stimulated by nonadherent marrow cell-derived conditioned medium. $1,25(OH)_2D_3$, but not dexamethasone, 17β-estradiol, or retinoic acid, increased both stromal cell proliferation and AP activity. The increased proliferation with $1,25(OH)_2D_3$ was nonadherent cell-dependent. BMP-2 also increased AP levels and acted in synergy with $1,25(OH)_2D_3$. These results suggest that (i) nonadherent marrow cells may support stromal cell development, and (ii) $1,25(OH)_2D_3$ as well as glucocorticoids may regulate osteogenesis from the bone marrow but a similar role for estrogen is not supported. (*Bone 16:671–678; 1995*)

Key Words: Marrow stromal cells; Osteoprogenitor; Steroid hormones; 1,25-dihydroxyvitamin D_3.

Introduction

Bone marrow comprises both developing hematopoietic cells and a heterogeneous nonhematopoietic component including fibroblasts, endothelial cells, reticular cells, adipocytes, and macrophages, which together comprise the marrow stroma. The multipotential stem cells in the marrow stroma can give rise to cells of different mesenchymal lineages and, at least in part, are thought to be the ultimate precursors of osteoblasts.[1] The synthetic glucocorticoid dexamethasone (dex) can induce at least a proportion of undifferentiated adherent fibroblast-like cells in bone marrow stroma (termed CFU-f and henceforward referred to as stromal cells) to differentiate into osteoprogenitor cells when suspensions of marrow cells are cultured in vitro.[2,3] The osteoprogenitor cells undergo subsequent maturation giving rise to cells with characteristics of differentiated osteoblasts.[3–6] We have previously shown[4] that the adherent cells from 4–5-week-old rats, when treated with dex and/or bone morphogenetic protein (BMP), develop high alkaline phosphatase and osteopontin mRNA within 5 days of culture, and express mRNAs for osteocalcin and bone sialoprotein by day 8. Addition of β-glycerophosphate to these cultures at day 7 produces mineralized nodules as seen by Alizarin red staining.

Abundant evidence suggests that other steroid hormones in addition to glucocorticoids, notably retinoic acid and $1,25(OH)_2D_3$, regulate the growth and differentiation of a variety of cell types including cells of the osteogenic lineage (for reviews, see Refs. 7 and 8). For example, retinoic acid directs pattern formation and limb bud development by acting on mesenchymal cells differentiating into cartilage and bone,[9] and preferentially induces multipotential mesenchymal cells to undergo osteoblastic rather than myogenic or adipogenic differentiation.[10] Involvement of $1,25(OH)_2D_3$ in early stages of osteogenesis is suggested by the observation that generation of a new bone organ following implantation of demineralized bone is disrupted in vitamin D-deficient animals,[11] and that $1,25(OH)_2D_3$ modulates calvaria-derived osteoprogenitor cell differentiation in a bone nodule formation assay.[12] Besides potential effects on osteoprogenitor cells, dex, retinoic acid, and $1,25(OH)_2D_3$, all induce expression of a more differentiated phenotype in preosteoblastic and osteoblastic cells.[13–15]

Although it remains controversial whether estrogen stimulates bone formation,[16,17] the failure to detect marked modulation by estrogen of differentiated osteoblast function in vitro[18,19] may imply that osteoprogenitor cells and not mature osteoblasts are the principal estrogen target cell in bone. In support of this, estrogen receptors and estrogen responses have recently been reported in bone marrow stromal cells and stromal cell lines.[20,21] We have investigated the effects of these steroid hormones on growth and the induction of alkaline phosphatase (AP)—one of the earliest markers of osteoblast differentiation—in rat bone marrow stromal cells cultured in charcoal-stripped serum. The rat bone marrow culture system comprises mostly nonadherent cells of hematopoietic origin and a smaller adherent cell population, which includes stromal fibroblasts and the CFU-f stem cell component of stromal fibroblasts. $1,25(OH)_2D_3$, but not dex, retinoic acid, or 17β-estradiol, consistently promoted stromal cell proliferation and induction of AP. In the course of defining a role for steroid hormones in the control of stromal cell development, culture in charcoal-stripped serum was found to considerably reduce stromal cell growth, an effect that could be at least partially reversed by soluble factors released by the nonadherent marrow cells. Therefore, although the importance of the stromal cell network for providing a microenvironment supportive of hematopoiesis is well recognized,[22–24] these findings

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suggest a potential for hematopoietic cells to sustain and stimulate the growth of stromal cells. Finally, the ability of $1,25(OH)_2D_3$ to induce stromal cell proliferation was shown to be nonadherent cell-dependent and it is therefore possible that systemic calcitropic hormones and hematopoietic cell-derived factors produced locally may act coordinately to regulate osteogenesis in bone marrow in vivo.

Materials and Methods

All reagents were purchased from Sigma unless otherwise stated. $1,25(OH)_2D_3$ was a gift from Dr. MR Uskokovic (Hoffmann La Roche, Nutley, NJ), and recombinant human BMP-2 (specific activity 3.1×10^4 U/mg) was generously provided by Genetics Institute, Andover, MA. [α-methyl ^{3}H]-thymidine was obtained from Amersham International (Arlington Heights, IL).

Marrow Cell Culture

Cell suspensions were produced from whole femoral marrow of 4–5-week-old female Wistar rats as described previously.[3] Briefly, femurs were scraped clean of soft tissue and muscle and the marrow flushed out from the midshaft using a syringe and needle with the appropriate culture medium (see below). Single cell suspensions of total marrow cells were produced by repeated aspiration through a syringe fitted with a 19 and then 21 gauge needle. Cells were seeded into 6 well plates (area 8.5 cm^2) at a density of 1 bone marrow aspirate per 60 cm^2 ($\sim 1.5 \times 10^6$ cells/cm^2) and cultured either in αMEM medium (Gibco BRL) supplemented with 15% fetal bovine serum (FBS) (HyClone Laboratories Inc., Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, or in αMEM without phenol red indicator and supplemented as above but with 15% FBS that had been charcoal stripped (CS-FBS). Medium lacking phenol red was used since phenol red or a component in the preparation has been demonstrated to possess mild estrogenic activity.[25] Charcoal stripping of FBS was employed to remove steroid hormones present in serum and thus reduce possible interference of serum factors in the actions of the exogenously added hormones.[26] The charcoal stripping procedure, in addition to removing steroids, has also been shown to reduce the concentration of certain peptide growth factors and nonsteroid hormones.[27,28] We have investigated the effects of these steroid hormones on growth and the induction of AP in rat bone marrow stromal cells cultured in charcoal-stripped serum which has diminished levels of both hormones and growth factors.

Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air. After 24 h (day 1), 50 μg/ml ascorbic acid was added to all cultures and appropriate cultures also received 10^{-8} M dex, 10^{-8} M $1,25(OH)_2D_3$, 10^{-9} M 17β-estradiol, 3×10^{-8} M all trans retinoic acid and/or 50–100 ng/ml rhBMP-2 as detailed in *Experimental Protocols*. Nonadherent cells were removed with the first medium change by vigorous washing 2× with phosphate buffered saline (PBS). Numbers of residual cells were further decreased with subsequent medium changes, but a small number of cells that were morphologically distinguishable from the fibroblast-like stromal cells and comprised not more than 5% of the total cell population, adhered and could not be removed by washing.

Experimental Protocols

The time course of experiments investigating the effects of factors on proliferation and AP activity of stromal cells cultured in medium containing CS-FBS is outlined in **Figure 1.** Experiments investigating the role of nonadherent cells in stromal cell development followed different time courses and are detailed below.

Protocol (i). The effect of culture in CS-FBS medium on marrow stromal cell growth: cells were plated directly into αMEM supplemented either with untreated FBS or with CS-FBS (day 0). Dex was added after 24 h (day 1), nonadherent cells were removed on day 3, and cell numbers were determined on day 6.

Protocol (ii). The effect of nonadherent cells on stromal cell growth: cells were plated into CS-FBS medium and nonadherent cells removed either on day 3 or day 5. Culture was continued in αMEM + 15% CS-FBS until day 6 when cell numbers and AP activities were measured. For some cultures in which nonadherent cells were removed on day 3, cultures to day 5 were continued in the presence of nonadherent cell conditioned medium (produced as described in the *Production of Nonadherent Cell Conditioned Medium.*

Protocol (iii). The interaction between nonadherent cells and $1,25(OH)_2D_3$ in stromal cell proliferation: for proliferation in the presence of nonadherent cells, marrow cells were plated in αMEM + 15% CS-FBS and $1,25(OH)_2D_3$ added after 24 h (day 1). Nonadherent cells were removed on day 5 and cultures continued in CS-FBS medium + $1,25(OH)_2D_3$ until measurement of cell proliferation (by [^{3}H]-thymidine incorporation) on day 7. For proliferation in the absence of nonadherent cells, nonadherent cells were removed from cultures on day 1 and cultures of adherent cells continued in αMEM + untreated FBS (to maximize the number of stromal cells). On day 3 medium was changed to αMEM + 15% CS-FBS and $1,25(OH)_2D_3$ added until assay on day 7.

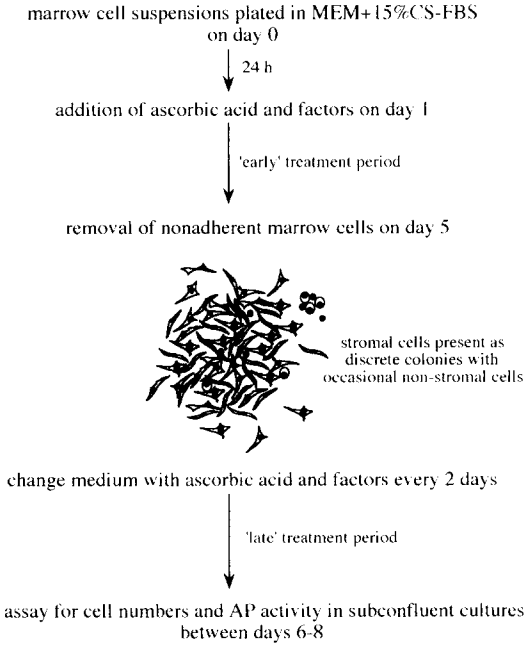

Figure 1. Experimental scheme for investigation of the effects of steroid hormones and BMP-2 on stromal cell development in marrow cultures maintained in medium with CS-FBS.

Measurement of Cell Proliferation

Stromal cell proliferation was assessed both by cell counting and by incorporation of [^{3}H]-thymidine into DNA. For cell counting, the adherent stromal cells were washed with PBS, resuspended by treatment with 0.25% trypsin, 1 mM EDTA (pH 7.8) in Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution (HBSS) and cell numbers determined using a hemocytometer. For radiolabeled thymidine incorporation, cells were pulsed with [^{3}H]-thymidine, 2 μCi per well, for 6 h. Wells were then washed 3× with PBS, cells resuspended by trypsinization, and the amount of radioactivity present in trichloroacetic acid precipitable material determined as described previously.[29]

Production of Nonadherent Cell Conditioned Medium

Nonadherent cells were removed on day 1 from control cultures of total marrow cells grown in medium containing untreated FBS. Pooled nonadherent cells were collected by centrifugation, resuspended in medium with CS-FBS, and replated into six well plates at a density of ~2×10^7 cells/2 ml/well. After a two-day incubation medium was pooled, cells removed by centrifugation, and the conditioned medium (CM) added to stromal cells without storage.

Measurement of Alkaline Phosphatase (AP) Activity

AP activity of the total cell layers was measured in parallel wells after extraction in cell digestion buffer (1.5 M Tris-HCl, 1 mM $ZnCl_2$, 1 mM $MgCl_2 \cdot 6H_2O$, pH 9.2 containing 1% Triton X-100) at 37°C for 30 min. Aliquots of the cell digest were mixed with assay buffer containing 7 mM p-nitrophenyl phosphate (Sigma 104) and the rate of production of p-nitrophenol monitored during the initial 5 min of reaction by absorbance at 410 nm. Enzyme activity is expressed as nmol p-nitrophenol formed/min/10^6 cells assuming 1 A_{410} = 64 nmol product.

Statistical Analyses

Results are presented as the mean ± SEM. Statistical significance between groups was calculated using the nonpaired Student's t-test, and the degree of significance is given in the figure and table legends. A p value <0.05 was considered statistically significant.

Results

The appearance of adherent marrow cells cultured in αMEM + 15% CS-FBS was similar to cells cultured with untreated serum; after removal of nonadherent cells, the adherent culture comprised discrete colonies of fibroblast-like stromal cells with occasional small rounded cells. As suggested by others,[30] these latter cells were most probably macrophages and/or endothelial cells. The numbers of nonstromal cells did not appear to change with time, and owing to the greater proliferative capacity of the stromal cell population, the extent of these "contaminating" cells decreased to less than 5% of the adherent culture and was therefore considered negligible.

Reduced Development of Marrow Stromal Cells Cultured in Medium Supplemented with CS-FBS

When cells from complete bone marrow were directly plated into αMEM + 15% CS-FBS, the development of the adherent stromal population was markedly reduced in comparison to identical marrow preparations grown in αMEM with 15% untreated FBS **(Figure 2).** For both serum types the nonadherent marrow cells were removed from cultures on day 3 and in all other respects cultures were identically treated, as described in protocol (i) (*Materials and Methods* section). By day 6 cell numbers in cultures grown in medium with CS-FBS were approximately 30% of those grown with untreated serum. Dex (10^{-8} M), added to cultures from day 1, reduced cell growth in both serum types. The poorer growth of stromal cells in CS-FBS medium was reflected in the greater time necessary to achieve confluence; typically confluency was reached by day 7 for cells in untreated FBS and days 9–10 for cells in CS-FBS.

Nonadherent Marrow Cells Stimulate Growth of the Stromal Cells

Growth of stromal cells in αMEM + 15% CS-FBS was substantially improved if removal of the nonadherent cells from the cultures was postponed from day 3 to day 5 of culture **(Table 1).** Medium conditioned by the nonadherent cells and added to cultures after removal of nonadherent cells on day 3 promoted stromal cell growth to the same extent as the nonadherent cells themselves, suggesting that a soluble factor(s) secreted by the nonadherent marrow cells is mitogenic for the stromal component. As a result of these findings, the nonadherent cells were not removed from cultures until day 5 in all subsequent experiments except where stated. AP activities in these cells cultured without inducer were low and were not affected by removal of nonadherent cells on different days. The effect of nonadherent cell-stromal cell coculture to different days was also investigated for marrow cells cultured in medium supplemented with untreated FBS and dex **(Table 2).** Stromal cell growth and AP activity were both stimulated by retaining the nonadherent cells in culture until day 3, compared to cultures in which nonadherent cells had been removed on earlier days. However, maintaining nonadherent cells in cultures beyond day 3 caused no additional enhancement in cell number or AP expression, in contrast to the effects observed with nonadherent cells in CS-FBS medium.

Effect of Steroid Hormones and BMP-2 on the Development of Stromal Cells Cultured in CS-FBS Medium

To determine which, if any, of the steroid hormones could reverse the impaired stromal cell development in CS-FBS contain-

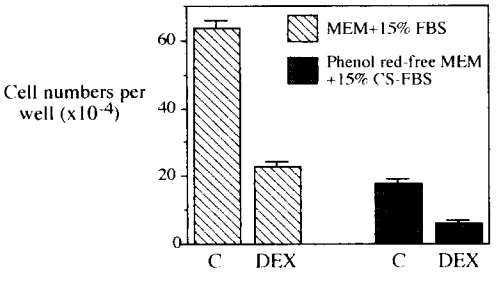

Figure 2. Impaired growth of marrow stromal cells in medium containing charcoal-stripped serum. Cell suspensions were plated directly in the medium indicated and dexamethasone was added to half the wells on day 1 of culture. Nonadherent marrow cells were removed on day 3 and cell numbers determined on day 6. Values represent the mean ± SEM of eight counts from one well per treatment, and represent a typical experiment repeated three times. C = control, DEX = dexamethasone 10^{-8} M.

Table 1. Stimulation of stromal cell growth by delaying removal of nonadherent marrow cells (N/A cells). Marrow cell suspensions were seeded in αMEM + 15% CS-FBS and cultured as described for protocol (ii) (*Materials and Methods* section). Nonadherent cells were removed either on day 3 or day 5, as indicated. After removal of the nonadherent cells on day 3 some wells received fresh medium ('day 3') and others received medium conditioned by nonadherent cells that had been separately cultured in CS-FBS medium ('day 3, then CM to day 5'). On day 5 both nonadherent cells and CM were removed and cell numbers and AP activity measured on day 6. Results of a representative experiment and pooled data are presented, where n = number of repeat experiments. In the representative experiment cell numbers were determined by eight counts from one well per treatment

Day of N/A cell removal	Typical experiment		Treated/day 3 control (mean ± SEM)	
	Cell number ($\times 10^{-4}$)	AP activity (nmol/min/10^6 cells)	Cell number	AP activity
Day 3	30.15 ± 1.35	8.49	1.0	1.0
Day 5	54.45 ± 1.10	8.23	2.23 ± 0.40[a] (n = 3)	0.98 (n = 2)
Day 3, then CM to day 5	48.05 ± 1.70	11.99	2.13 ± 0.49[a,b] (n = 6)	1.39 (n = 2)

[a]Significant difference $p < 0.05$ compared to day 3.
[b]Not significantly different compared to day 5.

ing medium, either dex (10^{-8} M), all trans retinoic acid (3×10^{-8} M), 17β-estradiol (10^{-9} M), or $1,25(OH)_2D_3$ (10^{-8} M) were added to cultures from day 1 (see Figure 1). For each hormone the dose used was within the concentration range known to modulate osteoblastic function in other in vitro culture systems. Assays for cell numbers and AP activities were performed when control cultures were 50–90% confluent (days 6–8). For a given culture, assays were performed on the same day. The effects of the steroids and of the osteoinductive protein BMP-2 on stromal cell growth and expression of AP are presented in **Table 3.** Relative to control cultures maintained in unsupplemented αMEM + 15% CS-FBS, dex reduced cell growth. Stromal cells cultured in standard medium for 6–10 days in the presence of dex typically exhibit AP activities of 200–400 nmol/min/10^6 cells, which is 5–10 fold higher than control-treated cells.[4] However, the dex-induction of AP in cells cultured in medium supplemented with CS-FBS was considerably blunted, reaching levels of only 20–50 nmol/min/10^6 cells. Consistently, 17β-estradiol produced no modulation of either the growth or AP activity. Retinoic acid appeared to be toxic to the cells despite its use at the lowest concentration observed to have an effect on osteoblastic cells[31,32] and a concentration considerably lower than that used with rat stromal cells cultured in untreated FBS.[3] The elevated AP levels of the surviving cells (when expressed per 10^6 cells) are a reflection of the very low cell numbers that remained. In contrast to all of the above agents, $1,25(OH)_2D_3$ reproducibly stimulated both stromal cell numbers and AP activity. The $1,25(OH)_2D_3$ stimulation of cell numbers was shown not to be due to increased initial cell adherence, because addition of the hormone on day 0 (at the time of plating) compared with addition on day 1 (at which time virtually all of the stromal cells have attached) did not affect cell number on subsequent days (data not shown). The magnitude of the AP induction with $1,25(OH)_2D_3$ was similar to that produced by rhBMP-2 (100 ng/ml); moreover, $1,25(OH)_2D_3$ and BMP-2 added simultaneously to the cultures produced a greater than additive increase in AP activity. The response to combinations of steroid hormones was also investigated. No hormonal mixture that lacked $1,25(OH)_2D_3$ was as effective at enhancing growth and AP activity as $1,25(OH)_2D_3$ alone, and $1,25(OH)_2D_3$ together with other steroids did not produce significant increases over single treatment with $1,25(OH)_2D_3$ (data not shown). Since $1,25(OH)_2D_3$ and the other steroids did not restore stromal cell development in αMEM + 15% CS-FBS to that observed when cells were cultured in medium containing untreated FBS, optimal

Table 2. Effect of nonadherent cell (N/A cell) removal on stromal cell development in medium with untreated FBS. Marrow cell suspensions were seeded in αMEM + 15% FBS (day 0) and dex (10^{-8} M) added after 24 h (day 1). Nonadherent cells were removed either on day 1, 2, 3, or day 5 as indicated and culture continued in the presence of dex until measurement of cell number and AP activity on day 8

Day of N/A cell removal	Cell number ($\times 10^{-6}$)	AP activity (nmol/min/10^6 cells)
Day 1	0.72	220
Day 2	0.97	406
Day 3	1.16	390
Day 5	1.15	383

Table 3. Effect of various steroid hormones and BMP-2 on the growth and alkaline phosphatase (AP) activity of stromal cells cultured in CS-FBS medium. Marrow cells were plated directly into αMEM + 15% CS-FBS and cultured as shown in Figure 1. The data represent values determined from different cultures with the assays performed between day 6 and 8. Measurements are the mean ± SEM where n = number of repeat experiments. DEX = dexamethasone, RA = all trans retinoic acid, 17β-E_2 = 17beta-estradiol, $1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3, and BMP-2 = recombinant human bone morphogenetic protein-2

Addition to medium (αMEM + 15% CS-FBS)	Relative number of cells	Relative AP activity (per 10^6 cells)
Control (no addition)	1.0	1.0
DEX 10^{-8} M (n = 4)	0.41 ± 0.10[d]	1.95 ± 0.43[b]
RA 3×10^{-8} M (n = 2)	0.15 ± 0.07[d]	10.18 ± 2.70[b]
17β-E_2 10^{-9} M (n = 3)	0.91 ± 0.01[a]	1.07 ± 0.02[a]
$1,25(OH)_2D_3$ 10^{-8} M (n = 6)	1.68 ± 0.15[d]	4.08 ± 0.93[c]
BMP-2 100 ng/ml (n = 4)	0.94 ± 0.16[a]	3.95 ± 0.61[c]
$1,25(OH)_2D_3$ + BMP-2 (n = 3)	1.78 ± 0.42[a]	17.75 ± 7.48[a]

[a]Not significantly different compared to control.
[b]Significant difference $p < 0.05$ compared to control.
[c]Significant difference $p < 0.01$ compared to control.
[d]Significant difference $p < 0.001$ compared to control.

growth of these cells probably requires nonsteroid factors removed from serum by the charcoal stripping.

Effect on Stromal Cells of 1,25(OH)$_2$D$_3$, Dex and BMP-2 Added at Different Times of Culture

To further define the requirement for 1,25(OH)$_2$D$_3$, dex, BMP-2, and combinations of these in stromal cell differentiation, agents were added to cultures in medium with CS-FBS either "early" (days 1–5) or "late" (days 5–8), as illustrated in Figure 1. As seen in **Figure 3a,** the ability of 1,25(OH)$_2$D$_3$ to stimulate proliferation was most apparent if cells were exposed to 1,25(OH)$_2$D$_3$ at the early time. In contrast, AP activity was influenced primarily by additions at late times; the presence of 1,25(OH)$_2$D$_3$ or BMP-2 at days 5–8 increased AP specific activity 2.5–3× over control values (Figure 3b). However, markedly greater AP expression in cultures with CS-FBS was only seen when cells were cultured simultaneously and continuously with 1,25(OH)$_2$D$_3$ + BMP-2; under these conditions, AP specific activity was comparable to that seen with stromal cells cultured with nonstripped serum + 10^{-8} M dex, generating AP activities of around 200 nmol/min/10^6 cells. Because 1,25(OH)$_2$D$_3$ induced stimulation of cell proliferation in CS-FBS cultures at early times of culture, and nonadherent cells were present during this period, it was important to ascertain whether the response of cells to 1,25(OH)$_2$D$_3$ was due to a direct effect on stromal cell division or was mediated indirectly via effects on nonadherent cells. Therefore, 1,25(OH)$_2$D$_3$ and CS-FBS was added at day 3 to stromal cell cultures in which nonadherent cells were removed at day 1. These cultures were compared with those in which 1,25(OH)$_2$D$_3$, nonadherent cells and CS-FBS were present simultaneously until day 5 when nonadherent cells were removed [see protocol (iii), *Materials and Methods*]. Cell proliferation, determined by [^{3}H]-thymidine incorporation, was measured in the presence of 1,25(OH)$_2$D$_3$ at concentrations ranging from 10^{-10}–10^{-7} M **(Figure 4).** In the presence of the nonadherent cells, the growth response of stromal cells to 1,25(OH)$_2$D$_3$ was dose-dependent, with 10^{-7} M 1,25(OH)$_2$D$_3$ inducing a 2.5-fold increase in DNA synthesis. In the same cultures 1,25(OH)$_2$D$_3$ caused a maximum twofold increase in cell number (control = 62 × 10^4; + 1,25(OH)$_2$D$_3$ = 116 × 10^4 cells). However, when stromal cells, grown without nonadherent cells from day 1, were subsequently cultured with a dose range of 1,25(OH)$_2$D$_3$ from days 3–6, no enhancement of DNA synthesis occurred. Cell numbers also did not increase under these conditions (data not shown). It is unlikely that increases in cell growth of this magnitude were the result of 1,25(OH)$_2$D$_3$-mediated macrophage proliferation because, firstly, very few nonstromal cells remained at the time of assay and, secondly, macrophages could not have been included in the cell counts as they do not readily detach with trypsin. These observations suggest that 1,25(OH)$_2$D$_3$ does not directly stimulate stromal cell proliferation.

The effects of early and late treatment with dex and/or 1,25(OH)$_2$D$_3$ on stromal cell differentiation was also investigated. When treatment at the early time with 1,25(OH)$_2$D$_3$ was followed by culture in any media containing dex at the late times, an increase in cell growth was no longer apparent (Figure 3c). In addition, these treatments produced little or no increase in AP expression (Figure 3d), which contrasts to the marked induction of AP activity by dex + 1,25(OH)$_2$D$_3$ when cells are cultured in standard medium.[4] Only when treated continuously with both dex + 1,25(OH)$_2$D$_3$ were the levels of AP activity in cells cultured in CS-FBS strongly increased, suggesting that under these culture conditions 1,25(OH)$_2$D$_3$ may be required for significant dex-induced osteoblastic differentiation.

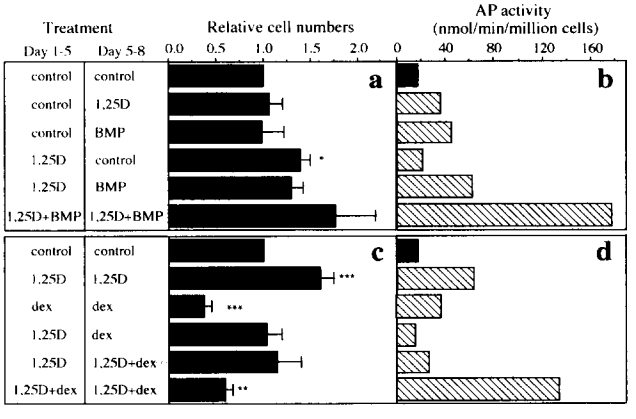

Figure 3. Effects of early and/or late addition of 1,25(OH)$_2$D$_3$ and BMP-2 [(a) and (b)] and 1,25(OH)$_2$D$_3$ and dex [(c) and (d)] on stromal cell growth and alkaline phosphatase (AP) activity. Cells were plated directly into αMEM + 15% CS-FBS and cultured as shown in Figure 1. On day 8, cell numbers and AP activity were measured. In (a) and (c) the relative cell numbers (mean ± SEM) obtained in three or more separate experiments are given. Statistical significance is indicated: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. In (b) and (d) AP activities (1 well per treatment) were determined in wells cultured in parallel to those used for cell counts. AP activities for a representative experiment are shown and similar results were obtained in two other experiments. Treatments: control = no treatment, 1,25D = 1,25(OH)$_2$D$_3$ 10^{-8} M, dex = dexamethasone 10^{-8} M, BMP = rhBMP-2 100 ng/ml.

Discussion

Cell culture in charcoal-stripped serum has frequently been used to study steroid requirements for growth and differentiation.[33–36] While many of these studies have indicated no adverse effects of the stripping procedure on cell proliferation,[33,36] it has been known for some time that, in addition to removal of steroids, this treatment also removes growth factors.[27] In addition, a recent report by HyClone Laboratories[28] has indicated that charcoal

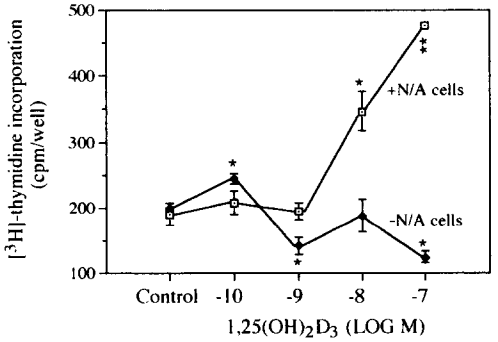

Figure 4. Effect of 1,25(OH)$_2$D$_3$ on proliferation ([^{3}H]-thymidine incorporation into DNA) of stromal cells in the presence and absence of nonadherent marrow cells (N/A cells). Stromal cells were cultured with a concentration range of 1,25(OH)$_2$D$_3$ with or without removal of the nonadherent cells as described in protocol (iii) (*Materials and Methods* section). [^{3}H]-thymidine incorporation was assayed on day 7 when cells of duplicate wells were pulsed with 2 μCi/well and harvested after 6 h incubation. Data are representative of two experiments. Statistically significant differences relative to the control values are given by: $*p < 0.05$ and $**p < 0.01$.

stripping of fetal bovine serum also markedly reduces (>50%) the levels of several hormones known to influence cell growth and differentiation including insulin, thyroid hormone, parathyroid hormone, and prostaglandins. Thus, the use of charcoal-stripped serum permits an initial assessment of growth and differentiation capacity under conditions of diminished growth factors and hormones as well as steroids. When rat bone marrow stromal cells were cultured in medium containing CS-FBS, both the proliferation of adherent cells and their ability to differentiate into osteoblasts was diminished. In addition to factors removed from serum by the charcoal stripping, it is also possible that the impaired development of stromal cells may in part be due to phenol red indicator which was present in medium supplemented with untreated serum but absent from CS-FBS medium. Phenol red may mimic the effects of estrogen on some cells.[25] However, exogenously added steroid hormones including $1,25(OH)_2D_3$ and 17β-estradiol were unable to fully reverse the effects of culture in CS-FBS medium, implying that other factors in addition to steroids were required for proliferation and osteogenesis. Growth of stromal cells in CS-FBS medium was increased by the presence of nonadherent marrow cells. Identical effects on growth were produced with medium conditioned by nonadherent cells indicating that diffusible factors released from these cells are mitogenic for stromal cells. It is well established that cells in marrow stroma are intimately involved in the regulation of hematopoiesis via cell-cell interactions and cytokine release,[22–24] but our finding corroborates existing evidence that hematopoietic cell types can support the growth of bone marrow stromal cells.[37–39] In vivo the sources of cytokines mitogenic for stromal cells may not be restricted to hematopoietic lineages but could also include: (i) other cell types present in marrow (e.g., reticular cells, adipocytes, endothelial cells), (ii) cells residing on endosteal bone surfaces (osteoblasts, osteoclasts, and lining cells), and (iii) bone matrix-bound cytokines. Thus, an array of cytokines may be derived from sources in close proximity to marrow stroma.

In cultures containing CS-FBS, $1,25(OH)_2D_3$ was the only steroid hormone of those investigated to enhance both stromal cell growth and expression of AP. That increased cell number was a result of stimulated proliferation and not enhanced adherence of colony-initiating cells was indicated by two observations; firstly, incorporation of [^{3}H]-thymidine was induced and, secondly, addition of $1,25(OH)_2D_3$ at the time of plating did not further enhance cell numbers. Increased proliferation was observed only if $1,25(OH)_2D_3$ was present during the early phase (days 1–5) of the culture before removal of the nonadherent cells. Furthermore, in cultures of stromal cells that had been depleted of nonadherent cells, the $1,25(OH)_2D_3$-induced increase in stromal cell proliferation was abolished. Therefore the $1,25(OH)_2D_3$ modulation of stromal cell proliferation is dependent on nonadherent cells. Since nonadherent cell-derived diffusible factors stimulate stromal cell growth, the $1,25(OH)_2D_3$ proliferative effect may be mediated by increasing nonadherent cell production of mitogenic cytokines (or by decreasing production of growth inhibitory cytokines or cytokine inhibitors, or both). Of the numerous cell types present in the nonadherent marrow cell population, monocytes, macrophages, and lymphocytes possess receptors for $1,25(OH)_2D_3$ and are consequently potential $1,25(OH)_2D_3$ responders in marrow cultures.[40] An alternative mechanism by which $1,25(OH)_2D_3$ may stimulate stromal cell proliferation, besides effecting release of mitogenic cytokines from nonadherent marrow cells, would be to render the stromal cells more responsive to cytokines through an up-regulation of cytokine receptors.

$1,25(OH)_2D_3$, when added to media with CS-FBS, not only stimulated cell proliferation but also increased levels of the osteoblast marker enzyme AP. These responses are in contrast to results obtained when $1,25(OH)_2D_3$ is added to stromal cell cultures with complete FBS. While adherent stromal cells in αMEM + 15% FBS show osteogenic differentiation with dex or BMP-2, and $1,25(OH)_2D_3$ potentiates the expression of several osteoblast markers, $1,25(OH)_2D_3$ alone has no influence on AP levels.[3,4] $1,25(OH)_2D_3$ added to cultures of more mature osteoblasts typically inhibits proliferation while increasing expression of differentiated markers such as AP, osteopontin, osteocalcin, and matrix Gla protein.[15,41–43] In the marrow stromal cells cultured with CS-FBS, maximal AP levels were induced by $1,25(OH)_2D_3$ only when added in the late phase of culture (days 5–8), suggesting that by this time a proportion of the cells have differentiated to a stage at which exposure to the vitamin can induce a response characteristic of more mature osteoblastic cells. $1,25(OH)_2D_3$ stimulated AP activity to approximately the same extent as rhBMP-2. In CS-FBS medium, BMP-2 was only a moderate inducer of AP activity, but the level of the enzyme could be increased synergistically by coaddition of $1,25(OH)_2D_3$. Interestingly, a synergistic increase in AP activity was also produced by simultaneous administration of BMP-2 with dex under standard culture conditions,[4] suggesting that the in vivo osteogenic actions of BMP-2 may be dependent upon the presence of a steroid hormone like $1,25(OH)_2D_3$ and/or glucocorticoid.

Expression of AP is not restricted to cells of the osteoblast lineage, although there is a close relationship between increased AP level and osteogenic differentiation, both in cultures containing osteoprogenitor cells such as those derived from bone marrow stroma and fetal calvaria,[3,4,44] and cloned preosteoblast cell lines.[14] In the adherent marrow culture preadipocytic stromal cells and bone endothelial cells exhibit low AP expression and therefore contribute to the measured AP activity. Despite some degree of cellular heterogeneity in rat stromal cultures under standard culture conditions, dex-stimulated expression of AP coincides with induction of other osteoblast phenotypic characteristics.[2,3] Similarly, cells grown in CS-FBS medium expressed mRNAs for AP, osteopontin, and osteocalcin, although their ability to synthesize a mineralized matrix was not assessed. The induction by dex of osteoblastic markers in stromal cells cultured in standard medium was markedly blunted when cells were maintained in CS-FBS medium. Moreover, treatment with dex + $1,25(OH)_2D_3$, which causes even more marked induction of osteoblastic characteristics in stromal cells cultured in standard medium, produced only modest increases in AP enzyme activities for cells maintained in CS-FBS. Only when cells were continuously exposed to both dex and $1,25(OH)_2D_3$ were AP levels considerably elevated. One possible explanation for this is that whereas $1,25(OH)_2D_3$ antagonizes dex induction of adipocyte formation in stromal cells grown under standard conditions,[45] significant adipogenesis occurs in cells cultured with CS-FBS in the presence of dex even with prior or simultaneous addition of $1,25(OH)_2D_3$ (our unpublished observations). In these circumstances, differentiation of stromal cells into adipocytes (which express low levels of AP) competes with osteoblastic differentiation, and consequently reduces the AP levels attained by the culture as a whole.

As well as being responsive to glucocorticoids and $1,25(OH)_2D_3$, some evidence indicates that stromal cells (or a subpopulation of such cells) are sensitive to the sex steroid estradiol. Recently stromal cell lines derived from murine bone marrow were demonstrated to possess estrogen receptors[20] and to respond to the hormone by a suppression of IL-6 production.[46] Moreover, estradiol modulates the expression of selected osteo-

genic markers in an osteoblastic cell line immortalized from stromal mouse bone marrow cultures.[21] In contrast to these observations and the responses of our cultures to dex and $1,25(OH)_2D_3$, estradiol consistently failed to exert any noticeable change in culture characteristics or to modulate proliferation and AP activity. Furthermore, estradiol did not act in synergy with BMP-2 to increase AP (our unpublished observations). While it is acknowledged that estrogen may act on cellular functions not investigated here, our preliminary findings do not support the notion that estrogen controls the process of osteoblast differentiation from the marrow stromal progenitor cell.

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