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The Effect of 1,25-Dihydroxyvitamin D_3 on Hematopoiesis in Long-Term Human Bone Marrow Cultures[1] (42566)

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Abstract. The modulatory effect of 1,25-dihydroxyvitamin D_3 (vit D) on the growth of myeloid progenitors and on the composition of the stromal layer in human bone marrow long-term cultures was studied. Vit D (2×10^{-8} *M*) caused an enhancement in myeloid progenitor cell (CFU-C) growth in the nonadherent and adherent layers during the entire 5-week incubation period. The vitamin did not alter the differentiation pattern of CFU-C (monocyte–macrophage progenitors CFU-M, granulocytic progenitors CFU-G, or monocyte–granulocyte progenitors CFU-GM). Vit D caused a marked increase in the percentage of lipid-containing cells in the adherent layer and an increase in the number of cells that specifically bound My4 monoclonal antibody (McAb), that reacted positively to fluoride-sensitive α-naphthyl acetate esterase, and that phagocytosed *Candida albicans* (CA). Concentrated supernatants harvested from control cultures showed significant levels of myeloid colony stimulating factor (CSF) activity. The addition of vit D to cultures for 5 weeks did not alter CSF levels. These results suggest that vit D may play a role in hematopoiesis by acting directly on the progenitor cells or via the stromal cell production of stimulatory factor(s).

The development of long-term murine marrow cultures by Dexter *et al.* (1, 2) and the adaptation of these techniques to the study of human marrow enabled the investigation of the role of interactions between stromal cells and hematopoietic progenitor cells (3–6). Stem cell maintenance in these cultures is known to depend on the formation of a heterogeneous adherent cell population that is believed to stimulate the hematopoietic microenvironment (7), but the precise designation of the cell types and the role of each cell type in hematopoietic maintenance remains uncertain. In recent years it has become apparent that a variety of physiological agents, such as retinoic acid (8, 9) or 1,25-dihydroxyvitamin D_3 (vit D) (10–12), can induce human normal and leukemic progenitor cells to differentiate along either granulocytic or monocyte–macrophage pathways. Based on these findings it has been suggested that these two agents may have a therapeutic role in preleukemia and some leukemias (11, 13, 14) and phase I clinical studies with these agents have started (13–16).

The long-term culture system permits the study of the effect of prolonged exposure to hematopoietic cells to drugs. It also provides the opportunity to study the effect of various drugs on the supporting stromal layer and the interaction of the supporting stroma with hematopoietic progenitor cells. Therefore, we investigated the effect of maturation inducing agents on hematopoiesis in a long-term culture system. In this report we have assessed the effect of vit D on the proliferative response of hematopoietic progenitors that are continuously exposed to the drug and on the composition and function of the adherent cell population.

Materials and Methods. *Drugs.* 1,25-Dihydroxyvitamin D_3 (a generous gift from Hoffmann–LaRoche, Basel, Switzerland) was stored at −20°C. A stock solution of 2×10^{-3} *M* vit D was prepared in 100% ethanol. The vitamin was diluted before use with phosphate-buffered saline (PBS) to the appropriate concentration. The final concentration of ethanol in culture was 0.001–0.003%. All experiments were performed in subdued light and the flasks containing vit D were covered with aluminum foil.

Bone marrow cell preparation. Ten marrow aspirates were obtained with informed consent

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from patients undergoing cardiac surgery. Specimens were collected in sterile tubes containing preservative-free heparin. Long-term cultures were established according to the method of Coulombel *et al.* (17). In brief: 2–3 $\times$ 10^7 nucleated marrow cells were seeded in 8 ml of growth medium in 25 cm^2 Corning tissue culture flasks (Corning Glass Works, Corning, NY). The growth medium consisted of Iscove's modified Dulbecco's medium (IMDM) supplemented with extra glutamine (400 mg/liter), inositol (40 mg/liter) and folic acid (10 mg/liter), horse serum (12.5% Flow Laboratories), fetal calf serum (FCS) (12.5%, HyClone Lab., Logan, UT), 2-mercaptoethanol (10^{-4} *M*), and hydrocortisone sodium succinate (10^{-6} *M*). The cultures were inoculated with cells from the unprocessed bone marrow aspirate and incubated at 33°C in an atmosphere of 5% CO_2 in air. After an initial 3–4 days, all nonadherent cells were removed and layered over 1077 g/cm^3 Ficoll–Hypaque. Red cells and mature granulocytes were discarded, and light density cells were washed and returned to their original flasks in fresh medium. The cultures were fed at weekly intervals by removing half of the medium containing half of the nonadherent cells. After 1 week in culture 2 $\times$ 10^{-8} *M* vit D was added to half of the flasks (this concentration of vit D was found by us to cause maximal enhancement of CFU-C growth in the semisolid culture system) (12). This concentration of vit D was added to the flasks weekly at the time they were refed.

Enzymatic detachment of adherent layer. Bacto trypsin (Difco, Detroit, MI) was dissolved in citrated saline (pH 7.0) to a concentration equivalent to 0.1% of trypsin Difco 1:250. Nonadherent cells and all of the growth medium were first removed from culture dishes. Cultures were washed twice with PBS and the detached cells were added to the nonadherent cell suspension. To detach adherent cells, 5 ml of the trypsin solution was pipetted onto the adherent layer and the cultures were incubated for 10 min at 37°C in an atmosphere of 5% CO_2 in air. At the end of the incubation period 1 ml of FCS was added to stop further trypsin action, and all adherent cells were collected.

CFU-C culture technique. The previously described agar culture system was used (18). Briefly, 1 $\times$ 10^5 cells (nonadherent or adherent) were plated in IMDM containing 15% FCS, 0.3% agar, and 5% placental-conditioned medium (PCM) in 35-mm petri dishes. Cultures were incubated at 37°C in humidified atmosphere of air–5% CO_2 for 10 days.

Staining of colonies. A recently developed *in situ* staining technique was used (19) to determine the type of myeloid colonies. Staining in sequence for α-naphthyl acetate esterase (ANA esterase) (monocyte–macrophages) and naphthol AS-D chloroacetate esterase (neutrophils) permitted typing of the myeloid colonies from the same culture dish.

Morphological studies of stroma. In situ studies. The adherent cell layer of each culture was evaluated weekly for the presence of lipid-containing cells by examination of the cultures using an inverted microscope. In each flask five random areas of 1 cm^2 were marked and the lipid-containing cells in each microscopic field were counted at 100× magnification. The number of cells per microscopic field was averaged for each culture flask and the mean number of cells and standard error were calculated. In order to determine the number of lipid-containing cells per flask the mean number of cells in a 1-cm^2 area were multiplied by the surface area of the flask (×25).

Studies of trypsinized adherent cells. After trypsinization a single cell suspension of the adherent cells was prepared. The cells were examined on cytocentrifuge preparations (Shandon Instruments, Sewickly, PA) after staining with Wright–Giemsa and α-naphthyl acetate esterase.

Immunofluorescent staining. To determine the percentage of monocyte–macrophages present in the adherent layer, 1 $\times$ 10^6 cells (in suspension) were incubated with (4°C for 30 min) 50 μl of My4 antibody (Coulter Immunology, Hialeah, FL), diluted 1:250 (in PBS + 0.02% sodium azide to prevent capping), washed twice, and further incubated (4°C for 20 min) with fluorescein-conjugated goat anti-mouse IgG (affinity purified, diluted 1:10), and washed three times in PBS (18). The percentage of cells with surface membrane fluorescence was determined under a Leitz Ortholux II fluorescence microscope. Background fluorescence was determined by using a non-reactive monoclonal IgG antibody.

Candida albicans phagocytosis. The per-

centage of mononuclear cells from the adherent layer phagocytosing CA was studied. *Candida albicans* was grown in Sabouraud's dextrose broth for 5–7 days, washed twice in saline, and resuspended in saline to a concentration of 10^6 yeast particles/ml. Our method for assessing phagocytosis has been described previously (20). Capped plastic 12 × 75-mm tubes containing 0.1 ml cell suspension (containing 1×10^5 cells), 0.1 ml AB serum, 0.1 ml Hanks' medium, and 0.2 ml yeast were incubated in a shaking water bath at 37°C. After 30 min incubation cytocentrifuge preparations were prepared and stained with Giemsa. Phagocytosis was enumerated as the percentage of cells containing two or more yeast particles (100 viable cells counted).

Assay of conditioned medium. Cell-free supernatants from long-term cultures incubated for 3–6 weeks were collected, combined, and assayed in an agar culture for colony-stimulating activity. Assays were performed using various volumes of unconcentrated supernatants and conditioned medium concentrated fivefold by ultrafiltration with Amicon PM 10 membrane (Lexington, MA). These supernatants were dialyzed using dialysis tubing with a molecular weight cutoff of 3500 (Thomas Scientific, Philadelphia, PA) and sterilized through filtration by 0.45-μm Millipore membranes. To inactivate vit D activity supernatants were exposed for 5 hr to direct sunlight. Normal human Ficoll–Hypaque buoyant mononuclear nonadherent bone marrow cells (5×10^4/ml) were used as target cells. The CFU-C culture technique was used, as described above.

Statistical analyses were performed utilyzing the Wilcoxon matched pairs signed rank test. Values were considered significantly different if $P < 0.05$.

Results. *Effect of vit D on nucleated cell and CFU-C numbers.* Long-term cultures were initiated from eight marrow specimens and terminated after varying periods of incubation for assessment of the effect of vit D on total cellularity and number of myeloid progenitors in both the adherent and nonadherent fractions. Results were obtained, for each time point investigated, in each experiment, from duplicate cultures assessed separately.

As can be seen in Fig. 1A the total number of cells in the nonadherent fraction of the vit

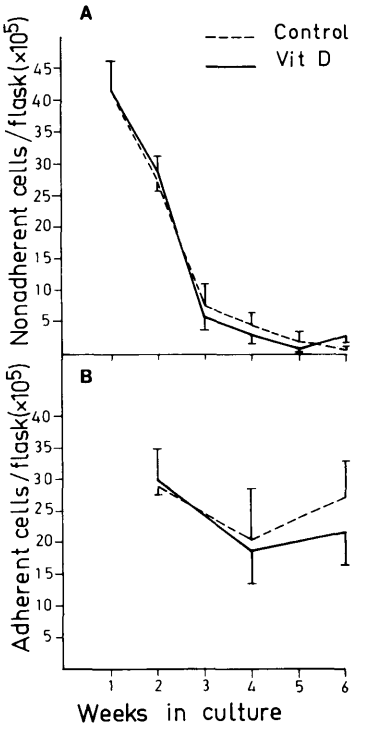

FIG. 1. Effect of vit D on survival of cells in the nonadherent (A) and adherent (B) compartments. Solid line indicates recovery of cells from cultures supplemented with 2×10^{-8} *M* vit D and the dashed line indicates recovery of cells from control cultures. The data represent the means ± SE of duplicate determinations from eight experiments. No corrections have been made for depopulation of the flasks with feeding.

D-treated and untreated cultures showed an initial rapid decline to an average value of 5.6 × 10^5 and 7.7 × 10^5, respectively, after 3 weeks. Over the next several weeks these values remained relatively stable. In contrast, the cellularity of the adherent fractions (Fig. 1B) remained relatively constant during the 6-week incubation period.

The results of CFU-C growth in the nonadherent and adherent layers and the effect of vit D on myeloid progenitor cell growth are shown in Fig. 2 (A and B). In all eight marrow specimens examined vit D enhanced CFU-C growth during the entire incubation period (Fig. 2B). The range of enhancement in the nonadherent and adherent layers was 157–215% and 121–145% of control, respectively, during Weeks 2 to 6 of culture ($P < 0.05$).

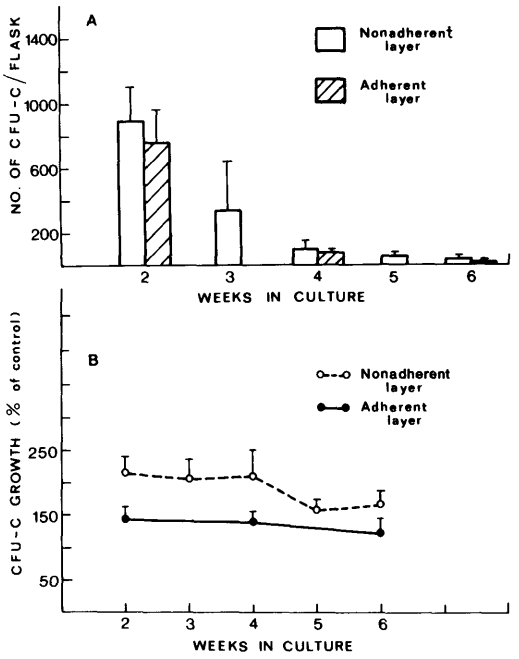

FIG. 2. CFU-C content (A) of human long-term cultures in the nonadherent and adherent compartments after varying periods of incubation and the effect of vit D (B) on CFU-C growth. Solid line indicates enhancement of CFU-C growth, as compared to control, in the adherent layer, and the dashed line indicates enhanced CFU-C growth in the nonadherent layer.

CFU-C subtypes. Table I depicts the effect of vit D on CFU-C subtypes assayed with time in long-term culture in the presence or absence of vit D. In control and vit D-supplemented cultures macrophage colonies predominated during the entire 6 weeks of culture, in both the nonadherent or adherent layers. The addition of vit D did not affect the relative proportions of CFU-G, CFU-M, or CFU-GM.

Composition of the adherent layer. Cultures were sequentially examined with a phase-contrast inverted microscope. Generally the adherent layer was established during the first 2–3 weeks of culture in the presence or absence of vit D, and reached confluency by the end of Week 3.

The cellular composition of the adherent population was heterogeneous. Elongated flattened cells with morphology typical of fibroblasts were observed as well as macrophages. Hematopoietic cells (small round cells) were observed attached to the surface of the adherent cells in islands. Cultures containing vit D showed a marked increase with time in the percentage of lipid-containing cells (7.5-fold increase at Week 6, Fig. 3A).

The effect of vit D on the development of macrophages in the adherent layer was investigated. Adherent cells were removed from the flasks by trypsin and morphological and functional studies were performed. Using the immunofluorescence assay, we found that in vit D-supplemented cultures (Fig. 3B) there was a 2.1-, 1.8-, and 1.8-fold increase at Weeks 2, 4, and 6, respectively ($P < 0.05$) in the percentage of cells reacting with the McAb My4. This McAb specifically binds to monocyte–macrophages (21). In Wright–Giemsa-stained cytocentrifuge preparations a higher number of macrophages were observed in vit D-supplemented cultures as compared to controls (data not shown). These findings were also supported by the results of nonspecific esterase stainings of the preparations (Fig. 3C) which showed a 1.2- to 1.6-fold increase ($P < 0.05$) in the number of cells reacting with α-naphthyl acetate. The percentages of mononuclear cells that phagocytosed CA in the presence or absence of vit D are shown in Fig. 3D. As can be seen in the presence of vit D a higher percentage of cells ($P < 0.05$) phagocytose CA as compared to controls during 6 weeks of culture.

Assays of conditioned medium. Supernatants were harvested from three different long-term bone marrow cultures after incubation for 3–6 weeks in the presence or absence of vit D. A sample of each supernatant was subjected to diafiltration (fivefold). Concentrated and unconcentrated supernatants were tested for activities affecting granulocyte–macrophage colony formation in the presence of various volumes of the supernatants. Results of a representative experiment are summarized in Table II. Unconcentrated CM had minimal CSF levels, however, fivefold-concentrated medium yielded significant levels of activity. Supernatants harvested from cultures that were supplemented with vit D showed the same CSF levels as controls. This CSF activity was compared to that of placental-conditioned medium which is a potent source of CSF (21, 22).

Discussion. In previous studies we have shown that 1,25-dihydroxyvitamin D (2

TABLE I. MORPHOLOGY OF MYELOID COLONIES

Weeks in culture	Nonadherent layer						Adherent layer					
	CFU-G		CFU-M		CFU-GM		CFU-G		CFU-M		CFU-GM	
	Control	Vit D	Control	Vit D	Control	Vit D	Control	Vit D	Control	Vit D	Control	Vit D
2	12 ± 2	16 ± 2	75 ± 4	72 ± 4	15 ± 3	9 ± 2	23 ± 4	17 ± 3	59 ± 7.9	66 ± 5.1	10 ± 3.6	12 ± 3.7
4	16 ± 3	10 ± 2	80 ± 5	85 ± 4	8 ± 3	7 ± 3	17 ± 3	23 ± 6	80 ± 8	68 ± 5.8	8 ± 2.1	8 ± 4.2
6	7 ± 2	3 ± 1	84 ± 8	88 ± 6	8 ± 2	6 ± 4	0	8 ± 2.3	90 ± 11.4	80 ± 12	7 ± 2.1	7 ± 1.9

Note. Comparison of type of myeloid colony growth assayed with time of marrow cells grown in long-term culture in the absence or presence of 2×10^{-8} *M* vit D. 1×10^5 nonadherent or adherent cells/ml were cultured for 10 days. Results expressed as means of percentage of colonies + SE. $n = 4$.

$\times 10^{-8}$ *M*) enhances CFU-C growth from normal and myelodysplastic patients in semisolid cultures (12, 18). In the present study we have investigated the modulatory effect of vit D on the growth and differentiation of hematopoietic progenitors and on the development of the stroma in long-term human bone marrow cultures. We have shown that in the presence of vit D an enhanced number of myeloid progenitors can be consistently detected in the nonadherent as well as in the adherent layer of long-term human marrow cultures for at least 5 weeks. The enhancing effect was already observed after exposure of the cultures to vit D for 1 week and was maintained during the entire 5 weeks of the study.

The development of CFU-C subtypes with time in long-term culture was also investigated by us. Toogood *et al.* (24) have reported that in human long-term cultures granulocytic progenitors predominate during the first 5 weeks in culture, while Slovick *et al.* (25) have demonstrated that after 3–4 weeks Day 7 CFU-G and Day 14 CFU-GM colony subtypes predominate in the cultures. Our study has shown that during the entire 6 weeks in culture CFU-M were the predominant Day 10 colony subtype and that vit D did not affect the relative proportion of myeloid progenitors in the long-term cultures.

Previous studies performed by us and by others in semisolid cultures have shown that vit D induces differentiation of myeloid progenitors along the monocyte–macrophage pathway (10–12). The discrepancies between these findings and our present findings might stem from different culture conditions (continuous suspension culture vs semisolid culture).

In the present experiments we have looked also at the effect of vit D on the development of the stromal layer. In human marrow cultures fat cell development is dependent on the presence of hydrocortisone (24). To our knowledge, this is the first report indicating that in the presence of hydrocortisone and vit D (2×10^{-8} *M*) there is enhanced development of lipid-containing cells in the adherent layer. It is tempting to relate the enhanced growth of CFU-C in our vit D-supplemented cultures to the increased number of lipid-containing cells. However, it cannot be excluded that the stimulating effect of vit D could be

via other cellular or humoral components present in the culture.

In the present study we have also shown that following continuous incubation of cultures with vit D the following changes occurred in the adherent layer, (a) a higher percentage of cells reacted with the McAb My4 that specifically binds to monocyte–macrophages, (b) a higher proportion of cells reacted positively for α-naphthyl esterase, and (c) a higher proportion of mononuclear cells phagocytosed CA. Therefore we conclude that vit D induces enhanced growth of monocyte–macrophages in the adherent layer. These studies are in agreement with previous studies performed by us and by others in which it was shown that vit D induces monocytic differentiation in normal human bone marrow cells maintained in short-term (7 day) liquid culture (10–12).

Our results with regard to the production of CSF in long-term culture support those of Heard *et al.* (26), Shadduck *et al.* (27), and Gualtieri *et al.* (28) and are contrary to the experience of Toogood *et al.* (24). The discrepancy may be attributed to the fact that the latter investigators did not concentrate the collected supernatants, thus low levels of CSF could not be detected.

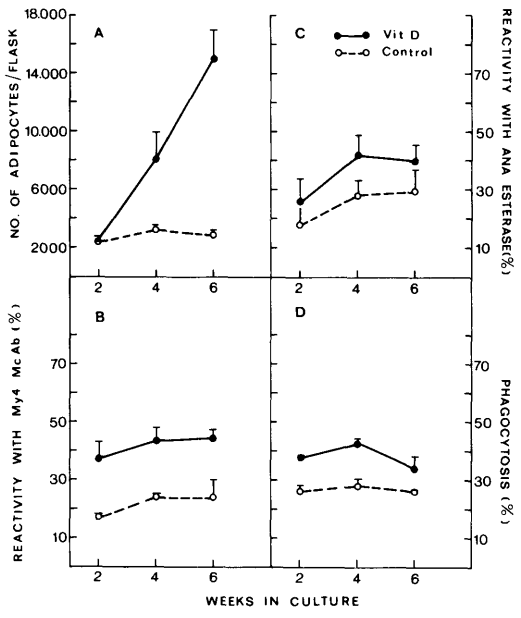

FIG. 3. (A) Number of lipid-containing cells per flask. Means ± SE. (The total number of adherent cells is indicated in Fig. 1B). (B) Percentage of cells reacting with McAb which recognize My4. Means ± SE. (C) Percentage of ANA esterase positive cells. Means ± SE. (D) Percentage of mononuclear cells that contain two or more yeast cells. Means ± SE.

TABLE II. EFFECT OF VIT D ON CSF ACTIVITY IN SUPERNATANTS OF LONG-TERM CULTURES

Type and concentration of conditioned medium (CM)	CSF activity determined in cultures containing:	
	Medium (control)	Vit D
—	0	0
PCM	80 + 0.6	ND
5% unconcentrated CM	2 + 0.3	4 + 0.8
15% unconcentrated CM	7 + 1	4 + 2
15% concentrated	30 + 8	30 + 6

Note. CSF activity in supernatants harvested from vit D-supplemented and control cultures following 3–6 weeks of incubation. 5×10^4 buoyant nonadherent fresh bone marrow cells were cultured in the presence of 5 or 15% of unconcentrated or concentrated CM. PCM served as control for CSF activity. Colonies were scored on Day 10.

In spite of our expectation that cultures containing a higher percentage of macrophages (vit D-treated cultures) will show higher colony stimulating activity (CSA) levels, the same activity was observed in control and vit D-treated cultures. Koeffler *et al.* (29) have shown that vit D decreased mRNA accumulation of CSF in mitogen-activated T lymphocytes. It might be speculated that vit D might inhibit CSF production by macrophages. These results as well as our present observations using the long-term culture system support the idea that vit D may play a role in hematopoiesis.

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