

## Vitamin D compounds. Effect on clonal proliferation and differentiation of human myeloid cells.

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### Research Article

We examined the effect of 1 alpha,25-dihydroxyvitamin D<sub>3</sub> (1 alpha,25(OH)<sub>2</sub>D<sub>3</sub>) and a variety of vitamin D analogs on proliferation and differentiation of normal and leukemic myeloid clonogenic cells. Only cells from myeloid leukemic lines that contained relatively mature cells (HL-60, U937, THP, HEL, M1) were induced to differentiate and were inhibited in their clonal growth by exposure to 1 alpha,25(OH)<sub>2</sub>D<sub>3</sub> (50% inhibition, 3 X 10<sup>-8</sup>-8 X 10<sup>-10</sup> M). A fluorinated analog of vitamin D was 5-10-fold more potent than 1 alpha,25(OH)<sub>2</sub>D<sub>3</sub>. Cells from a human myeloblast line (KG-1) and normal human granulocyte-monocyte stem cells (GM-CFC), both of which depend on colony-stimulating factor (CSF) for clonal growth, were stimulated in their clonal proliferation by 1 alpha,25(OH)<sub>2</sub>D<sub>3</sub> in the presence of suboptimal concentrations of CSF. Leukemic cells from 10 of 14 patients with myeloid leukemia, but not normal GM-CFC from 12 patients in remission, were markedly inhibited in their clonal proliferation by 1 alpha,25(OH)<sub>2</sub>D<sub>3</sub>. Our results suggest that 1 alpha,25(OH)<sub>2</sub>D<sub>3</sub> may be a cofactor in hematopoiesis and that vitamin D analogs may have a differential effect on normal versus leukemic growth.

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# Vitamin D Compounds

## Effect on Clonal Proliferation and Differentiation of Human Myeloid Cells

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### Abstract

We examined the effect of  $1\alpha,25$ -dihydroxyvitamin  $D_3$  ( $1\alpha,25(OH)_2D_3$ ) and a variety of vitamin D analogs on proliferation and differentiation of normal and leukemic myeloid clonogenic cells. Only cells from myeloid leukemic lines that contained relatively mature cells (HL-60, U937, THP, HEL, M1) were induced to differentiate and were inhibited in their clonal growth by exposure to  $1\alpha,25(OH)_2D_3$  (50% inhibition,  $3 \times 10^{-8}$ – $8 \times 10^{-10}$  M). A fluorinated analog of vitamin D was 5–10-fold more potent than  $1\alpha,25(OH)_2D_3$ . Cells from a human myeloblast line (KG-1) and normal human granulocyte-monocyte stem cells (GM-CFC), both of which depend on colony-stimulating factor (CSF) for clonal growth, were stimulated in their clonal proliferation by  $1\alpha,25(OH)_2D_3$  in the presence of suboptimal concentrations of CSF. Leukemic cells from 10 of 14 patients with myeloid leukemia, but not normal GM-CFC from 12 patients in remission, were markedly inhibited in their clonal proliferation by  $1\alpha,25(OH)_2D_3$ . Our results suggest that  $1\alpha,25(OH)_2D_3$  may be a cofactor in hematopoiesis and that vitamin D analogs may have a differential effect on normal versus leukemic growth.

### Introduction

The active form of vitamin D is  $1\alpha,25$ -dihydroxyvitamin  $D_3$  ( $1\alpha,25(OH)_2D_3$ ),<sup>1</sup> which results from sequential hydroxylation in the liver and kidney of the parental vitamin  $D_3$ .  $1\alpha,25(OH)_2D_3$  is generally accepted as the principal form of vitamin D responsible for calcium homeostasis (1). The classic target organs of this secosteroid are the intestine, bone, and kidney. Recently, the vitamin D endocrine system was found to interact with the hematopoietic system. The evidence for these interactions is that:

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1. *Abbreviations used in this paper:*  $1\alpha,25(OH)_2D_3$ ,  $1\alpha,25$ -dihydroxyvitamin  $D_3$ ; AML, acute myelogenous leukemia; CFU-GM, colony-forming unit-granulocyte-macrophage; CML, chronic myelogenous leukemia; CSF, colony-stimulating factor;  $24,24-F_2-1\alpha,25(OH)_2D_3$ ,  $24,24$ -difluoro- $1\alpha,25$ -dihydroxyvitamin  $D_3$ ; FCS, fetal calf serum; GM-CFC, granulocyte-macrophage colony-forming cell; NBT, nitroblue tetrazolium.

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(a) hematopoietic cells have receptors for  $1\alpha,25(OH)_2D_3$  (2, 3); (b)  $1\alpha,25(OH)_2D_3$  modulates myeloid stem cell colony-forming unit-granulocyte-macrophage (CFU-GM) differentiation towards macrophages (4, 5); (c) very low concentrations of  $1\alpha,25(OH)_2D_3$  ( $10^{-10}$  M) induce neoplastic cells from several myeloid cell lines (HL-60, U937) to differentiate to macrophage-like cells (6–9); and (d) activated, normal macrophages can synthesize  $1\alpha,25(OH)_2D_3$  (10).

Acute myelogenous leukemia (AML) arises from neoplastic transformation of a myeloid stem cell. Most acute myelogenous leukemia cells are unable to undergo terminal differentiation. Instead, these cells remain in the proliferative pool and rapidly accumulate. In the present study, we investigated the effects of  $1\alpha,25(OH)_2D_3$  and a variety of other vitamin D analogs on the clonal proliferation of both myeloid leukemia cell lines blocked at different stages of maturation and leukemic myeloid stem cells harvested from patients. The results were contrasted with the effects of the same vitamin D compounds on proliferation of normal myeloid progenitors harvested from leukemic patients in remission. Further studies were performed to attempt to elucidate the mechanism by which vitamin D compounds affect hematopoietic proliferation.

### Methods

*Cells.* Several myeloid cell lines were used in this study; details and references are given in Table I. These cell lines were cultured in tissue culture flasks (Miles Laboratories, Naperville, IL) in alpha medium (Flow Laboratories, Inc., McLean, VA) with 10% fetal calf serum (FCS; Irvine Scientific, Santa Ana, CA). For plating experiments, only cells in logarithmic growth were used. The calcium concentration in the media was 1.8 mM.

Bone marrow was obtained from healthy volunteers and leukemic patients by aspiration from the iliac crest after written consent was obtained. For anticoagulation, preservative-free heparin prepared from porcine intestinal mucosa (5 U/ml; O'Neal, Jones & Feldman, St. Louis, MO) was used. The mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients, washed twice in phosphate-buffered saline (PBS), and suspended in alpha medium containing 10% FCS and 1% penicillin and streptomycin (Irvine Scientific). Peripheral blood mononuclear cells were obtained with similar techniques from consenting leukemia patients who had a high concentration of leukemia cells in their blood (>30,000 per microliter). Details about the leukemia patients are given in Table III. The bone marrow samples usually contained >95% leukemic cells by morphology. In the samples from the peripheral blood, >80% of the mononuclear cells were neoplastic. The bone marrows from 12 leukemia patients in remission were also studied. Remissions were determined independently by a pathologist and one author (Dr. Munker) using morphology and karyotype analysis. The remission patients were off therapy for at least 30 d.

*Vitamin  $D_3$  compounds.*  $1\alpha,25(OH)_2D_3$  and  $24,24$ -difluoro- $1\alpha,25$ -dihydroxyvitamin  $D_3$  ( $24,24-F_2-1\alpha,25(OH)_2D_3$ ) and other analogs of vitamin  $D_3$  that are listed in Fig. 2 were obtained as a gift from Dr. Milan Uskokovic and Dr. Gary Truitt (Hoffmann-LaRoche Inc., Nutley, NJ). The compounds ( $10^{-3}$  M) were dissolved in pure ethanol and stored

Table I. Effects of  $1\alpha,25(\text{OH})_2\text{D}_3$  and  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  on Clonal Growth of Cells from Myeloid Leukemia Lines

| Cell line   | Description                  | Reference | 50% Inhibitory concentration        |                                                             |
|-------------|------------------------------|-----------|-------------------------------------|-------------------------------------------------------------|
|             |                              |           | $1\alpha,25(\text{OH})_2\text{D}_3$ | $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ |
|             |                              |           | <i>M</i>                            | <i>M</i>                                                    |
| HL-60       | human promyelocytes          | (11)      | $8 \times 10^{-10}$                 | $4 \times 10^{-11}$                                         |
| U937        | human monoblasts/histiocytes | (12)      | $4 \times 10^{-9}$                  | $7 \times 10^{-9}$                                          |
| HEL         | bipotent*                    | (13)      | $2 \times 10^{-8}$                  | $3 \times 10^{-9}$                                          |
| THP-1       | human monoblasts             | (14)      | $3 \times 10^{-8}$                  | $8 \times 10^{-10}$                                         |
| M1          | mouse myeloid leukemia       | (15)      | $1 \times 10^{-8}$                  | $5 \times 10^{-10}$                                         |
| HL-60 blast | human early myeloblasts      | (16)      | no inhibition                       | no inhibition                                               |
| KG-1A       | human early myeloblasts      | (17)      | no inhibition                       | no inhibition                                               |
| KG-1        | human myeloblasts            | (18)      | no inhibition‡                      | no inhibition‡                                              |
| K562        | bipotent*                    | (19)      | no inhibition                       | no inhibition                                               |

\* Monoblast and erythroblast characteristics. ‡ Stimulation at suboptimal concentrations of CSF.

protected from light at  $-20^\circ\text{C}$ . Immediately before use, dilutions of the stock material in alpha medium with 10% FCS were made. The maximal concentrations of ethanol in the culture (0.05%) did not influence cell growth or differentiation.

**Colony formation in soft agar and differentiation studies.** Cells were plated in Lux culture dishes in a two-layer soft agar system according to previously described methods (20, 21). The underlayer contained 0.5% agar; the upper layer, 0.3% agar (Difco Laboratories, Inc., Detroit, MI). The culture medium was alpha medium; in some experiments, Iscove's medium was substituted for alpha medium with similar results. For each plating experiment,  $2\times$  stock solutions of agar were prepared by autoclaving with distilled water and the agar was kept at  $43^\circ\text{C}$ . For each layer, these stock solutions were mixed with prewarmed alpha medium (which contained a final concentration of 17% FCS and 1% penicillin-streptomycin) and 1 ml was carefully pipetted into each culture dish. This mixture became semisolid at room temperature within 20 min. The colony-stimulating factor (CSF) and the compounds to be tested were mixed into the underlayer before solidification of the agar. As a source of CSF, different concentrations of conditioned medium from a human T lymphocyte line (Mo) were used (22). For human bone marrow,  $2 \times 10^5$  cells were plated. Myeloid cell lines were plated at 1,000–5,000 cells/plate. The plating efficiency varied between 5% (KG) and 38% (K562). The KG cell line (passages 12–20) formed colonies only in the presence of CSF. FCS was present in all liquid and soft agar cultures. Cultures were placed in a humidified atmosphere, 5%  $\text{CO}_2$ , at  $37^\circ\text{C}$ . Colonies ( $\geq 40$  cells) were scored after 10–12 d with an inverted microscope. All experiments were done using triplicate or quadruplicate plates per experimental point. Control plates with no CSF were performed for each experiment.

The production of colony-stimulating activity under the influence of  $1\alpha,25(\text{OH})_2\text{D}_3$  was tested by culturing various cell lines (HL-60, HEL, KG-1, THP-1, U937) with  $5 \times 10^{-7}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$ . After 5 d, the supernatants were harvested and stored at  $4^\circ\text{C}$  until used. When tested, the supernatants (0.1–10%) were mixed into the lower agar layer and assayed for stimulation of granulocyte-macrophage colony-forming cell (GM-CFC).

The induction of differentiation was measured by morphology, histochemistry, and function, including adherence to plastic and reduction of nitroblue tetrazolium (NBT). The NBT reduction was studied as previously described (23). Briefly, the cells were mixed with an equal volume of a solution containing 1.25 mg/ml NBT (Sigma Chemical Co., St. Louis, MO), 17 mg/ml bovine serum albumin, and 1  $\mu\text{g}/\text{ml}$  12-O-tetradecanoylphorbol 13-acetate (Miles Laboratories) for 30 min. at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . They were then washed in PBS, cytocentrifuged, fixed in methanol, and stained with Giemsa. For statistical comparisons between different points, Student's *t* test was used.

## Results

**Effect of vitamin D metabolites on proliferation and differentiation of myeloid leukemia cell lines.** We examined the effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  and  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  on the clonal proliferation of myeloid leukemia cell lines blocked at different stages of differentiation (Fig. 1, Table I). The clonal growth of those

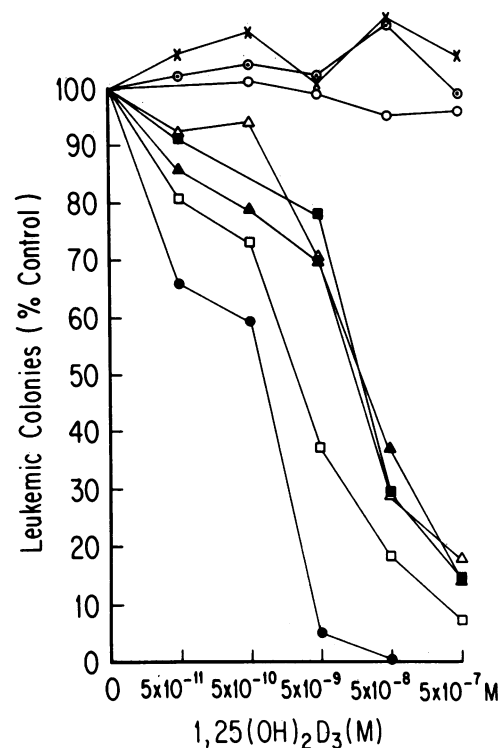


Figure 1. Effect of various concentrations of  $1\alpha,25(\text{OH})_2\text{D}_3$  on clonal growth of myeloid cell lines (●, HL-60; □, U937; △, M1; ▲, THP-1; ○, HEL; ○, HL-60 blast; ×, KG-1A; ○, K562). Cells were plated in soft agar containing different concentrations of  $1\alpha,25(\text{OH})_2\text{D}_3$  and the number of colonies were enumerated on day 12 of culture. Results are expressed as a percent of control cells not exposed to  $1\alpha,25(\text{OH})_2\text{D}_3$ . Each point represents the mean of three experiments with triplicate dishes per point.

Table II. Effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  and  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  on the Ability of Cells from Leukemic Lines to Reduce NBT

| Cell line                                                   | Percentage NBT-positive cells |             |      |       |      |      |       |      |
|-------------------------------------------------------------|-------------------------------|-------------|------|-------|------|------|-------|------|
|                                                             | HL-60                         | HL-60 blast | KG-1 | KG-1A | U937 | HEL  | THP-1 | M1   |
| Control                                                     | 2±2                           | 0±1         | 0±0  | 0±0   | 1±1  | 3±2  | 35±5  | 2±1  |
| $1\alpha,25(\text{OH})_2\text{D}_3$                         | 60±7                          | 1±1         | 0±0  | 0±0   | 49±8 | 27±9 | 48±5  | 16±3 |
| $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ | 61±3                          | 1±1         | 0±0  | 0±0   | 50±4 | NT‡  | NT    | NT   |

Results represent the mean of four separate experiments (±SD). \* Cells cultured in liquid media containing  $5 \times 10^{-7}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  for 5 d, washed, and tested for their ability to reduce NBT. ‡ NT, not tested.

lines that contain relatively immature cells (K562, KG-1a, KG-1, HL-60 blasts) was not inhibited by the vitamin D metabolites. Likewise, cells of these lines were not induced to differentiate as determined by NBT reduction when cultured with the vitamin D metabolites (Table II). Previous studies showed that the ability to reduce NBT was a sensitive reflection of differentiation of myeloid cells (23). In contrast, the clonal proliferation of those lines that contain relatively mature cells (HL-60, U937, THP-1, HEL, M1) was inhibited by the vitamin D metabolites. 50% inhibition of colony formation occurred in the concentration range of  $3 \times 10^{-8}$ – $8 \times 10^{-10}$  M. Likewise, cells of these lines were induced to differentiate when cultured with the vitamin D metabolite (Table II).

In four of the five sensitive lines, the fluorinated vitamin D compound was about 10–50-fold more potent than  $1\alpha,25(\text{OH})_2\text{D}_3$  in the inhibition of clonal proliferation (Table I). 50% inhibition of colony formation occurred in the concentration range of  $3 \times 10^{-9}$ – $4 \times 10^{-11}$  M  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  for these four myeloid cell lines. Cell lines that could not be induced to differentiate with  $1\alpha,25(\text{OH})_2\text{D}_3$  also could not be induced to differentiate with the fluorinated compound (Table II). The concentration that resulted in 50% of the maximum NBT reduction was  $4 \times 10^{-9}$  M when the HL-60 cells were cultured with  $1\alpha,25(\text{OH})_2\text{D}_3$  and  $2 \times 10^{-9}$  M when the cells were cultured with the fluorinated analog (data not shown).

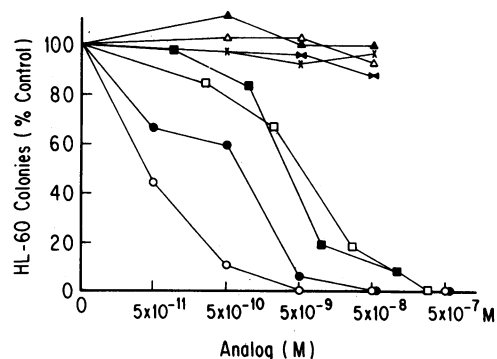


Figure 2. Effect of various concentrations of different analogs of vitamin D on clonal growth of HL-60 promyelocytes. The analogs are: ●,  $1\alpha,25(\text{OH})_2\text{D}_3$ ; ■,  $1\alpha,24\text{S}25(\text{OH})_3\text{D}_3$ ; ▲,  $24\text{S},25(\text{OH})_2\text{D}_3$ ; △,  $1\alpha(\text{OH})\text{D}_3$ ; ○,  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ ; □,  $1\alpha,24\text{R}25(\text{OH})_3\text{D}_3$ ; ×,  $24\text{R}25(\text{OH})_2\text{D}_3$ ; ▶,  $25(\text{OH})\text{D}_3$ . Cells were plated in soft agar with various vitamin D analogs and the number of colonies enumerated after 10 d of culture. Results are expressed as a percent of control cells not exposed to the vitamin D analog. Each point represents the mean of three experiments with triplicate dishes per point.

We examined the effect of six other vitamin D compounds on the clonal proliferation of the HL-60 promyelocytes (Fig. 2). The inhibition of colony formation paralleled the known ability of the analog to bind to the cellular  $1,25(\text{OH})_2\text{D}_3$  receptor (6). The rank order of potency of the active compounds was:  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3 > 1\alpha,25(\text{OH})_2\text{D}_3 > 1\alpha,24\text{R},25(\text{OH})_3\text{D}_3 = 1\alpha,24\text{S},25(\text{OH})_3\text{D}_3$ . In contrast,  $1\alpha\text{-OH-D}_3$ ,  $25\text{-OH-D}_3$ ,  $24\text{S},25(\text{OH})_2\text{D}_3$ , and  $24\text{R},25(\text{OH})_2\text{D}_3$  had no effect on clonal growth in the concentrations that we tested (Fig. 2).

We performed time-response experiments in order to determine the rapidity by which  $1\alpha,25(\text{OH})_2\text{D}_3$  inhibited the clonal growth of HL-60 cells. The cells were exposed to  $1\alpha,25(\text{OH})_2\text{D}_3$  ( $10^{-6}$  M) for various periods of time, washed thoroughly three times, plated in soft agar, and colony formation was determined after 10 d of culture. Colony formation was inhibited by 50% within 20 h of exposure to  $1\alpha,25(\text{OH})_2\text{D}_3$  and was totally inhibited by 48 h (Fig. 3). Paradoxically, we noted a consistent, significant stimulation of colony formation if the cells were exposed to  $1\alpha,25(\text{OH})_2\text{D}_3$  for a short time (1–10 h) ( $P \leq 0.05$ ). The stimulation was in the range of 50%. A similar enhancement of clonal growth was noted when HL-60 promyelocytes were exposed to  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  and when U937 monoblasts were briefly (8 h) exposed to  $1\alpha,25(\text{OH})_2\text{D}_3$  ( $10^{-6}$  M) (data not shown).

We have developed a unique human myeloblast cell line, KG-1, whose clonal proliferation in soft agar culture is dependent on CSF. We examined in detail the effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  on the clonal growth of the KG-1 cells (Fig. 4). In the presence of

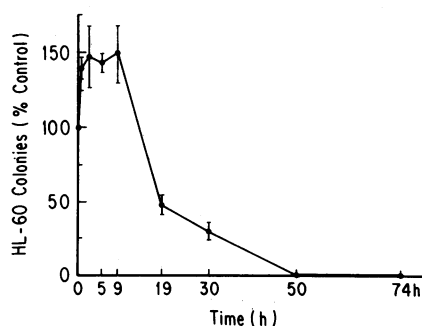
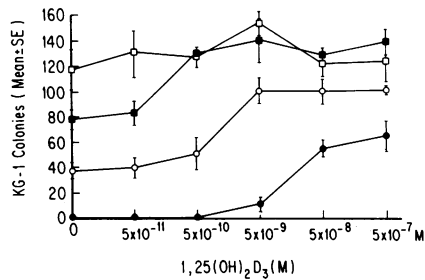


Figure 3. Effect of pulse exposures of  $1\alpha,25(\text{OH})_2\text{D}_3$  on the clonal growth of HL-60 promyelocytes. The HL-60 cells were exposed to  $10^{-6}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$  for the indicated time periods, washed, plated ( $1 \times 10^3$  cells/dish) in soft agar and colonies were enumerated after 10 d of culture. The results are expressed as a percent of control cells not exposed to  $1\alpha,25(\text{OH})_2\text{D}_3$ . Each point represents the mean±SD of three experiments with quadruplicate dishes per point.



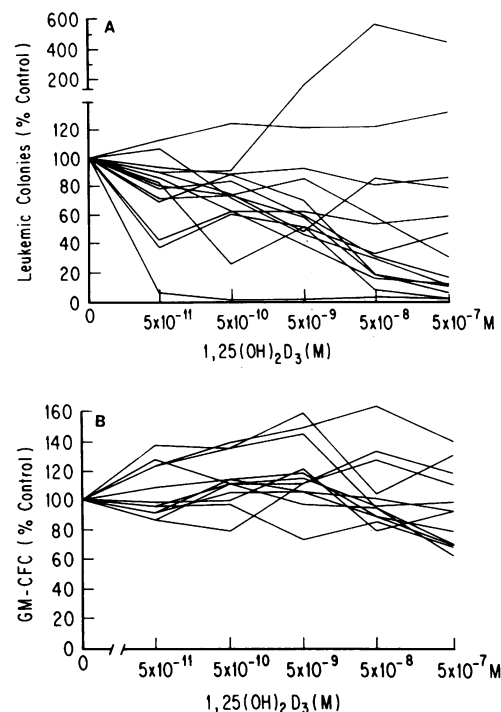
**Figure 4.** Effect of both  $1\alpha,25(\text{OH})_2\text{D}_3$  and colony-stimulating factor on clonal growth of KG-1 myeloblasts. The KG-1 cells ( $3 \times 10^3$ /dish) were plated in soft agar with various concentrations of  $1\alpha,25(\text{OH})_2\text{D}_3$  and 0, ●; 0.2%, ○; 0.5%, ■; and 2%, □ Mo-conditioned medium as source of CSF. Colonies were enumerated on day 10 of culture and the data are expressed as the mean number of colonies  $\pm$  SD from three experiments using quadruplicate dishes per point.

maximally stimulating concentrations of CSF, we noted no effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  on clonal proliferation of the KG-1 cells. In contrast, in the presence of either submaximally stimulating concentrations of CSF or in the absence of CSF,  $1\alpha,25(\text{OH})_2\text{D}_3$  significantly ( $P \leq 0.05$ ) enhanced KG-1 clonal proliferation in a dose-dependent fashion. The KG-1 cells formed less than 5 colonies in the absence of both CSF and  $1\alpha,25(\text{OH})_2\text{D}_3$ . In contrast, a mean  $62 \pm 21$  ( $\pm$ SE) colonies were formed in cultures containing  $5 \times 10^{-7}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$ . In cultures containing one-third maximally stimulating concentrations of CSF, the KG-1 cells formed a mean  $40 \pm 5$  ( $\pm$ SE) colonies. The addition of  $5 \times 10^{-9}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$  to these culture plates increased colony formation to a mean  $80 \pm 10$  ( $\pm$ SE), a significant ( $P < 0.01$ ) stimulation. The enhanced colony formation observed in the presence of  $1\alpha,25(\text{OH})_2\text{D}_3$  and submaximally stimulating concentrations of CSF was additive, not synergistic.

**Effect of vitamin D metabolites on proliferation of fresh leukemic and normal human myeloid colony-forming cells.** We examined the effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  and  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  on the clonal growth of leukemic blast cells harvested from peripheral blood or bone marrow of 14 individuals with active myeloid leukemia. The patients had acute or chronic myelogenous leukemia (CML) (Fig. 5 A, Table III). 10 of the 14 leukemic patients had neoplastic cells that were at least 50% inhibited in their colony formation in the presence of  $5 \times 10^{-7}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$ , and 5 of the 14 leukemic patients had at least a 50% inhibition of leukemic colony formation at  $5 \times 10^{-9}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$ . Clonal growth of AML and CML cells was inhibited approximately equally by  $1\alpha,25(\text{OH})_2\text{D}_3$ . However,  $1\alpha,25(\text{OH})_2\text{D}_3$  stimulated the clonal proliferation of blast cells from one patient (No. 3) by  $>400\%$ . This 33-yr-old patient had M1 acute myeloblastic leukemia. The cytogenetic examination of his blast cells showed 46 chromosomes plus a consistent extra-chromosomal fragment.

The  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  dose-response curves of clonal growth inhibition generally paralleled the  $1\alpha,25(\text{OH})_2\text{D}_3$  curves (data not shown); however, the fluorinated analog was 10–100-fold more inhibitory than  $1\alpha,25(\text{OH})_2\text{D}_3$  on the clonal growth of cells from 4 of 12 patients (Nos. 8, 9, 11, 12) (Table III).

The  $1\alpha,25(\text{OH})_2\text{D}_3$  and  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  had little effect on the clonal growth of myeloid stem cells (GM-CFC) harvested from 12 myeloid leukemia patients who were in remission postchemotherapy (Fig. 5 B). The GM-CFC from leu-



**Figure 5.** Effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  on clonal growth of leukemic and normal myeloid colony-forming cells. Marrow or peripheral blood leukemic cells were obtained from 14 patients with AML (Fig. 5 A); also, marrow cells were harvested from 12 AML patients in remission (Fig. 5 B). The light density mononuclear cells were cultured in the presence of various concentrations of  $1\alpha,25(\text{OH})_2\text{D}_3$  and 5.0% Mo-conditioned medium (source of colony-stimulating factor). Colonies were counted on day 12 of culture and each point represents the mean of triplicate cultures. Results are expressed as a percent of control cells not exposed to  $1\alpha,25(\text{OH})_2\text{D}_3$ . Control and AML cultures contained a mean of  $135 \pm 67$  and  $148 \pm 208$  ( $\pm$ SD) myeloid colonies, respectively.

kemic patients in remission were not inhibited more than 30% at any concentration of the two vitamin D compounds ( $5 \times 10^{-7}$ – $5 \times 10^{-11}$  M). These experiments were performed in the presence of maximally stimulating concentrations of CSF.

We investigated the effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  on the clonal growth of normal human myeloid stem cells cultured in the presence of varying concentrations of CSF. These studies were initiated in view of our finding that KG-1 colony formation was enhanced by  $1\alpha,25(\text{OH})_2\text{D}_3$ . In contrast to the KG-1 results,  $1\alpha,25(\text{OH})_2\text{D}_3$  did not stimulate normal myeloid colony formation in the absence of CSF (Fig. 6). However, in the presence of 15% maximally stimulating concentrations of CSF, the addition of  $5 \times 10^{-9}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$  to the dishes enhanced myeloid colony formation by a mean of  $143 \pm 7\%$  ( $\pm$ SE) as compared with plates containing CSF alone ( $P \leq 0.05$ ). A similar stimulation was observed in plates containing either  $5 \times 10^{-8}$  M or  $5 \times 10^{-7}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$  and 15% maximally stimulating concentrations of CSF. The  $1\alpha,25(\text{OH})_2\text{D}_3$  did not enhance colony formation in the presence of 50 and 100% of maximum stimulating concentrations of CSF (Fig. 6).

Experiments were performed to investigate if the enhancement of clonal proliferation of KG and normal human myeloid stem cells by  $1\alpha,25(\text{OH})_2\text{D}_3$  in the presence of suboptimal concentrations of CSF is caused by cell production of CSF. The KG-1 and several other cell lines (HL-60, HEL, THP-1, U937)

Table III. Effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  and  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  on Clonal Growth of Myeloid Leukemia Cells Freshly Obtained from Patients

| Patient No. | Diagnosis* | FAB-type | Age<br>yr | Cell source | L-CFC‡ | $1\alpha,25(\text{OH})_2\text{D}_3$ | $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ |
|-------------|------------|----------|-----------|-------------|--------|-------------------------------------|-------------------------------------------------------------|
|             |            |          |           |             |        | $ED_{50}\S$                         | $ED_{50}\S$                                                 |
| 1. M.F.     | AML        | M 1      | 64        | B           | 22±5   | $2 \times 10^{-9}$ M                | not tested                                                  |
| 2. W.P.     | AML        | M 1      | 44        | B           | 72±34  | no effect                           | no effect                                                   |
| 3. W.M.     | AML        | M 1      | 33        | M           | 12±4   | stimulation <sup>  </sup>           | stimulation <sup>  </sup>                                   |
| 4. P.O.     | AML        | M 2      | 50        | M           | 810±91 | $5 \times 10^{-11}$ M               | $3 \times 10^{-11}$ M                                       |
| 5. C.L.     | AML        | M 2      | 42        | M           | 23±4   | $5 \times 10^{-7}$                  | not tested                                                  |
| 6. B.T.     | AML        | M 2      | 40        | M           | 131±34 | $5 \times 10^{-11}$ M               | $6 \times 10^{-11}$ M                                       |
| 7. K.R.     | AML        | M 2      | 44        | M           | 16±2   | no effect                           | no effect                                                   |
| 8. M.L.     | AML        | M 4      | 31        | M           | 22±3   | $4 \times 10^{-9}$ M                | $5 \times 10^{-11}$ M                                       |
| 9. W.D.     | AML        | M 5      | 37        | M           | 112±20 | $2 \times 10^{-8}$ M                | $2 \times 10^{-9}$ M                                        |
| 10. B.A.    | CML        |          | 11        | B           | 295±89 | no effect                           | no effect                                                   |
| 11. H.J.    | CML        |          | 12        | B           | 36±12  | $4 \times 10^{-9}$ M                | $2 \times 10^{-10}$ M                                       |
| 12. M.D.    | CML        |          | 27        | M           | 60±11  | $8 \times 10^{-9}$ M                | $9 \times 10^{-10}$ M                                       |
| 13. C.A.    | CML        |          | 34        | B           | 129±3  | $6 \times 10^{-9}$ M                | $5 \times 10^{-9}$ M                                        |
| 14. G.S.    | CML-BC     |          | 25        | B           | 337±45 | $1 \times 10^{-7}$ M                | $2 \times 10^{-8}$ M                                        |

\* CML-BC, chronic myelogenous leukemia, blast crisis; B, peripheral blood; M, bone marrow. ‡ L-CFC, leukemic colony-forming cells per  $2 \times 10^5$  cells plated in soft agar with maximally stimulating concentrations of CSF. §  $ED_{50}$ , effective dose that inhibited 50% clonal growth of leukemic cells. || Stimulation, 62 colonies formed in the presence of  $1\alpha,25(\text{OH})_2\text{D}_3$  ( $5 \times 10^{-8}$  M), 49 formed in the presence of  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  ( $5 \times 10^{-8}$  M).

as well as normal human bone marrow mononuclear cells were cultured for 5 d with  $5 \times 10^{-8}$ – $5 \times 10^{-9}$  M  $1,25(\text{OH})_2\text{D}_3$ . The conditioned media (0.1–10%) were tested in soft agar culture with either KG-1 or normal human bone marrow in the absence of CSF or the presence of one-third submaximally stimulating concentrations of CSF. No colony formation and only rare cluster formation was observed (data not shown).

## Discussion

Using eight myeloid cell lines, we have found that the cell lines that were induced to differentiate by  $1\alpha,25(\text{OH})_2\text{D}_3$  were always inhibited in their clonal growth by  $1\alpha,25(\text{OH})_2\text{D}_3$ . The responsive cells were relatively more mature (HL-60, promyelocytes; U937 and THP-1, monoblasts; HEL, bipotent erythroblasts-monoblasts; M1, late myeloblasts) than the unresponsive cells

(KG-1A, KG-1, early myeloblasts; HL-60 blast, early myeloblastic clone of HL-60; K562, early bipotent myeloerythroblasts). The 50% inhibition of colony formation of the responsive lines occurred in the concentration range of  $3 \times 10^{-8}$ – $4 \times 10^{-10}$  M of  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ . These concentrations are comparable to the concentrations required for induction of differentiation in liquid culture (8, 9, 24, 25, 26). Our experiments are supportive of the concept that myeloid blast cells have limited capabilities for replication when induced to differentiate. The vitamin D-responsive progenitor cells differentiated in vitro and lost their potential for clonal growth. The vitamin D-unresponsive leukemic cells did not differentiate and remained in the proliferative pool, giving rise to colonies of similar cells.

The  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  was 2–50-fold more potent in clonal inhibition than  $1\alpha,25(\text{OH})_2\text{D}_3$ . An explanation for the increased potency of the fluorinated analog is not immediately available. Studies that examined the induction of the vitamin D-induced Ca-binding protein, calbindin, in another cell culture system, found that the fluorinated compound was four times more potent than  $1\alpha,25(\text{OH})_2\text{D}_3$  (27); the authors speculated that the fluorinated analog had increased affinity for the cellular  $1\alpha,25(\text{OH})_2\text{D}_3$  receptor. Among other possible explanations for the increased potency of the fluorinated vitamin D compound might be a slower in vitro degradation of the analog or a decreased binding to serum-binding proteins as compared with  $1\alpha,25(\text{OH})_2\text{D}_3$ , thus resulting in a higher free concentration.

We and others have found that the inhibition of clonal growth and induction of differentiation of HL-60 by various vitamin D compounds parallels their known ability to bind to cellular  $1\alpha,25(\text{OH})_2\text{D}_3$  receptors (6, 8). We have also shown that the concentration of  $1\alpha,25(\text{OH})_2\text{D}_3$  ( $5 \times 10^{-9}$  M) that saturates 50% of  $1\alpha,25(\text{OH})_2\text{D}_3$  receptors in HL-60 was nearly identical to the concentration of the sterol that induced 50% of the HL-60 cells to differentiate, as measured by their ability to bind *N*-formyl-

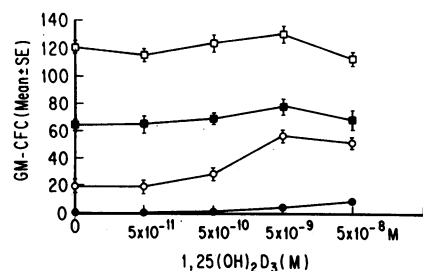


Figure 6. Effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  and colony-stimulating factor on normal myeloid clonal growth. Normal human bone marrow ( $2 \times 10^5$  cells/dish) was plated in the presence of different concentrations of both CSF (●, 0; ○, 7; ■, 15; □, 40  $\mu\text{l}$  Mo-conditioned medium/plate) and  $1\alpha,25(\text{OH})_2\text{D}_3$ . Number of myeloid colonies (GM-CFC) were counted after 10 d of culture. Results represent the mean of three experiments with quadruplicate dishes per point.

methionyl-leucyl-[<sup>3</sup>H]phenylalanine (8). Likewise, clones of HL-60 with decreased receptor protein (HL-60 blast) can neither be induced to differentiate into macrophages nor inhibited in their clonal growth by  $1\alpha,25(\text{OH})_2\text{D}_3$  (8, 28). Taken together with other studies (6), the data are consistent with  $1\alpha,25(\text{OH})_2\text{D}_3$  inhibiting clonal proliferation and inducing differentiation of HL-60 through cellular  $1\alpha,25(\text{OH})_2\text{D}_3$  receptors. However, as held by Olsson et al. (7), presence of these cellular receptors does not assure that the myeloid leukemia cells can be induced to differentiate by vitamin D compounds.

Time-response experiments showed that HL-60 cells required ~20 h exposure to  $1\alpha,25(\text{OH})_2\text{D}_3$  ( $5 \times 10^{-7}$  M) to produce a 50% clonal inhibition of growth. Another study has found that the transcription of the *c-myc* oncogene, which is associated with cell division, begins to decrease within 4 h of exposure of HL-60 to  $10^{-8}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$  (29). We and others previously showed that HL-60 cells must be exposed to  $1\alpha,25(\text{OH})_2\text{D}_3$  for at least 18–24 h to induce substantial differentiation (8, 26). These experiments suggest that the ability of  $1\alpha,25(\text{OH})_2\text{D}_3$  to inhibit clonal growth of leukemia cells is temporally associated with the ability of  $1\alpha,25(\text{OH})_2\text{D}_3$  to induce differentiation of the cells (8).

Paradoxically, we found that brief exposure ( $\leq 10$  h) to  $10^{-6}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$  significantly enhanced clonal growth of the HL-60 cells. We do not as yet understand why this occurs. We showed that HL-60 does not synthesize detectable CSF after brief exposure to  $1\alpha,25(\text{OH})_2\text{D}_3$ . We are now examining alteration in CSF receptor number or affinity in these cells after brief exposure to  $1\alpha,25(\text{OH})_2\text{D}_3$ .

We found that clonal growth of the leukemic cells from about 70% of the AML and CML patients that we examined was inhibited by either  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$  in vitro. In most cases, the clonal growth was markedly inhibited at rather low concentrations of the vitamin D compounds (range,  $5 \times 10^{-7}$ – $3 \times 10^{-11}$  M). An exception was patient No. 3, whose leukemic cells increased their clonal growth about fourfold in the presence of either  $5 \times 10^{-8}$  M  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ . The neoplastic cells of this patient were relatively immature (M1, FAB classification) and had an unusual chromosomal abnormality. Further patients with similar neoplastic cells must be studied to know if such a stimulation also occurs in other cases.

In contrast to leukemia cells from the majority of patients, normal bone marrow myeloid progenitors (CFU-GM) harvested from 12 leukemic patients in remission were not inhibited in their clonal growth when cultured with either  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $24,24\text{-F}_2\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ . Why these vitamin D compounds preferentially inhibited in vitro the proliferation of leukemic, but not normal, human myeloid stem cells is not clear. Differences in receptor number or affinity, or the activation of different genes and metabolic pathways may account for this differential effect. Since  $1\alpha,25(\text{OH})_2\text{D}_3$  causes alterations in Ca transport in several types of cells, one might speculate that leukemic colony-forming cells are particularly sensitive to these alterations.

We have previously studied normal and malignant myeloid colony formation in the presence of all-*trans*-retinoic acid (21). Retinoic acid also suppresses the clonal growth of neoplastic cells from most myeloid leukemia lines and patients, but stimulates normal CFU-GM by >100%. Evidence suggests that retinoic acid acts upon normal and malignant hematopoietic progenitor cells through a mechanism different from that of  $1\alpha,25(\text{OH})_2\text{D}_3$  (8, 20).

Our study suggests that  $1\alpha,25(\text{OH})_2\text{D}_3$  can act as a hematopoietic cofactor that promotes clonal growth of myeloid stem cells. We found that in the presence of submaximal concentrations of CSF,  $1\alpha,25(\text{OH})_2\text{D}_3$  stimulated the clonal growth of normal human bone marrow GM-CFC. A similar result was obtained with the KG-1 cell line, which is dependent on CSF for its clonal growth. This cell line was also stimulated to form colonies when only  $1\alpha,25(\text{OH})_2\text{D}_3$  was added to the cultures.  $1\alpha,25(\text{OH})_2\text{D}_3$  did not stimulate the marrow or KG-1 cells to produce detectable CSF. The increased clonal growth in the presence of  $1\alpha,25(\text{OH})_2\text{D}_3$  and submaximal concentrations of CSF may reflect an increased responsiveness to CSF due to effects on the number and/or affinity of CSF receptors on the target cells.  $1\alpha,25(\text{OH})_2\text{D}_3$  may be stimulating KG-1 clonal growth in cultures in the absence of exogenous CSF by making the cells more responsive to endogenous CSF present in FCS. Work is in progress in our laboratory to examine these hypotheses.

Vitamin D analogs might eventually be useful in treating patients with myeloid leukemias, either alone or in combination with other agents. Other studies showed that administration of  $1\alpha,25(\text{OH})_2\text{D}_3$  significantly prolonged the life of mice injected with the M1 transplantable leukemia cells (30). Our present study found, however, that the 50% effective concentration of  $1\alpha,25(\text{OH})_2\text{D}_3$  that is required both for clonal inhibition and differentiation of human leukemic cells in vitro is 10–100-fold higher than the serum level of  $1\alpha,25(\text{OH})_2\text{D}_3$  present in vivo (31). In addition, a smaller percentage of  $1\alpha,25(\text{OH})_2\text{D}_3$  is bound to binding proteins in our culture conditions than is bound in normal human plasma, which might preferentially increase the in vitro as compared with the in vivo potency of  $1\alpha,25(\text{OH})_2\text{D}_3$ . We have treated a series of patients with the myelodysplastic syndrome with oral  $1\alpha,25(\text{OH})_2\text{D}_3$  with no apparent success; however, theoretically desirable levels of  $1\alpha,25(\text{OH})_2\text{D}_3$  could not be achieved without the patients developing hypercalcemia (32). Several new vitamin D analogs, including  $1\alpha,25\text{S},26\text{-trihydroxy}\Delta 22$  vitamin  $\text{D}_3$ , have been developed that induced differentiation of HL-60 cells but that have less ability than  $1\alpha,25(\text{OH})_2\text{D}_3$  to cause hypercalcemia (33). Future trials with these new vitamin D analogs may have therapeutic potential.

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