VITAMIN D ANALOGS INHIBIT ERYTHROID DIFFERENTIATION AND INDUCE MONOCYTIC DIFFERENTIATION OF LEUKEMIC CELLS WITH THE SAME RELATIVE POTENCY

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ABSTRACT

Myeloid leukemia cells of human and murine origin can be induced to differentiate into more mature forms which lose their neoplastic properties. The hormonal form of vitamin D is a powerful inducer of monocytic differentiation, but its therapeutic use is limited by hypercalcemia. It was recently reported that a novel derivative of vitamin D, 1,25-dihydroxy-16-ene-23-yne-vitamin D_3, is an exceptionally potent inducer of monocytic differentiation, and prolongs survival of mice bearing leukemia cells. We now show that this compound is also a most potent inhibitor of erythrodifferentiation. This finding has important implications for the control of hematopoiesis.

INTRODUCTION

Analogs of vitamin D have recently become available which induce monocytic differentiation of myelogenous leukemia cells with potency greater than 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), the hormonal form of vitamin D, yet have relatively low effects on calcium metabolism (1-6). Since the potential for the therapeutic use of vitamin D and its analogs in human leukemia is limited by the hypercalcemia which results from an increased intestinal calcium absorption and mobilization of calcium from bones (7,8), analogs with a higher ratio of differentiation to calcium mobilization activities offer promise as adjuncts to the chemotherapy of leukemia. Indeed, increased survival time of leukemic mice treated with 1,25-dihydroxy-16-ene-23-yne-vitamin D_3($1,25(OH)_2$-16-ene-23-yne-D_3) has recently been reported (9).

Work in this laboratory has demonstrated that $1,25(OH)_2D_3$ inhibits chemically induced erythroid differentiation of human leukemic cells K562 (10). This inhibition was maximized by an exposure of the cultured cells to this vitamin-hormone prior to the induction of differentiation with chemotherapeutic agent 1-beta-D-arabinofuranosylcytosine (Ara-C) or other inhibitors of DNA synthesis, and was evident at concentrations of $1,25(OH)_2D_3$ which are optimal for the monocytic differentiation of HL60 cells (10,11).

The molecular mechanisms by which $1,25(OH)_2D_3$ induces monocytic and inhibits erythroid differentiation are not known, in spite of a considerable accumulation of data on the accompanying changes in gene expression (12-15). Studies of these mechanisms would be accelerated if it could be shown that the signals generated by $1,25(OH)_2D_3$ or its analogs are the same in HL60 and K562 cells, even though they result in opposite effects on the phenotype of differentiation. We report here the evidence suggesting that this is the case.

0037-9727/91/1973-0214$3.00/0

MATERIALS AND METHODS

Cell Cultures. K562 cell line established from a case of chronic myeloid leukemia in blast crisis and which can be induced to erythroid differentiation was obtained from Dr. P.F. Bocarsly (Department of Pathology, UMD-New Jersey Medical School). HL60-G1 cell line, an early passage subline of promyelocytic origin which differentiates into monocytic cells, was cloned from stock obtained from Dr. R. Wilson (Department of Biochemistry, UMD-New Jersey Medical School). The stock cultures were grown in 7% CO_2 at 37°C in RPMI 1640 medium (Hazleton Research Products, Denver, PA.) supplemented with 1% glutamine (Grand Island Biological Co. (GIBCO), Grand Island, N.Y.) and 15% heat inactivated (56°C for 1 hr) fetal bovine serum (Hyclone Laboratories, Logan UT). Absence of mycoplasma contamination was confirmed by an autoradiographic method (16).

Experiments were initiated by resuspending cells in fresh medium containing 1% final concentration of penicillin (50 IU) and streptomycin (50 ug/ml) solution (GIBCO, Grand Island, N.Y.) at a density of 300×10^3 K562 cells/ml and 500×10^3 HL60 cells/ml. Cell vibility was determined by counting 300 cells on a Neubauer's hemocytometer after exposure to 0.2% trypan blue stain. Staining for nonspecific esterase (NSE) activity and nitro blue tetrazolium (NBT) dye reduction assay were all performed as previously described (13). The presence of hemoglobin in the differentiating K562 cells was determined by the benzidine reaction (17).

Chemicals. $1,25(OH)_2D_3$ and its analogs 26,27-F6-$1,25(OH)_2D_3$, $1,25(OH)_2$-16-ene-23-yne-D_3, 1,25s,26-trihydroxy-delta^{22}-cholecalciferol, 1,25-dihydroxy-22-ene-26,27-hexafluoro-cholecalciferol and 1,25-dihydroxy-23-yne-cholecalciferol were provided by Dr. Milan Uskokovic, Hoffmann LaRoche Inc., Nutley, N.J.

Ara-C, aphidicolin and other chemicals required were obtained from Sigma Chemical Co. Genistein (4',5,7-trihydroxyisoflavone) was obtained from ICN Biochemicals, Cleveland, Ohio.

RESULTS

Relative Potency of Induction of Monocytic Differentiation. We compared the efficiency of induction of differentiation by six derivatives of cholecalciferol listed in the Materials and Methods section above. One, $1,25(OH)_2D_3$, is the physiologically active form of vitamin D (1), and another two, 26,27-F6-$1,25(OH)_2D_3$ and $1,25(OH)_2$-16-ene-23-yne-D_3 were reported to have greater anti-leukemic activity than $1,25(OH)_2D_3$ (4,7-9). The compounds were used at 2.4×10^{-8} M, a concentration previously found sufficient to induce near maximal monocytic differentiation of these cells in five days. Time-course studies of the induction of two phenotypic markers of monocytic differentiation, the positivity for the enzyme NSE and the evidence of the oxidative burst capability by the NBT reaction, showed that $1,25(OH)_2$-16-ene-23-yne-D_3 did indeed markedly accelerate the rate of monocytic differentiation of HL60-G1 cells over that obtained with $1,25(OH)_2D_3$ (Fig 1 A and B). However, exposure to equimolar 26,27-F6-$1,25(OH)_2D_3$ resulted in slower and less complete differentiation (Fig 1). This difference from the previously reported high potency of 26,27-F6-$1,25(OH)_2D_3$ may be due to the fact that an early passage subline of HL60 cells was used in our studies. Other analogs of vitamin D that we used in these experiments showed rates and extent of HL60-G1 cell differentiation essentially the same as those obtained with 26, 27-F6-$1,25(OH)_2D_3$ (data not shown).

Relative Potency of Inhibition of Erythroid Differentiation. K562 leukemia cells exposed to variety of antimetabolites acquire phenotypic markers of erythroid differentiation, and can be conveniently scored as positive for hemoglobin by the benzidine reaction (17). When pretreated for 24 hrs with 2.4×10^{-8} M concentration of the vitamin D analogs, induction of erythroid phenotype by 1 uM ara-C was reduced with the same relative potency as induction of HL60-G1 cells to monocytic differentiation. This is illustrated for $1,25(OH)_2D_3$, 26,27-F6-$1,25(OH)_2D_3$ and $1,25(OH)_2$-16-ene-23-yne-D_3 in Fig 2A. Essentially similar results were obtained when 1 uM aphidicolin, an inhibitor of DNA polymerases alpha and

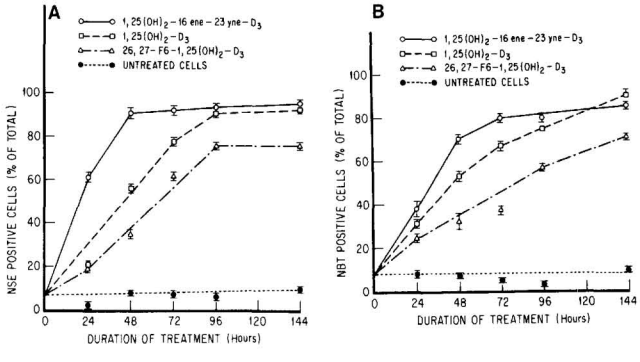

Figure 1. Time course curves showing the relative potency of $1,25(OH)_2D_3$ and its analogs 26,27-F6-$1,25$-$(OH)_2D_3$ and $1,25(OH)_2$-16-ene-23-yne-D_3, all at 2.4×10^{-8} *M*, on monocytic differentiation of HL60-G1 cells. Differentiation was measured by: (A) Nonspecific esterase activity (NSE) and (B) Nitro blue tetrazolium reduction (NBT). Each point represents the mean ± SE of three independent determinations. SE less than 0.35 are not shown.

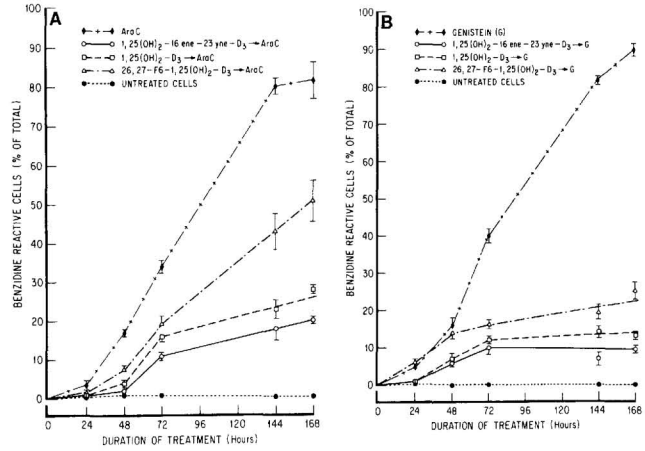

Figure 2. Erythroid differentiation of K562 cells measured by percentage of benzidine-positive cells after exposure to chemical inducers, alone, and after 24-hr pretreatment with $1,25(OH)_2D_3$ and its analogs 26,27-F6-$1,25(OH)_2D_3$ and $1,25(OH)_2$-16-ene-23-yne-D_3. All steroids were used at 2.4×10^{-8} *M*. (A) Erythroid differentiation induced by Ara-C (1 μM). (B) Erythroid differentiation induced by genistein (111 μM). Each point represents the mean ± SE of three independent determinations. SE less than 0.35 are not shown.

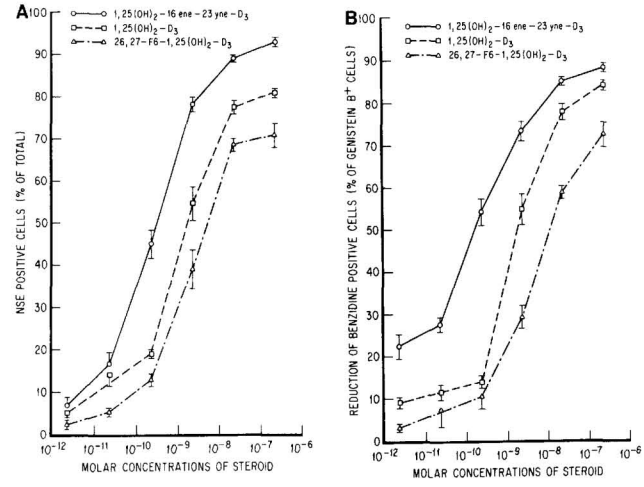

Figure 3. Dose-response curves comparing the relative potency of $1,25(OH)_2D_3$ and its analogs 26,27-F6-$1,25(OH)_2D_3$ and $1,25(OH)_2$-16-ene-23-yne-D_3 on induction of monocytic and inhibition of erythroid differentiation. (A) Induction of monocytic differentiation by vitamin D analogs in HL60-G1 cells. (B) Inhibition by vitamin D analogs of erythroid differentiation induced by (111 μM) genistein in K562 cells. Each point represents the mean ± SE of three independent determinations.

$$\% \text{ Inhibition of erythrodifferentiation} = 100 - \frac{\text{B+ cells with 24-hr steroid pretreatment}}{\text{B+ cells without 24-hr steroid pretreatment}} \times 100$$

delta, was used (data not shown). Interestingly, preexposure to vitamin D analogs inhibited genistein-induced erythroid differentiation of K562 cells (18) to a greater extent, but the relative potency of the analogs remained the same (Fig 2 B).

Comparison of the Dose-Dependence of the Effects of Vitamin D Analogs on Monocytic and Erythroid Differentiation. The physiological serum levels of $1,25(OH)_2D_3$ in humans are in the

range of 0.5-1.5 x 10^{-10}M (19-21), while the concentration necessary for the maximal differentiation of human hematopoietic cells under culture conditions approach 10^{-7}M (11). We therefore examined the effects on differentiation of HL60-G1 and K562 cells of vitamin D analogs in this entire range. The profiles of dose-dependency of the induction of monocytic differentiation and the inhibition of erythroid differentiation were remarkably similar for each analog (Fig 3 A and B). This figure also shows that in the low range of physiological concentrations, where calcium-mobilizing activities of the steroids are minimal, the effects of $1,25(OH)_2$-16-ene-23-yne-D_3 on leukemic cell differentiation clearly exceed that of the other derivatives of cholecalciferol studied here.

DISCUSSION

Data presented above indicate that the most potent steroid inducer of monocytic differentiation that we have studied, $1,25(OH)_2$-16-ene-23-yne-D_3, is also the most potent inhibitor of erythroid differentiation in K562 cells, and that compounds with lower potency for monocytic differentiation than $1,25(OH)_2D_3$ are also weaker inhibitors of erythrodifferentiation (compare Fig 1 with Fig 2). In addition to the same rank order of potency with respect to induction (of monocytic) and inhibition (of erythroid) differentiation, the dose-dependency curves of these effects are parallel (compare Fig 3 A and B). This suggests a similar molecular basis for both of these effects, so that data obtained in either system may be used to obtain a composite picture of the mechanisms by which $1,25(OH)_2D_3$ affects hematopoietic stem cells. Furthermore, the parameters of the S-shaped curves for dose dependency indicate that at the physiological concentrations (approximately 10^{-10}M, refs (19-21) $1,25(OH)_2D_3$ has little effect on HL60 or K562 cell differentiation. However, a relatively modest increase in the concentration of $1,25(OH)_2D_3$ produces a rapidly escalating effect on reducing erythroid, and promoting monocytic, differentiation (Fig 3 A and B). To the extent that HL60 and K562 cells are similar to the hematopoietic precursor cells in the bone marrow, these findings have implications for the role of $1,25(OH)_2D_3$ in the control of hematopoiesis. More specifically, our results provide an explanation for the greater effect of $1,25(OH)_2$-16-ene-23-yne-D_3 over $1,25(OH)_2D_3$ on the survival of mice bearing leukemia cells (9). We demonstrate that $1,25(OH)_2$-16-ene-23-yne-D_3 has a clear effect on the differentiation of HL60 and K562 cells at concentrations too low (approximately 10^{-10}M) to have an appreciable hypercalcemic effect. These findings may point the way to the design of vitamin D analogs with even greater potential as anti-leukemic agents.

ACKNOWLEDGEMENTS

We are grateful to Dr. Milan Uskokovic for gifts of vitamin D analogs. This research was supported by an NIH Grant CA-44722 from the National Cancer Institute.

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Received January 30, 1991
Accepted March 25, 1991