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INHIBITION BY 1α,25-DIHYDROXYVITAMIN D_3 OF DIMETHYL SULFOXIDE-INDUCED DIFFERENTIATION OF FRIEND ERYTHROLEUKEMIA CELLS

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SUMMARY: Regulation of erythroid differentiation by vitamin D_3 derivatives was examined in Friend erythroleukemia cells. After Friend cells were cultured for 5 days with 1.5% dimethyl sulfoxide (DMSO), as much as 70% of the cells became benzidine-positive and the hemoglobin content increased in parallel with the increase of benzidine-positive cells. The DMSO-induced erythroid differentiation was markedly inhibited by concurrent addition of the active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25(OH)$_2$D$_3$]. Of the vitamin D_3 derivatives tested, 1α,25(OH)$_2$D$_3$ was the most potent in inhibiting DMSO-induced erythroid differentiation. 1α,25(OH)$_2$D$_3$ alone was totally ineffective in both cell growth and erythroid differentiation. These results together with our previous reports indicate that 1α,25(OH)$_2$D$_3$ is somehow involved not only in myeloid differentiation, but also in erythroid differentiation.

Erythroid differentiation has been extensively studied in the murine erythroleukemia cells established by Friend et al. (1). Morphologically, these cells resemble proerythroblasts and show only a small percentage (< 1%) of spontaneous erythroid differentiation (1). The cells can be induced to differentiate into erythrocytes by such inducers as dimethyl sulfoxide (DMSO) (2), butyric acid (3), and hexamethylene bisacetamide (4). Glucocorticoids (5) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (6) inhibit DMSO-induced differentiation of Friend cells. Erythroid differentiation can be detected

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Abbreviations used: 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D_3; 25(OH)D_3, 25-hydroxyvitamin D_3; 1α(OH)D_3, 1α-hydroxyvitamin D_3; 24R,25(OH)$_2$D$_3$, 24R,25-dihydroxyvitamin D_3; DMSO, dimethyl sulfoxide; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; Ml, a murine myeloblastic leukemia cell line; HL-60, a human promyelocytic leukemia cell line; U937, a human histiocytic monoblast-like lymphoma cell line.

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by chromatin condensation and other morphological changes resulting in non-dividing cells (2), appearance of specific antigens in erythrocyte membranes (7), heme and hemoglobin syntheses (2), and accumulation of globin mRNA (8).

In 1981, Abe et al. (9) found that the active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], suppresses proliferation and induces differentiation of murine myeloblastic leukemia cells (M1) into monocyte-macrophages. Subsequently, several laboratories including ours reported that 1α,25$(OH)_2D_3$ is capable of inducing differentiation of not only M1 cells, but also human promyelocytic leukemia cells (HL-60) and human histiocytic monoblast-like lymphoma cells (U937) preferentially along the monocyte-macrophage pathway (10-14).

In this work, we examined the effect of vitamin D_3 derivatives on erythroid differentiation using Friend erythroleukemia cells. 1α,25$(OH)_2D_3$ markedly inhibited the DMSO-induced differentiation of the cells.

MATERIALS AND METHODS

Vitamin D_3 derivatives: 25-Hydroxyvitamin D_3 [25$(OH)D_3$] was purchased from Phillips-Duphar, Amsterdam, The Netherlands. 1α,25$(OH)_2D_3$, 24R,25-dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$] and 1α-hydroxyvitamin D_3 [1α$(OH)D_3$] were the gifts of Dr. I. Matsunaga, Chugai Pharmaceutical Co., Tokyo, Japan. 1α,24R,25-Trihydroxyvitamin D_3 [1α,24R,25$(OH)_3D_3$] was synthesized by incubating with 24R,25$(OH)_2D_3$ the kidney homogenates from vitamin D-deficient chicks by the method of Yamada et al. (15).

Cells and cell culture: Friend erythroleukemia cells (clone 745A) were provided by Dr. S. Hata, Chugai Pharmaceutical Co., Tokyo. Cells were cultured at 37°C in Eagle's minimal essential medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY) in a humidified atmosphere of 5% CO_2 in air. Cells in the logarithmic phase of growth were seeded at 3 x 10^4/ml. The doubling time under these conditions was 11 h. Graded concentrations of DMSO (Wako Pure Chemicals, Osaka, Japan) were added at the time of seeding. Each vitamin D_3 derivative dissolved in ethanol was added to keep a final ethanol concentration of less than 0.1% at the time of seeding.

Benzidine staining and assay for hemoglobin contents: After cells were washed once in Hanks' balanced salt solution (HBSS) and then suspended in the solution, the suspension was mixed with an equal volume of the freshly prepared staining solution (3% benzidine in 90% acetic acid : H_2O : 30% H_2O_2 = 1 : 5 : 0.1, v/v) and the number of benzidine-positive cells was determined using a phase contrast microscopy (16). At least 200 cells were counted.

The hemoglobin content of cultured cells was measured by optical absorption (17). After the cells were cultured with DMSO, they were chilled on ice and washed twice in HBSS. The washed cell pellet was resuspended in a lysing buffer [0.8% NaCl, 0.03% Mg-acetate, 5% NP-40 (Shell), 0.12% Tris (pH 7.4)]. After standing for 15 min on ice, the nuclei were spun out at 1500 rpm for 15 min. The visible absorbance spectrum of the supernatant and the absorbance at 414 nm were recorded, and readings were also taken at 403 and 425 nm to correct for nonspecific absorption due to light scattering.

The absorbing material was positively identified as hemoglobin by the presence of absorption maxima at 414, 540 and 576 nm, which shifted to 417, 537 and 568 nm, respectively, after carbon monoxide treatment. Hemoglobin was then quantified at 414 nm, assuming an absorbance of 1.0 at 414 nm to be equivalent to 0.13 mg/ml.

RESULTS AND DISCUSSION

Untreated Friend cells showed only a small percentage (< 1%) of spontaneous erythroid differentiation. When cells were cultured with DMSO, cell growth was significantly inhibited by 1.5 and 2.0% DMSO, and percentages of the benzidine-positive cells increased on day 2, attained maximum on day 5, and decreased thereafter. The maximal values were 37.6% at 1.0% DMSO, 68.1% at 1.5% and 56.8% at 2.0% on day 5. Therefore, cells were cultured with 1.5% DMSO and erythroid differentiation was examined on day 5 in following experiments.

Figure 1 shows the effect of 1α,25(OH)$_2$D$_3$ on erythroid differentiation and restricted growth of Friend cells induced by DMSO. 1α,25(OH)$_2$D$_3$ inhibited markedly DMSO-induced erythroid differentiation (Fig. 1B), but it did not affect cell growth appreciably (Fig. 1A). On day 5, percentages of the benzidine-positive cells were decreased to 61% at 0.12 nM, 53% at 1.2 nM, 22%

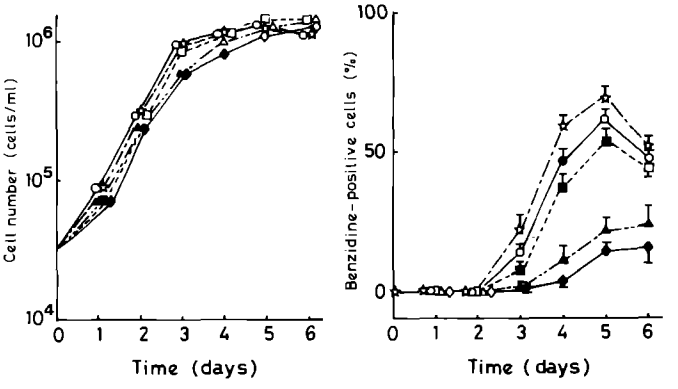

Fig. 1 Dose response effects of 1α,25(OH)$_2$D$_3$ on growth (A) and differentiation (B) of Friend cells induced by 1.5% DMSO. Cells were cultured with 1.5% DMSO and graded concentrations of 1α,25(OH)$_2$D$_3$ for 6 days. Panel A shows a typical dose response curve of 1α,25(OH)$_2$D$_3$ in cell growth in the presence of 1.5% DMSO. The standard error of each value was less than 6.2% in 4 replicates. Panel B shows inhibitory effects of graded concentrations of 1α,25(OH)$_2$D$_3$ on 1.5% DMSO-induced increase of benzidine-positive cells. Each point represents means ± S.E. of 8 experiments. Symbols: 1.5% DMSO alone (☆), 1.5% DMSO plus 0.12 nM (○ and ●), 1.2 nM (□ and ■), 12 nM (△ and ▲), and 120 nM (◇ and ◆) of 1α,25(OH)$_2$D$_3$. Black symbols: significantly different from 1.5% DMSO alone on each day ($p < 0.05$).

Table I. Inhibitory effect of 1α,25(OH)$_2$D$_3$ on DMSO-induced increase of hemoglobin content

Addition		Hemoglobin content
DMSO %	1α,25(OH)$_2$D$_3$ nM	μg/dish
0	0	7.0 ± 0.9
1.5	0	199 ± 6
1.5	0.12	204 ± 6
1.5	1.2	164 ± 24
1.5	12	107 ± 23[a]
1.5	120	68 ± 11[a]

Cells were cultured for 5 days with 1.5% DMSO alone or 1.5% DMSO plus graded concentrations of 1α,25(OH)$_2$D$_3$. Data are means ± S.E. of 6 experiments.
[a] Significantly different from 1.5% DMSO alone ($p < 0.05$)

at 12 nM, and 14% at 120 nM of 1α,25(OH)$_2$D$_3$. Table I shows the amounts of hemoglobin synthesized by Friend cells cultured with 1.5% DMSO and graded concentrations of 1α,25(OH)$_2$D$_3$. DMSO increased hemoglobin content markedly, but its content was decreased significantly by adding concentrations higher than 12 nM of 1α,25(OH)$_2$D$_3$. The inhibitory effect of 1α,25(OH)$_2$D$_3$ on the hemoglobin synthesis was well correlated with that on the percentages of benzidine-positive cells. A regression curve was obtained with a highly significant correlation ($\gamma = 0.955$) between the inhibitory effect of 1α,25-(OH)$_2$D$_3$ on the hemoglobin synthesis and the percentages of benzidine-positive cells. The inhibitory effect of the vitamin on DMSO-induced erythroid differentiation is similar to that of dexamethasone (5) and TPA (6).

DMSO inhibited proliferation and enhanced differentiation of Friend erythroleukemia cells. On the other hand, 1α,25(OH)$_2$D$_3$ inhibited DMSO-induced erythroid differentiation, but it did not stimulate cell growth. Thus, cell growth and differentiation may be regulated separately. 1α,25(OH)$_2$D$_3$ alone affected neither growth nor differentiation of Friend cells (data not shown).

Of the vitamin D$_3$ derivatives tested, 1α,25(OH)$_2$D$_3$ was the most potent in inhibiting DMSO-induced erythroid differentiation, followed successively by 1α,24R,25(OH)$_3$D$_3$, 1α(OH)D$_3$, 25(OH)D$_3$, and 24R,25(OH)$_2$D$_3$ (Fig. 2). The order of the potency of vitamin D$_3$ derivatives in inhibiting erythroid dif-

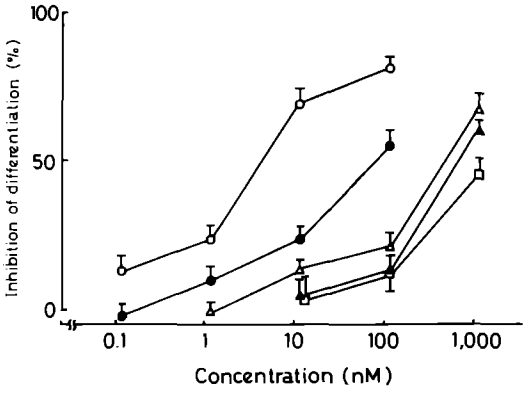

Fig. 2 Inhibition of DMSO-induced differentiation of Friend cells by various derivatives of vitamin D_3. Cells were cultured for 5 days with 1.5% DMSO and graded concentrations of $1\alpha,25(OH)_2D_3$ (○), $1\alpha,24R,25(OH)_3D_3$ (●), $1\alpha(OH)D_3$ (△), $25(OH)D_3$ (▲), or $24R,25(OH)_2D_3$ (□). Cell differentiation was expressed as percentages of benzidine-positive cells in total cells, and inhibition of differentiation was expressed as percentages of the control value (1.5% DMSO alone). Each point represents means ± S.E. of 10 experiments.

ferentiation is closely related to the binding affinity of the vitamin D_3 derivatives for the cytosol receptor found in chick intestine (18) and human myeloid leukemia cells (HL-60) (19), suggesting that the vitamin inhibits DMSO-induced erythroid differentiation by a receptor-mediated mechanism.

To further examine the inhibitory effect of $1\alpha,25(OH)_2D_3$ on DMSO-induced erythroid differentiation, we investigated the effect of time of exposure to $1\alpha,25(OH)_2D_3$. Treatment of Friend cells with $1\alpha,25(OH)_2D_3$ for 24 - 72 h prior to the addition of DMSO was totally ineffective in inhibiting DMSO-induced erythroid differentiation (Table II). Addition of $1\alpha,25(OH)_2D_3$ 72 h after adding DMSO also failed to inhibit differentiation. Thus it is likely that the vitamin acts in an early step of the commitment by DMSO. It has been reported that glucocorticoids inhibit globin gene expression induced by DMSO at a transcriptional level (20).

In conclusion, the naturally occurring hormone, $1\alpha,25(OH)_2D_3$, is involved not only in myeloid differentiation as reported previously (9 - 14), but also in erythroid differentiation. $1\alpha,25(OH)_2D_3$ induces differentiation of myeloid leukemia cells such as Ml, HL-60 and U937 preferentially along the monocyte-macrophage pathway (9 - 14). In contrast, the vitamin inhibits erythroid

Table II. Effect of time of exposure to $1\alpha,25(OH)_2D_3$ on DMSO-induced differentiation of Friend cells

Time of exposure to $1\alpha,25(OH)_2D_3$ (h)	Benzidine-positive cells (BPC) (%)	Percent inhibition (%)
	65.4 ± 2.4	0
-72 ~ 0	64.4 ± 3.5	1.5
-48 ~ 0	66.1 ± 3.3	-1.1
-24 ~ 0	64.8 ± 3.4	0.9
0 ~120	22.4 ± 3.0	65.7
24 ~120	22.7 ± 2.4	65.3
48 ~120	47.5 ± 3.3	27.4
72 ~120	64.4 ± 1.4	1.5
96 ~120	65.0 + 1.6	0.6

1.5% DMSO was added at time 0. Cells were exposed to 12 nM of $1\alpha,25(OH)_2D_3$ for indicated times before or after adding DMSO. Benzidine-positive cells (BPC) were counted 120 h after adding DMSO. Percent inhibition was calculated according to the following formula:

$$\frac{\text{BPC (\%) with DMSO alone} - \text{BPC (\%) with DMSO plus } 1\alpha,25(OH)_2D_3}{\text{BPC (\%) with DMSO alone}} \times 100$$

Data are means ± S.E. of 4 experiments.

differentiation induced by DMSO. Thus, it is conceivable that $1\alpha,25(OH)_2D_3$ plays a critical role in determining differentiation of bone marrow progenitor cells.

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