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Vitamin D_3 Derivatives Inhibit the Differentiation of Friend Erythroleukemia Cells

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SUMMARY

A number of vitamin D_3 metabolites inhibit benzodiazepine- and dimethyl sulfoxide-induced differentiation of Friend erythroleukemia cells. The inhibition is dose dependent and occurs at nM concentrations. The order of potency of these compounds is 1,25-dihydroxycholecalciferol > 1,25,26-trihydroxycholecalciferol > 1,24*R*,25-trihydroxycholecalciferol > 1α-hydroxycholecalciferol> 24*R*,25-dihydroxycholecalciferol > 25*S*,26-dihydroxycholecalciferol. The inhibition is maximal when the vitamin D_3 analogs are added together with the inducer, and becomes progressively decreased with delayed addition. These results suggest that the vitamin D_3 metabolites may play a regulatory role in erythropoiesis.

Metabolites of vitamin D_3 are circulating hormones that play a major role in calcium homeostasis. Their primary sites of action are in the intestine and bone, where a specific cytosolic receptor mediates their effects on calcium transport and mobilization (1, 2). It is also known that an active metabolite of vitamin D_3 stimulates erythropoiesis in aparathyroid rats (3). Furthermore, the vitamin D_3 receptor is present in various tumor cell lines (4–7), and the active metabolites induced differentiation of both the mouse M-1 and the human HL-60 myeloid leukemia cells into monocytes (8–11), suggesting that the hormones may have novel actions on cellular functions (12). The Friend erythroleukemia cells, derived from erythroid precursor cells blocked in an early stage of erythroid development by the Friend virus complex (13), can be induced by a variety of agents to undergo a program of differentiation very much like that of normal erythropoiesis (14). Some key events in this program are terminal cell division and the synthesis of hemoglobin. We now report that vitamin D_3 analogs, at nM concentrations, inhibit the differentiation of Friend erythroleukemia cells with a rank order of potencies similar to that reported for their induction of myeloid leukemia cells (8, 11). This finding should facilitate studies of the mechanism of action of vitaminD_3 metabolites, which is presumably similar in the two systems although their biological effects are seemingly opposite, and may also be of use in understanding the regulation of cellular differentiation.

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Materials and Methods

The DS-19 subclone (gift of Dr. R. A. Rifkind, Memorial Sloan-Kettering Cancer Center, New York) of Friend erythroleukemia cell line 745A was grown in continuous suspension culture in Eagle's Minimal Essential Medium (MEM-α, GIBCO) supplemented with 10% heat-inactivated fetal calf serum, as described elsewhere (15). Drugs were dissolved in ethanol and diluted into culture medium to achieve the desired final concentrations. Ethanol concentrations of up to 0.2%, the highest amount used, had no effect on any of the parameters studied (data not shown). Differentiation of the cells, seeded at 1×10^4 per ml, was induced by 5 days of exposure to the benzodiazepine Ro 7-3351 (Hoffman-La Roche), as described (15). Aliquots of the culture were then treated with 0.2% benzidine dihydrochloride (Sigma) and counted for cells that stained blue, an indication of hemoglobin accumulation and a convenient quantitative assay of erythroid differentiation (15, 16). Normally, the cells are capable of proliferation and are not benzidine reactive (<1%), but about 70% become benzidine-positive and cease cell division after 5 days of treatment with 40 μM Ro 7-3351 (15). The vitamin D_3 analogs (Hoffman-La Roche) were added together with or without the inducer on day 0 and their effect on the induction of differentiation was examined on day 5. The vitamin D_3 analogs alone at all concentrations did not alter the percentage of benzidine-positive cells.

Results

The vitamin D_3 analogs inhibited the benzodiazepine-stimulated hemoglobin synthesis in a concentration-dependent manner (Fig. 1). The most potent blocker was the dihydroxy metabolite 1,25$(OH)_2D_3$, which half-maximally inhibited at 1.5 nM, followed closely by two trihydroxy derivatives

ABBREVIATIONS: 1,25$(OH)_2D_3$, 1,25-dihydroxy vitamin D_3; 1,25,26$(OH)D_3$, 1,25,26-trihydroxy vitamin D_3; 1,24*R*,25$(OH)_3D_3$, 1,24*R*,25-trihydroxy vitamin D_3; 1$\alpha$$(OH)D_3$, 1$\alpha$-hydroxy vitamin D_3; 24*R*,25$(OH)_2D_3$, 24*R*,25-dihydroxy vitamin D_3; 25*S*,26$(OH)_2D_3$, 25*S*,26-dihydroxy vitamin D_3; DMSO, dimethyl sulfoxide.

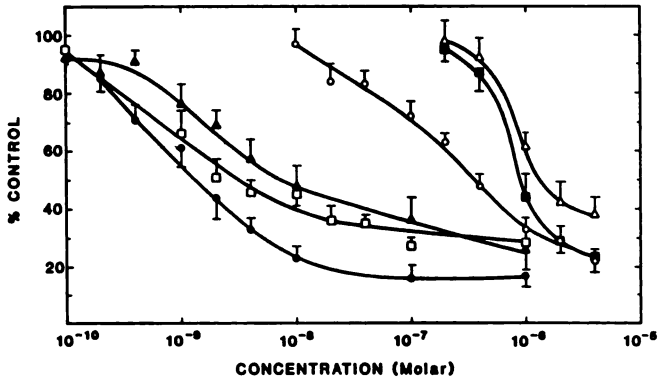

Fig. 1. Concentration dependency of the inhibition of Friend erythroleukemia differentiation by vitamin D_3 derivatives. Friend erythroleukemia cells (subclone DS-19) were cultured as described in the text. The benzodiazepine inducer Ro 7-3351 (40 μM) and various concentrations of vitamin D_3 derivatives were added on day 0. On day 5 the cells were harvested and stained with benzidine as described (15). Cells that stained blue were scored, and control was taken as 100%. Results are expressed as mean ± standard deviation from at least four experiments. ●, $1,25(OH)_2D_3$; □, $1,25,26(OH)_3D_3$; ▲, $1,24R,25(OH)_3D_3$; ○, $1\alpha(OH)D_3$; ■, $24R,25(OH)_2D_3$; △, $25S,26(OH)_2D_3$.

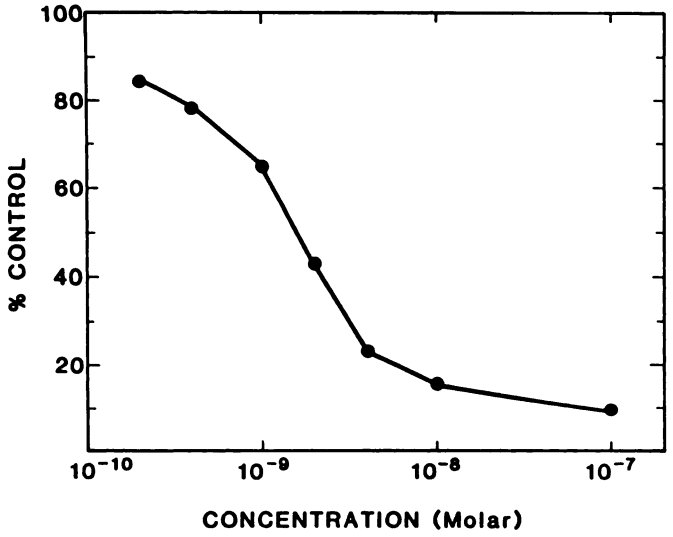

Fig. 2. Inhibition of DMSO-induced Friend erythroleukemia differentiation by $1,25(OH)_2D_3$. DS-19 cells were cultured as described in Fig. 1 except that 1% DMSO was used as the inducer. Cells were harvested and scored for benzidine staining on day 5. Results are the average of two experiments.

[$1,25,26(OH)_3D_3$, 3 nM, and $1,24R,25(OH)_3D_3$, 8 nM] and three others that were active at higher nM concentrations [$1\alpha(OH)D_3$, 450 nM; $24R,25(OH)_2D_3$, 900 nM; and $25S,26(OH)_2D_3$, 1500 nM]. This rank order of potencies is similar to that reported for the vitamin D_3 metabolite-induced differentiation of M-1 (8) and HL-60 (11) cells, which, although a rather different biological outcome, does suggest a similar mechanism of action. It is also of interest that the vitamin D_3 analogs could maximally inhibit only about 80% of the inducible cells (Fig. 1). A similar heterogeneity in response has been observed for the inhibition of erythroleukemia differentiation by phorbol esters (17) and dexamethasone (18).

The peripheral-type benzodiazepines do not appear to affect differentiation by virtue of an interaction with their high affinity binding site (15, 19). This conclusion is supported by two further observations relating to the inhibitory actions of vitamin D_3 analogs. 1) All analogs of vitamin D_3 fail to displace radiolabeled benzodiazepines from the peripheral-type site. 2) Vitamin D_3 analogs inhibit the induction of differentiation of Friend cells by DMSO, which does not interact with the benzodiazepine site (Fig. 2).

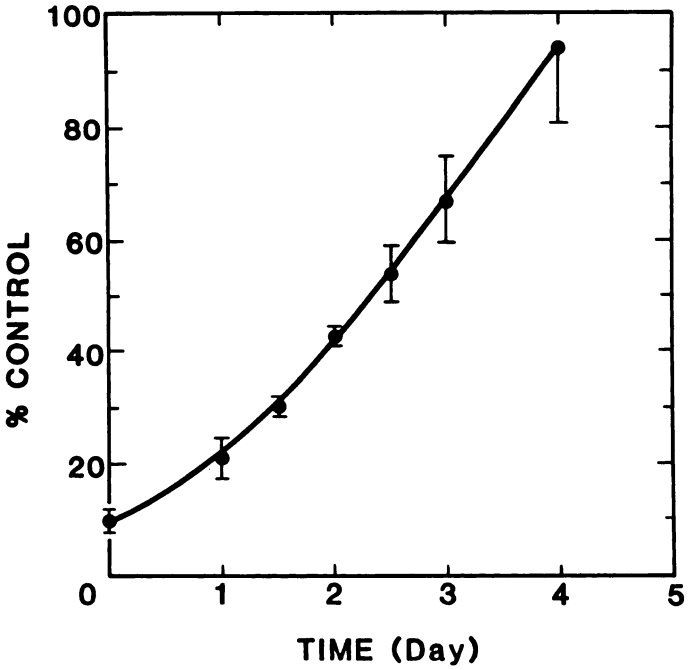

Fig. 3. Effect of time of addition of $1,25(OH)_2D_3$ on its inhibition of Friend erythroleukemia differentiation. DS-19 cells were cultured as described in Fig. 1. Ro 7-3351 (40 μM) was added on day 0 and $1,25(OH)_2D_3$ was added into the culture at the indicated times from day 0 to day 4. On day 5 the cells were harvested and scored for benzidine staining as described in Fig. 1. Control is defined as cultures exposed to the inducer (Ro 7-3351) for 5 days in the absence of the inhibitor [$1,25(OH)_2D_3$], and the level of differentiation is normalized as 100%. Results are expressed as mean ± standard deviation from four experiments.

The inhibition of hemoglobin synthesis was most effective when $1,25(OH)_2D_3$ was added to the cell culture simultaneously with the inducer (day 0). Progressive delay of addition of the vitamin D_3 metabolite resulted in decreasing inhibition of differentiation (Fig. 3). When added on day 2.5, $1,25(OH)_2D_3$ was only half as effective as when it was added on day 0, and by day 4 most of the cells were no longer sensitive to the inhibitor.

The effect of the vitamin D_3 analogs is reversible since induction of differentiation by Ro 7-3351 or DMSO is not affected by a 3-day preincubation with 5 nM $1,25(OH)_2D_3$ (data not shown).

Discussion

We have shown in this report that the vitamin D_3 analogs are potent inhibitors of erythroleukemia differentiation. The inhibition was concentration dependent and showed a rank order of potencies similar to that observed for other biological effects of these compounds, suggesting a single mechanism of action presumably involving the vitamin D_3 receptor. The inhibition was not limited to a single class of inducers. The inhibition was maximal when the vitamin D_3 analog was added together with the inducer, and became increasingly ineffective with progressive delay of addition. This time course is consistent with a stochastic model of cellular differentiation (20–22), which states that in every cell generation there is a discrete

probability for a cell to irreversibly commit itself to differentiation. This probability remains the same even though the number of cells available for commitment decreases with time. Thus, when $1,25(OH)_2D_3$ was added 2.5 days after the inducer, when about half the cells had already been recruited (15), it was only half as effective as when it was added on day 0, when none of the cells had been committed yet. By day 4 most of the cells had already been committed and were no longer sensitive to the inhibitor. These results also suggest that the vitamin D_3 metabolites interfered with some events in the multistep process of commitment rather than with the synthesis of hemoglobin per se. It is possible that the hormones act at the same step as dexamethasone and the tumor promoters, which have been shown not to suppress the earliest events of commitment but rather some later ones that involved the expression of the differentiated state (20, 23).

The potent inhibition of erythroleukemia differentiation by the vitamin D_3 analogs is in contrast to their equally potent induction of differentiation in other cell lines of reticuloendothelial origin, namely, the M-1 and the HL-60 cells. They are thus bipotential compounds that induce differentiation in some cell lines and suppress it in others. Other agents with such bidirectional actions include the tumor-promoting phorbol esters, which inhibit the differentiation of murine erythroleukemia (24, 25) and neuroblastoma cells (26), but stimulate the myeloid leukemia cells to develop into monocytes (27). This raises the possibility that the vitamin D_3 metabolites and the phorbol esters, which are well known as specific activators of protein kinase C (28), may act through a common pathway in these cell lines to affect differentiation.

Our data further suggest that the vitamin D_3 metabolites might play a regulatory role in erythropoiesis. Although the prime regulator of erythroid differentiation *in vivo* is the hormone erythropoietin (29, 30), the calcium homeostatic system has also been shown to play a positive role in erythroid maturation (31–33). Furthermore, it has been demonstrated that erythropoietin may elicit some of its erythropoietic activity by stimulating the release of parathyroid hormone, which results in an increase in circulating $1,25(OH)_2D_3$ and plasma Ca^{2+} levels (34). The elevated $1,25(OH)_2D_3$ levels may act as a negative feedback modulator of erythropoiesis by sparing the precursor cells from depletion. By preventing the immediate terminal commitment of precursor cells, it may affect the erythropoietin-sensitive precursor pool size or indirectly promote mitosis of certain elements of the erythroid series.

The mechanism of action of vitamin D_3 metabolites is unknown but is presumably similar in the different cell lines and involves the vitamin D_3 receptor. Recently, it was reported that $1,25(OH)_2D_3$ suppressed the amplified expression of the c-myc oncogene in HL-60 cells (35), thus suggesting a possible molecular mechanism for its induction of HL-60 differentiation, and raises the interesting question of whether an analogous mechanism operates in DS-19 cells. Indeed, it has been demonstrated that c-myc is expressed in erythroleukemia cells and the expression is rapidly and biphasically regulated by a differentiation inducer such as DMSO (36). Detailed studies along these lines should be of great value in understanding both the mechanism of action of vitamin D_3 hormones and the regulation of a process as complex as cellular differentiation.

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