1,25-Dihydroxyvitamin D₃ and its Analogs Inhibit Acute Myelogenous Leukemia Progenitor Proliferation by Suppressing Interleukin-1β Production

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Abstract

We hypothesized that 1,25-dihydroxyvitamin D₃ (1,25D₃) and its analogues may inhibit acute myelogenous leukemia (AML) proliferation by interrupting IL-1β–mediated growth-stimulatory signals. The incubation of the IL-1β–responsive AML cell line OCIM2 with 10 nM 1,25D₃ reduced growth 80% in liquid culture, and a 100–1000-fold lower concentration of 20-epi analogues (MC1288 and MC1301) was sufficient to achieve similar growth inhibition. The growth inhibition was associated with a rapid but transient downregulation of IL-1β and IL-1β–converting enzyme (ICE) mRNAs in 1,25D₃- and 20-epi analogue–treated cells, and the 20-epi analogue was more effective than 1,25D₃ in repressing ICE expression. An examination of long-term changes in the levels of mature IL-1β and its precursor revealed that 24-h incubation of OCIM2 with either 1,25D₃ or its 20-epi analogues abolished the production of mature IL-1β. The effect of 1,25D₃ and its analogues on growth of fresh bone marrow cells from seven AML patients was tested by a clonogenic assay. Growth inhibition of 60% was reached in only one of seven 1,25D₃-treated samples, but all seven samples were inhibited 60–90% by the 20-epi analogue MC1301. Growth inhibition by 1,25D₃ and the analogue was reversible by addition of IL-1β. These results suggest that 1,25D₃ and its 20-epi analogues interrupt IL-1β autocrine growth regulation by inhibiting IL-1β production and processing but not the response to IL-1β. (J. Clin. Invest. 1997. 100:1716–1724.) Key words: vitamin D • analogues • IL-1β • IL-1β–converting enzyme • acute myelogenous leukemia

Introduction

The physiological role of 1,25-dihydroxyvitamin D₃ (1,25D₃) is regulation of calcium homeostasis (1), but at pharmacological concentrations, 1,25D₃ can also inhibit growth and induce differentiation of normal and malignant hematopoietic cells (2–4). The mechanisms by which 1,25D₃ exerts its growth-inhibitory effects are not clear. Because the proliferation of malignant cells has been shown to be regulated by various cytokines in an autocrine and paracrine fashion (5–10), we have hypothesized that 1,25D₃ acts by interfering with these mechanisms. To test this hypothesis, it is necessary to identify a disease model with well-characterized autocrine growth requirements and then to assess the effect of 1,25D₃ on those requirements. Acute myelogenous leukemia (AML) is such a model, because IL-1β has been found to be produced at high concentrations by AML marrow cells and to stimulate AML growth (10, 11). In normal monocytes and macrophages, the unprocessed IL-1β is cell associated, and processing and secretion of the cleaved bioactive IL-1β occurs almost simultaneously in response to inflammatory stimuli (for a review, see reference 12). In leukemia cells, where IL-1β is expressed constitutively and acts as a growth factor for the cells that produce it, the regulation of IL-1β processing and secretion is less understood. Previous studies from our laboratory showed that in low cell density cultures (conditions essential for the growth-stimulatory action of IL-1β and the growth-inhibitory action of IL-1β inhibitors), both pro– and mature IL-1β are cell associated (13). Under these conditions, there is no detectable secretion of IL-1β into the culture medium. Studies from other laboratories show that AML patient blasts grown at high density may or may not secrete pro– and mature IL-1β (14, 15). However, even in samples that do not secrete IL-1β, the growth of the blasts is IL-1β dependent and can be inhibited by IL-1β antibodies (14). Therefore, in leukemia, the constitutively produced bioactive IL-1β probably remains membrane associated and may function in an autocrine fashion, or, alternatively, may be available for the receptors of the neighboring cells by cell–cell interaction (juxtacrine mechanism). Interference with IL-1β production or action is being considered as a therapy for AML. For example, it is possible to partially inhibit growth of AML cells by using inhibitors of IL-1β processing or an IL-1β receptor antagonist (16–20).

In the study reported here, we examined the effect of 1,25D₃ on the expression of IL-1β and the action of IL-1β–converting enzyme (ICE), an essential component in the maturation of pro–IL-1β into a bioactive cytokine (21–24). We reasoned that by identifying the step at which 1,25D₃ interferes with IL-1β action, it may be possible to improve the potency of 1,25D₃ by combining it with other agents that facilitate its growth-inhibitory action through the IL-1β pathway.

The cell line chosen for these experiments, OCIM2, was isolated from a patient with AML (French-American-British classification M6). These cells express markers of multiple cell lineages, including myeloid/monocytic, erythroid, and megakaryocytic cells (25). We report here that OCIM2 cells produce high concentrations of IL-1β and proliferate in response to this cytokine. Furthermore, the growth of OCIM2 is inhib-
ited by antibodies to IL-1β, soluble IL-1 receptor, and by IL-1β receptor antagonist. We demonstrate that 1,25D₃ inhibits the expression and processing of but not the response to IL-1β in OCIM2 cells. Analogues with a growth-inhibitory potency 100–1000-fold greater than that of 1,25D₃ inhibit processing of pro-IL-1β to its mature form more effectively than does the natural hormone.

Methods

Reagents. The vitamin D metabolite 1α,25-dihydroxyvitamin D₃ (1,25D₃) and the analogues 24a,26a,27a-tri-homo-1α,25-dihydroxyvitamin D₃ (CB-966), 20-epi-1α,25-dihydroxyvitamin D₃ (MC1288), 20-epi-24a,26a,27a-tri-homo-1α,25-dihydroxyvitamin D₃ (MC1301) (Fig. 1), and 1β,25-dihydroxyvitamin D₃ were the generous gifts of Dr. L. Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). [α,32P]-dATP was obtained from ICN Biomedicals, Inc. (Irvine, CA), and a high-sensitivity human IL-1β ELISA system was from Amersham Corp. (Arlington Heights, IL).

Cell culture and transfections. OCIM2 cells were maintained in RPMI 1640 supplemented with 10% FBS. For the studies of growth regulation, OCIM2 cells were grown in liquid cultures at a density of 10⁵ cells/ml in 100-mm dishes with or without the ligands at various concentrations. More ligand was added after 48 and 96 h, and the cells were counted after 2, 4, and 6 d.

For transfections, the cells were plated in 60-mm dishes at a density of 5 × 10⁴ cells/dish. The cells were transfected with 4 μg plasmid containing the vitamin D–responsive element (VDRE, GGTGACT-CACCGGGTGAAACGGGGCATT) from the human osteocalcin gene (26). This response element was attached to a thymidine kinase promoter and chloramphenicol acetyltransferase (CAT) reporter. All transfections were performed by the DEAE dextran method (27). 1,25D₃ was added immediately after transfection. For measurement of CAT activity, cells were washed in PBS 48 h after transfection, re-suspended in cold 0.25 M Tris-HCl (pH 7.5), lysed by three freeze-thaw cycles, and centrifuged. Aliquots of supernatant containing 50 μg protein were incubated with 1 mM acetylcoenzyme A and [14C]chloramphenicol for 24 h at 37°C. Substrate and products were resolved by TLC as described by Gorman et al. (28), and autoradiographed for 24–48 h.

RNA extraction and Northern blot analysis. Total RNA was extracted from OCIM2 cells by the acid-guanidinium thiocyanate–phenol–chloroform method (29). Briefly, the cells were homogenized in denaturing solution containing 4 M guanidinium thiocyanate and mixed sequentially with 2 M sodium acetate (pH 4), phenol, and chloroform. This mixture was centrifuged, and the upper layer was collected, precipitated with isopropanol, redissolved in denaturing solution, reprecipitated in isopropanol, washed with 75% ethanol, and dissolved in water.

Total RNA (10 μg/lane) was separated by formaldehyde denaturing agarose gel electrophoresis. The separated RNA was then transferred to Duralon membranes (Stratagene Inc., La Jolla, CA), prehybridized in formamide/dextran sulfate solution for 4 h at 42°C, and hybridized overnight with the indicated 32P-labeled probe. The membranes were then washed twice at room temperature in 1× SSC, once at 68°C in 0.1× SSC, sealed in plastic bags, and mRNA was detected by autoradiography after 1–96 h exposure to x-ray film.

Cell line clonogenic assay. The clonogenic assay was performed as previously described (30). Briefly, OCIM2 cells were cultured in 0.8% methylcellulose (Fluka Chemical Corp., Ronkonkoma, NY), 10% FCS, and RPMI 1640 at 2–4 × 10⁵ cells/ml. When indicated, the following reagents were added: IL-1β neutralizing antibodies (100 ng/ml; Genzyme Corp., Boston MA), soluble IL-1 receptor (500 ng/ml; Immunex Corp., Seattle, WA), or IL-1 receptor antagonist (100 ng/ml; Synergen Inc., Boulder, CO). 1,25D₃ or its analogues were added at the initiation of culture at a final concentration of 10 nM, with or without 100 U/ml recombinant human IL-1β (mol wt 17,500; Boehringer Mannheim Biochemicals, Indianapolis, IN). Each culture mixture was placed in four 35-mm petri dishes (Nunc, Inc., Naperville, IL) and maintained at 37°C with 5% CO₂ in air in a humidified atmosphere. Colonies were counted after 7 d with an inverted microscope. A colony was defined as a cluster of > 40 cells.

Western immunoblotting. Cell lysates were assayed for protein concentration by the BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL). Each set of paired samples was then adjusted to the same protein concentration. The proteins in the cell lysates (500 μg/lane) were denatured in Laemmli sample buffer and separated by SDS-PAGE. Electrophoresis was conducted at constant wattage (10 W) in running buffer cooled to 4°C. The stacking gels contained 4% (wt/vol) acrylamide-bisacrylamide (29:1), and the separating gels contained 12% (wt/vol) acrylamide-bisacrylamide (29:1). After electrophoresis, the separated proteins were transferred to nitrocellulose membranes overnight at 30 V in a cooled (4°C) reservoir containing transfer buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol, pH 8.3). The nitrocellulose membranes were stained for 5 min in 0.5% Ponceau S and 1% glacial acetic acid in H₂O to verify equal loading of protein in control and treated samples (31). After staining, the membranes were rinsed for 2 min, examined for protein staining, then rinsed again for 10 min.

For immunodetection, the membranes were incubated in 5% dried milk dissolved in 50 mM PBS for 1 h at room temperature, washed three times in PBS plus 0.5% Tween 20, and incubated for 1 h with polyclonal rabbit anti–IL-1β antibody (Endogen, Inc., Boston, MA) or normal rabbit IgG (used as a control) diluted 1:200 in PBS containing 0.5% Tween 20. After incubation, the membranes were subjected to three 15-min rinses in PBS containing 0.5% Tween 20. Bound antibody was detected with the ECL Western Blotting Detection System (Amersham Corp.) The membranes were incubated at room temperature for 1 h with anti–rabbit horseradish peroxidase–labeled antibody diluted 1:2,000 in PBS plus 0.5% Tween 20. The membranes were then washed in PBS containing 0.5% Tween 20, and
bound antibody was detected by the ECL system. Chemiluminescence of the membranes was detected by exposure to X-OMAT AR5 film (Eastman Kodak Co., Rochester, NY).

**Patient samples.** Bone marrow aspirates were obtained at diagnosis from seven patients with AML. The studies were performed with the patients’ informed consent and were approved by the Human Experimentation Committee of our institution. Adherent cells and T cells were removed from the bone marrow samples as described below.

**Adherent cell fractionation.** Low-density bone marrow mononuclear cells obtained by fractionation with Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ) were incubated in plastic tissue-culture dishes or flasks (Falcon Plastics; Becton Dickinson, San Jose, CA) with 10% FCS in α-medium (GIBCO BRL, Gaithersburg, MD). The fractionation procedure was repeated until no cells adhered to the tissue-culture dishes. The nonadherent cells harvested in this way contained <3% monocytes as confirmed by the following techniques: (a) microscopic differential counting of at least 100 cells prepared with Wright’s stain; (b) nonspecific esterase (α-naphthyl butyrate) staining; (c) immunocytochemical analysis with CD14 (My-4) mAb (Coulter Corp., Hialeah, FL) to identify monocyte–promonocyte cells, as previously described (32, 33).

**T cell depletion.** T cells were depleted from the nonadherent fraction by negative immunomagnetic selection with modifications (34). Nonadherent bone marrow cells were incubated with CD3 mAb (Becton Dickinson) at a concentration of 1 μg/10⁶ cells in PBS with 0.25% FCS for 30 min at 4°C. The labeled cells were washed three times and then incubated at a ratio of 20:1 beads/cell with goat anti-mouse IgG-conjugated immunomagnetic beads (Advanced Magnetics, Inc., Cambridge, MA) at 4°C for 60 min in an end-over-end rotation. Immunomagnetic bead–rosetted cells were removed by using a magnetic particle concentrator (Advanced Magnetics, Inc.), and the unrosetted cells remaining in suspension were harvested with a Pasteur pipette. In some experiments, this procedure was repeated twice. The T lymphocyte–depleted population contained <3% CD3+ cells, as assessed by an immunocytochemical staining performed on cytospin cells (32, 33).

**AML blast colony assay.** A method described previously was used to assay AML blast colony formation (35, 36). Briefly, 10⁶ nonadherent T cell–depleted bone marrow cells were plated in 0.8% methylcellulose in α-medium supplemented with 10% FCS and with 15 ng/ml recombinant human GM-CSF (Immunex Corp.). Vehicle, 1,25D₃, or analogs at a final concentration of 10 nM were added in the absence or presence of 100 U/ml IL-1β. The cultures were incubated in 35-mm petri dishes in duplicate for 7 d at 37°C in a humidified atmosphere of 5% CO₂ in air. The number of AML blast colonies was evaluated microscopically on day 7 of culture. A blast colony was defined as a cluster of 20 or more cells. We have shown previously that the AML blast colony assay identifies blasts rather than normal progenitors by cytogenetic analysis of individual colonies (37).

**Results**

**Characterization of the autocrine regulation of OCIM2 proliferation by IL-1β.** To confirm the role of IL-1β as a growth factor of the AML cell line OCIM2, we performed a clonogenic assay in the presence of exogenous IL-1β, or in the presence of inhibitors of IL-1β action, including neutralizing antibody to IL-1β, IL-1 receptor antagonist, or soluble IL-1 receptor. The results of these experiments (Fig. 2) show that the addition of each inhibitor of IL-1β action caused a 40–50% inhibition of OCIM2 colonies. These results suggest that bioactive IL-1β is produced by OCIM2 and functions in an autocrine fashion as growth factor. The addition of exogenous IL-1β induced a slight growth stimulation of OCIM2 in the control group, and reversed the effect of IL-1β inhibitors on OCIM2 prolifera-

![Figure 2. Growth inhibition of OCIM2 cells by inhibitors of IL-1β action. OCIM2 cells were plated in four plates in medium containing methyl cellulose at a density of 10⁵ cells/ml, in the presence or absence of IL-1β (100 U/ml) and the following reagents: IL-1β neutralizing antibodies (Ab, 100 ng/ml), soluble IL-1 receptor (sIL-1R, 500 ng/ml), and IL-1 receptor antagonist (IL-1RA, 100 ng/ml). The effect of these treatments on formation of colonies was evaluated after 7 d by microscopic examination.](image-url)
performed dose–response experiments. OCIM2 cells were incubated with 1,25D₃ and counted after 2, 4, and 6 d. Significant growth inhibition was detected first on day 4, and maximal growth inhibition (80%) occurred on day 6 (data not shown). The dose–response plots show that the sensitivity of OCIM2 cells to the growth-inhibitory effect of 1,25D₃ was comparable to that of the highly sensitive leukemia cell line, HL60: both cell lines had an ED₅₀ of 0.2–0.5 nM (Fig. 4 B and reference 40). Therefore, even though OCIM2 cells have a mixed phenotype of early myelomonocytic and erythroid lineage whereas the HL60 cells are characterized as monocyte/granulocyte progenitors, the two leukemia cell lines responded similarly to the growth-inhibitory action of 1,25D₃.

Effect of 1,25D₃ analogue structure on growth-inhibition of OCIM2 cells. Recent studies from our laboratories showed that VDR-mediated transcriptional activity of vitamin D analogues correlated with their potency as growth-inhibitory agents in the leukemia cell line HL60 (40). To determine if this pattern was also true for the AML cell line OCIM2, we measured the growth-inhibitory effect of 1,25D₃, and three side-chain–modified analogues (Fig. 1 and Fig. 4 B) and compared the results with our previously reported measurements of VDR-mediated transcriptional activity for these compounds (41). The results of these titrations show the following: (a) all side-chain modifications increased both transcriptional activity and growth-inhibitory action; (b) side-chains with a 20-epi configuration (analogues MC1288 and MC1301) were more potent than side-chains with natural orientation (1,25D₃ and analogue CB966); (c) the analogue MC1301, which has both chemical and stereochemical modifications in its side-chain, had the highest VDR-mediated transcriptional activity (ED₅₀ = 6 × 10⁻¹³ M; reference 41) and the highest growth-inhibitory activity in AML cells (ED₅₀ = 4 × 10⁻¹³ M). These results suggest that the relative potency of 1,25D₃ and the analogues used in these experiments does not change in different types of myeloid leukemia cells, and is well correlated with their VDR-mediated transcriptional activity.

The side-chain modification may affect not only the overall potency of the analogue but also the spectrum of gene-regulatory events induced by it, thus leading to diverse cellular response. To determine if the growth-inhibitory actions induced by the various analogues involved the IL-1β pathway, we examined whether IL-1β could reverse growth inhibition by the analogues as it does inhibition by 1,25D₃. All ligands were given at a concentration sufficient to induce maximal growth inhibition in liquid culture (10 nM), and colony formation in the presence and absence of IL-1β was examined (Fig. 4 A). Our experiments showed that the actions of the three analogues were at least partly reversible by IL-1β, and suggest that the growth-inhibitory actions of the analogues, like that of 1,25D₃, do not involve inhibiting the response of the cells to IL-1β.

Effect of 1,25D₃ on IL-1β expression. Because the growth of OCIM2 cells depends on IL-1β, we examined the effect of 1,25D₃ on IL-1β expression and processing. This was done by plating OCIM2 cells at low density, treating them with 10 nM 1,25D₃, and comparing the steady-state levels of IL-1β and ICE mRNA in control and 1,25D₃-treated cultures at various
The effect of the two ligands on ICE mRNA was evident even in the presence of cycloheximide, suggesting that this action of the ligands does not depend on synthesis of new proteins. The effect of the two ligands on ICE mRNA was assessed by Northern blotting as described in Methods.

Northern blot analysis using RNA from OCIM2 cells treated with 10 nM and 1 nM 1,25D₃ and collected 3 h after plating. Expression of IL-1β, ICE, and β-actin mRNA was assessed by Northern blotting.

Because our ELISA assay detects both pro–IL-1β and bioactive IL-1β, we also examined the cell-associated immunoreactive IL-1β by Western blotting (Fig. 6). Analysis of cell extracts showed a significant amount of cell-associated pro–IL-1β and mature IL-1β in untreated cells. A 24-h incubation of the cells with 10 nM 1,25D₃, or its analogues completely abolished the cell-associated mature IL-1β but significantly increased the amounts of pro–IL-1β. In conclusion, immunoreactive IL-1β suggest that the greater potency of the 20-epi analogue is associated with a more effective repression of ICE mRNA expression.

Figure 5. Time course for repression of IL-1β and ICE mRNA by 1,25D₃. OCIM2 cells were plated in liquid culture medium at a density of 10⁵ cells/ml, with and without 10 nM 1,25D₃, and collected after 3, 6, and 24 h. For 1-h treatment, 1,25D₃ was added 2 h after plating, and cells were harvested 3 h after plating. Expression of IL-1β, ICE, and β-actin mRNA was assessed by Northern blotting.

Figure 6. Dose-dependent repression of IL-1β and ICE mRNA expression by 1,25D₃ and MC1288. OCIM2 cells were plated in liquid culture medium at a density of 10⁵ cells/ml with and without the indicated doses of 1,25D₃, MC1288, or 10 μg/ml cycloheximide (CHX). After 1 h (experiments without cycloheximide) or 3 h (experiments with cycloheximide), IL-1β, ICE, and β-actin mRNA were assessed by Northern blotting.

Figure 7. Quantification of immunoreactive IL-β production by OCIM2 cells. OCIM2 cells were plated in liquid culture at a density of 10⁵ cells/ml, with or without 10 nM 1,25D₃ and its analogues. Medium was collected after 1, 3, 6, 24, and 48 h, and cell extracts were prepared after 24 h. Immunoreactive IL-β was quantified by an ELISA assay with 0.1 ml of medium or extract from 10⁴ cells. The sensitivity of the assay is 0.3 pg/ml. The dots shown are results from cell extracts obtained from duplicate cultures. Immunoreactive IL-β was undetectable in the medium at any of the time points and after any of the treatments indicated.
was not detected in culture medium of control OCIM2 cells, but both pro–IL-1β and mature IL-1β were found in extracts from these cells. Therefore, we conclude that neither pro–IL-1β nor mature IL-1β are released to the medium by these cells. Furthermore, because culture medium from 1,25D₃-and analogue-treated OCIM2 cells did not contain detectable immunoreactive IL-1β and the cell-associated proteins did not contain any detectable mature IL-1β, we conclude that the hormone and its analogues induced complete and long-lasting repression of mature IL-1β production.

The intracellular accumulation of pro–IL-1β suggests that synthesis of pro–IL-1β protein is not blocked in 1,25D₃- and analogue-treated cells. Therefore, the absence of mature IL-1β in these cells is not due to depletion of the substrate but more likely to repression of mature IL-1β production by a very effective inhibition of ICE activity. We do not know if inhibition of ICE activity by 1,25D₃ and its analogues is due to repression of ICE protein synthesis or cleavage, or to direct repression of enzyme activity.

**Growth inhibition of leukemia progenitors from AML patients by 1,25D₃ and its analogues.** We detected differences in the potency and mode of action of 1,25D₃ and its analogues with respect to growth inhibition and cytokine production in an AML cell line. To determine whether there is a similar effect in primary cultures of leukemia progenitors, we obtained fresh bone marrow samples from AML patients. It was especially important to determine the degree of reversibility of treatment by exogenous IL-1β, because it stimulates the growth of leukemia cells in both autocrine and paracrine fashion. Samples from seven patients were treated with 1,25D₃ and the three analogues, with or without IL-1β, and AML progenitor proliferation was assessed by the AML blast colony assay (Fig. 9). There was a great degree of heterogeneity in the responsiveness of individual patient cells to the growth-stimulatory action of exogenous IL-1β (Fig. 9 B): the growth of two samples was stimulated significantly by IL-1β, the growth of three was slightly stimulated, and the growth of two samples was not. Likewise, the growth-inhibitory response to 1,25D₃ was diverse (Fig. 9 A): one patient sample did not respond, five

responded poorly (20–40% growth inhibition), and one was inhibited 60%. All of the IL-1β-responsive samples responded to the exogenous cytokine by growth stimulation in the presence of 1,25D₃, and the growth-inhibitory action of 1,25D₃ was reversed significantly by IL-1β in five patients. These results suggest that the growth-inhibitory effect of 1,25D₃ is, at least in part, mediated by suppression of the IL-1β pathway. However, 1,25D₃ action in AML is probably not mediated through repression of the response to IL-1β.

The responses of the patient samples to the analogues MC1288 and CB966 were not significantly different from their
response to the natural hormone: samples that did not respond to 1,25D$_3$ also did not respond to these analogues, and samples that responded poorly to 1,25D$_3$ also responded poorly to these two analogues (data not shown). In contrast, all of the patient samples responded to analogue MC1301 by 60–90% growth inhibition. Six of the samples treated with MC1301 responded to IL-1β by partial reversal of growth inhibition (Fig. 9A). These results support our findings from the AML cell line, that MC1301 is the most potent growth-inhibitory analogue, and that growth inhibition by the analogues did not prevent the response of AML cells to IL-1β. In fact, in four patient samples, treatment with MC1301 increased the growth-stimulatory action of IL-1β (Fig. 9B). These results suggest that MC1301 may be useful for treatment of AML because it is a potent inhibitor of cell growth and, more importantly, because it acts on all patient samples. However, because MC1301 treatment stimulated somewhat the response to IL-1β, a combination of MC1301 and an inhibitor of IL-1β action (IL-1 receptor antagonist or soluble IL-1 receptor) may increase effectiveness of this therapeutic approach.

Discussion

It has been shown that the growth-inhibitory action of 1,25D$_3$ in hematopoietic precursors is associated with an induction of sequence of gene regulatory events that lead to a cell-cycle arrest at G$_0$–G$_1$ (3, 42). This sequence of molecular events involves downregulation of the oncogenes c-myc (43) and c-myb (44) and upregulation of gene products that are associated with inhibition of cell cycle, such as the cyclin-dependent kinase inhibitor waif/p21 (45). However, it is not clear whether these are primary target genes for 1,25D$_3$ action, and their repression (c-myc, c-myb) or induction (p21) overcomes the constitutive growth stimulus of the leukemia cells. Alternatively, these gene-regulatory events may be the consequence of earlier 1,25D$_3$–induced molecular events that directly inhibit the production and action of growth factors. The AML cell culture model we used has the advantage of providing a clear-cut starting point for studying the mechanism of growth-inhibitory and differentiating actions of 1,25D$_3$ and its analogues. Because the cells produce and depend on a specific cytokine, IL-1β, they can be used to answer a very simple question: Do 1,25D$_3$ and its analogues inhibit production of or response to the cytokine? Alternatively, does their growth-inhibitory effect bypass the IL-1β pathway?

We found that in an IL-1β–producing and –responsive AML cell line, 1,25D$_3$ inhibited various aspects of IL-1β production: it decreased the steady-state levels of IL-1β and ICE mRNAs, and it stopped completely the production of mature IL-1β protein. The inhibitory effect of the hormone and its analogues on IL-1β processing was longer lasting than the repression of IL-1β and ICE mRNA. Furthermore, the synthesis of pro–IL-1β protein was not repressed at all. Therefore, we hypothesize that the inhibition of ICE action in AML cells is the predominant mechanism by which 1,25D$_3$ and its analogues inhibit the IL-1β–mediated growth response.

What is the mechanism by which 1,25D$_3$ and its analogues inhibit ICE action? Our experiments suggest that it is not suppression of ICE gene expression, because it lasted only 1–3 h. Furthermore, there are other lines of evidence that suggest that the primary mode of regulation of ICE activity is not regulation of ICE gene expression and protein synthesis. For example, ICE mRNA is expressed constitutively in most tissues (21, 24, 46), and large quantities of the immunoreactive unprocessed protein can be detected in those tissues. On the other hand, the processed enzyme cannot be detected, suggesting that active ICE does not normally accumulate (47). More importantly, in biological conditions that require rapid upregulation of ICE activity, such as endotoxin stimulation, ICE mRNA levels do not change (46, 48). It has been proposed that ICE activity is dependent on the availability of the substrate (pro–IL-1β). However, the results of the ELISA assays and Western blots suggest that in our system the availability of the substrate (pro–IL-1β) cannot be the limiting factor in the action of ICE in 1,25D$_3$–treated cells. Another mechanism for regulation of ICE activity is a cleavage by ICE homologue type 3 (ICH-3) (48). The upregulation of ICH-3 mRNA by endotoxin and in vitro evidence that ICH-3 can cleave ICE suggest that ICH-3 is a likely upstream candidate for regulation of ICE activity. Thus, the short term repression of ICE activity by 1,25D$_3$ and its analogues may be due to the rapid and transient downregulation of ICE mRNA, but the long-term repression of the enzyme may be due to a late and longer lasting repression of a putative upstream activator of ICE, ICH-3. Another possibility is that 1,25D$_3$ and its analogues induce natural inhibitors of ICE action, such as a eukaryotic homologue of the cytokine response modifier (crmA) or a specific cystatin (49).

1,25D$_3$ is a classic growth-inhibitory and differentiating agent for myeloid leukemia. This hormone, however, has not been used in the treatment of leukemia because in physiological concentration its action is moderate and reversible and in pharmacological amounts its toxic side-effects are severe and irreversible (42, 50). Several approaches have been used to increase its potency, including synthesis of analogues with extensive structural changes (reviewed in reference 51). The study presented here points to the possibility that one analogue of 1,25D$_3$, MC1301, is indeed significantly more potent than 1,25D$_3$ with respect to inhibition of AML growth in both cell line and primary leukemia progenitor cultures. The molecular mechanisms that promote the greater potency of this 20-epi analogue have been shown recently to be associated with the induction of an extremely stable complex with the VDR (41). Because this analogue is active even at picomolar concentrations, it is possible that it can be used in vivo in small quantities sufficient to induce significant repression of leukemia cell growth without inducing toxic hypercalcemia.

Another approach to increase the potency of 1,25D$_3$ therapy is combining 1,25D$_3$ analogues with other drugs to facilitate growth-inhibitory actions (42). However, combination therapy requires a comprehensive understanding of the mechanism by which 1,25D$_3$ or its analogues inhibits the growth of leukemia cells. The results of this study suggest a rational approach to combination therapy for AML. The reversibility of the action of 1,25D$_3$ and its analogues by exogenous IL-1β in both a cell line and patient samples supports the hypothesis that the growth-inhibitory effect of these agents on AML is mediated through inhibition of IL-1β production and not by inhibition of IL-1β action. Because the hormone and its analogues did not suppress the response to IL-1β, it is likely that in the natural environment, 1,25D$_3$- or analogue-treated leukemia cells are still susceptible to the trophic actions of IL-1β produced by neighboring cells. Therefore, it may be possible to treat AML by combining a potent vitamin D analogue and an inhibitor of IL-1β action such as IL-1β receptor antagonist.
or soluble IL-1β receptor. This use of combination therapy has two advantages: it may inhibit both the autoimmune and paracrine trophic action of IL-1β, and it may potentiate analogue action, so that small amounts of analogue can be used effectively with a minimal risk of toxic calcemic side-effects.

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