1,25-Dihydroxyvitamin D_3 and its Analogues Inhibit Acute Myelogenous Leukemia Progenitor Proliferation by Suppressing Interleukin-1β Production

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Abstract

We hypothesized that 1,25-dihydroxyvitamin D_3 ($1,25D_3$) 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 $1,25D_3$ 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 $1,25D_3$- and 20-epi analogue–treated cells, and the 20-epi analogue was more effective than $1,25D_3$ 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 $1,25D_3$ or its 20-epi analogues abolished the production of mature IL-1β. The effect of $1,25D_3$ 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 $1,25D_3$-treated samples, but all seven samples were inhibited 60–90% by the 20-epi analogue MC1301. Growth inhibition by $1,25D_3$ and the analogue was reversible by addition of IL-1β. These results suggest that $1,25D_3$ 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 D_3 ($1,25D_3$)[1] is regulation of calcium homeostasis (1), but at pharmacological concentrations, $1,25D_3$ can also inhibit growth and induce differentiation of normal and malignant hematopoietic cells (2–4). The mechanisms by which $1,25D_3$ 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 $1,25D_3$ 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 $1,25D_3$ 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 $1,25D_3$ 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 $1,25D_3$ interferes with IL-1β action, it may be possible to improve the potency of $1,25D_3$ 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-

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1. *Abbreviations used in this paper:* AML, acute myelogenous leukemia; CAT, chloramphenicol acetyltransferase; $1,25D_3$, 1α,25-dihydroxyvitamin D_3; ICE, IL-1β–converting enzyme; RSV, Rous sarcoma virus; VDR, vitamin D receptor; VDRE, vitamin D–responsive element.

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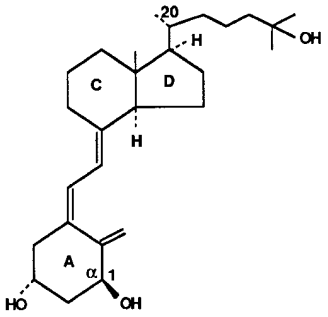
ited by antibodies to IL-1β, soluble IL-1 receptor, and by IL-1β receptor antagonist. We demonstrate that $1,25D_3$ inhibits the expression and processing of but not the response to IL-1β in OCIM2 cells. Analogues with a growth-inhibitory potency 100–1000-fold greater than that of $1,25D_3$ inhibit processing of pro–IL-1β to its mature form more effectively than does the natural hormone.

Methods

Reagents. The vitamin D metabolite 1α,25-dihydroxyvitamin D_3 ($1,25D_3$) and the analogues 24a,26a,27a-tri-homo-1α,25-dihydroxyvitamin D_3 (CB-966), 20-epi-1α,25-dihydroxyvitamin D_3 (MC1288), 20-epi-24a,26a,27a-tri-homo-1α,25-dihydroxyvitamin D_3 (MC1301) (Fig. 1), and 1β,25-dihydroxyvitamin D_3 were the generous gifts of Dr. L. Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). [α-^{32}P]-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 10^5 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 5×10^5 cells/dish. The cells were transfected with 4 μg plasmid containing the vitamin D–responsive element (VDRE, GGTGACT-CACCGGGTGAACGGGGGCATT) 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). $1,25D_3$ was added immediately after transfection. For measurement of CAT activity, cells were washed in PBS 48 h after transfection, resuspended 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 acetylcoenyzme A and [^{14}C]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.



1α,25-dihydroxyvitamin D3

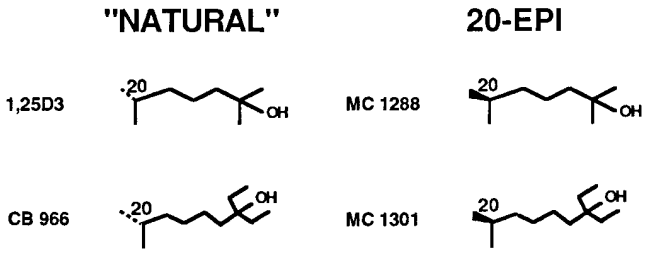


Figure 1. Structural formulas of $1,25D_3$ and its analogues. *Natural*, natural stereochemistry of the side-chain; *20-epi*, 20-epi orientation of the side-chain.

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 ^{32}P-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 $2–4 \times 10^4$ 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). $1,25D_3$ 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% CO_2 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 H_2O 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/10^6 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 cytospun cells (32, 33).

AML blast colony assay. A method described previously was used to assay AML blast colony formation (35, 36). Briefly, 10^5 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, $1,25D_3$, or analogues 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% CO_2 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-1β 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 proliferation. These results indicate that OCIM2 cells are also responsive to exogenously added IL-1β.

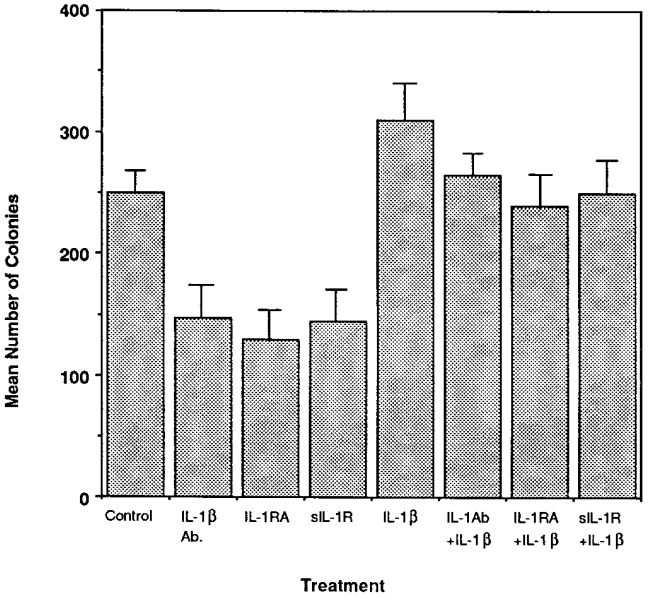


Figure 2. Growth inhibition of OCIM2 cells by inhibitors of IL-1β action. OCIM2 cells were plated in four plates in medium containing methyl cellulose at a density of 10^5 cells/ml, in the presence or absence of IL-1β (100 U/ml) and the following reagents: IL-1β neutralizing antibodies (*Ab*, 100 ng/ml), 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.

Transcriptional and growth-inhibitory responses of the AML cell line OCIM2 to $1,25D_3$. $1,25D_3$ can inhibit the growth of cells only if they have functional nuclear receptors for vitamin D (38, 39). Therefore, we first tested OCIM2 for the presence of transcriptionally active vitamin D receptor (VDR). The cells were transfected with a reporter gene (CAT) containing a thymidine kinase promoter and the osteocalcin VDRE (Fig. 3). As a control, we used a fusion gene containing a CAT reporter and a promoter that is not responsive to vitamin D, Rous sarcoma virus (RSV). The transfected cells were grown with and without $1,25D_3$, and CAT expression was measured 48 h after transfection. There was a 5–10-fold induction of the transfected osteocalcin VDRE/CAT gene by $1,25D_3$ (Fig. 3 *B*), whereas the RSV/CAT gene did not respond to the hormone (Fig. 3 *A*). We concluded that OCIM2 cells contained ligand-responsive, transcriptionally active VDRs.

Next, we used clonogenic assays to examine the effect of $1,25D_3$ on the growth of the cells. In these assays, we asked two questions: Does $1,25D_3$ inhibit colony formation by these cells? and, can IL-1β prevent the growth-inhibitory action of the hormone? The results showed that $1,25D_3$ inhibited OCIM2 colony formation and that the growth-inhibitory effect of $1,25D_3$ was partially reversible by IL-1β (Fig. 4 *A*). These findings suggested that $1,25D_3$ effectively inhibits the growth of these AML cells without interfering with their response to the growth-stimulatory action of exogenous IL-1β.

To examine growth-inhibitory action of $1,25D_3$ in greater detail, we repeated the experiments in liquid culture (Fig. 4 *B*),

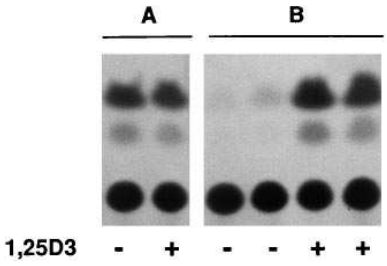


Figure 3. Transcriptional activity of the VDR in OCIM2 cells. OCIM2 cells were transfected with the control plasmid RSV/CAT (*A*), or a reporter gene containing the osteocalcin VDRE attached to the thymidine kinase promoter and CAT reporter (*B*). The transfected cells were treated with vehicle (ethanol) or 10 nM $1,25D_3$ for 48 h. CAT activity was assayed in extracts from the transfected cells, and the products were separated by TLC and detected by autoradiography.

performing dose–response experiments. OCIM2 cells were incubated with $1,25D_3$ 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 $1,25D_3$ was comparable to that of the highly sensitive leukemia cell line, HL60: both cell lines had an ED_{50} 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 $1,25D_3$.

Effect of $1,25D_3$ 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 $1,25D_3$ 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 ($1,25D_3$ 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 ($ED_{50} = 6 \times 10^{-13}$ M; reference 41) and the highest growth-inhibitory activity in AML cells ($ED_{50} = 4 \times 10^{-13}$ M). These results suggest that the relative potency of $1,25D_3$ 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 $1,25D_3$. All ligands were given at a concentration sufficient to induce maximal growth

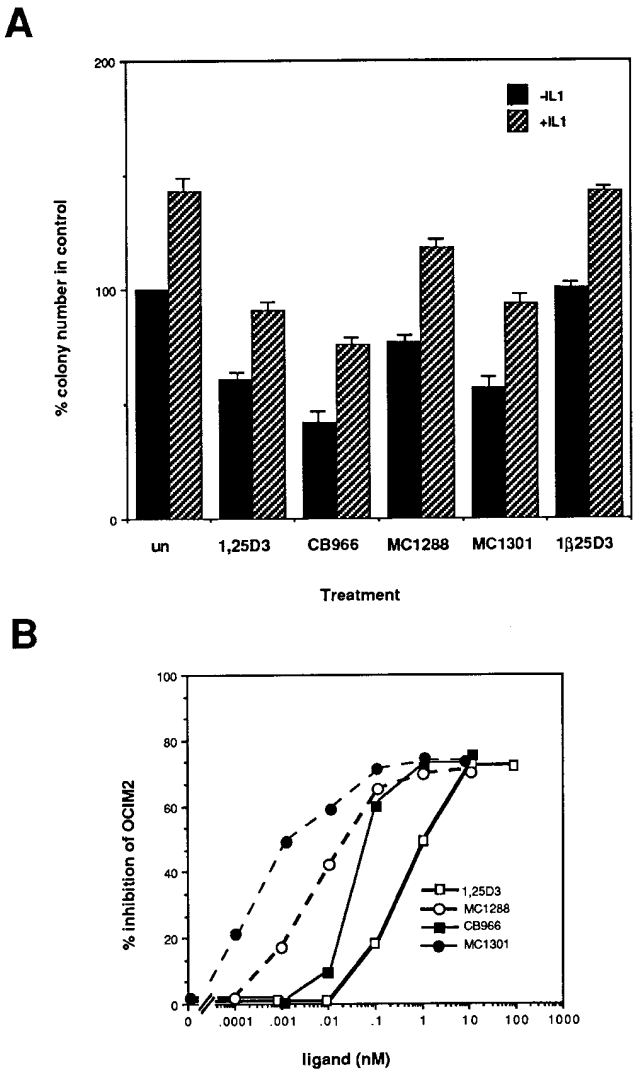


Figure 4. Growth inhibition of OCIM2 cells by $1,25D_3$ and its analogues. (*A*) OCIM2 cells (10^5/ml) were plated in four plates in medium containing methyl cellulose, with or without IL-1β (100 U/ml) and 10 nM $1,25D_3$ or its analogues. Colony formation was measured after 7 d by microscopic examination. (*B*) OCIM2 cells were plated in two plates at 10^5 cells/ml in RPMI 1640 and 10% FBS, with or without increasing concentrations of $1,25D_3$ or its analogues. Fresh ligand was added after 48 and 96 h of culture. The cells were counted after 6 d of incubation.

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 $1,25D_3$, do not involve inhibiting the response of the cells to IL-1β.

Effect of $1,25D_3$ on IL-1β expression. Because the growth of OCIM2 cells depends on IL-1β, we examined the effect of $1,25D_3$ on IL-1β expression and processing. This was done by plating OCIM2 cells at low density, treating them with 10 nM $1,25D_3$, and comparing the steady-state levels of IL-1β and ICE mRNA in control and $1,25D_3$-treated cultures at various

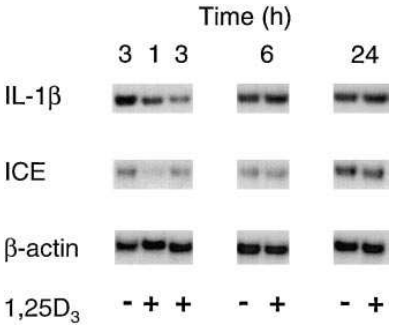


Figure 5. Time course for repression of IL-1β and ICE mRNA by $1,25D_3$. OCIM2 cells were plated in liquid culture medium at a density of 10^5 cells/ml, with and without 10 nM $1,25D_3$, and collected after 3, 6, and 24 h. For 1-h treatment, $1,25D_3$ was added 2 h after plating, and cells were collected 3 h after plating. Expression of IL-1β, ICE, and β-actin mRNA was assessed by Northern blotting as described in Methods.

times by Northern blot analysis (Fig. 5). These experiments showed that there was a slight decrease in IL-1β mRNA in control cultures after 6–24 h and a significant increase in ICE mRNA in control cultures after 24 h. $1,25D_3$ induced rapid but transient downregulation of both genes. This repression was first observed within 1 h of treatment, but diminished after 6 h.

Because the 20-epi analogue of $1,25D_3$ (MC1288) is a hundred times more potent in inhibiting growth than the natural hormone, we examined whether MC1288 was also a more efficient repressor of IL-1β and ICE expression. We repeated the Northern blot analysis using RNA from OCIM2 cells treated with 10 nM and 1 nM $1,25D_3$ and MC1288 (Fig. 6). The experiments showed that 1 nM $1,25D_3$ reduced IL-1β mRNA expression 60%, but 10 nM $1,25D_3$ had no further effect. Similarly, maximal repression (70%) of IL-1β mRNA was seen with 1 nM MC1288, and there was no further inhibition by 10 nM MC1288. Significant repression of IL-1β mRNA by these ligands 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 different: at 1 nM, $1,25D_3$ had no inhibitory effect on ICE mRNA, and 10 nM repressed expression only 40%. On the other hand, 1 nM MC1288 induced a maximal repression of ICE mRNA (70%). These results clearly indicate differential gene regulation by $1,25D_3$ and MC1288. Furthermore, they suggest that the greater potency of the 20-epi analogue is associated with a more effective repression of ICE mRNA expression.

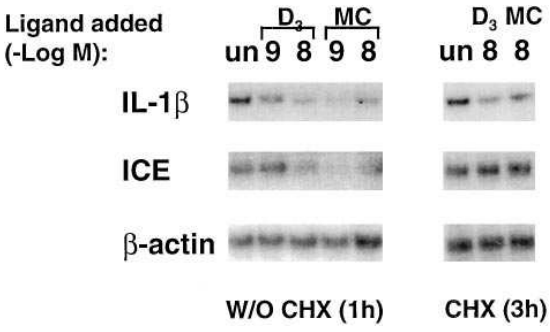


Figure 6. Dose-dependent repression of IL-1β and ICE mRNA expression by $1,25D_3$ and MC1288. OCIM2 cells were plated in liquid culture medium at a density of 10^5 cells/ml with and without the indicated doses of $1,25D_3$, MC1288, or 10 μg/ml cycloheximide (*CHX*). After 1 h (experiments without cycloheximide) or 3 h (experiments with cycloheximide), IL-1β, ICE, and β-actin mRNA were assessed by Northern blotting.

Effect of $1,25D_3$ and its analogues on expression of IL-1β protein. The repression of IL-1β and ICE mRNA by $1,25D_3$ and MC1288 was transient, and at later time points [24 (Fig. 5) and 48 h (data not shown)], these mRNAs returned to control levels. To determine if the IL-1β protein levels also change and if the change lasts longer than the mRNA changes, we performed several assays. First, we quantified the overall immunoreactive IL-1β produced by OCIM2 cells 24 h after treatment, using an ELISA (Fig 7). This assay showed that 10^4 OCIM2 cells contained 1 pg of immunoreactive IL-1β, and a 24-h incubation induced 1.5-, 2-, 2.5-, and 4-fold upregulation of immunoreactive IL-1β protein by $1,25D_3$, MC1288, CB966, and MC1301, respectively. We also examined the immunoreactive IL-1β secreted to the medium by these cells. We were unable to detect secretion of IL-1β by untreated and ligand-treated cells after up to 48 h of incubation even with an ELISA assay system that can detect as little as 0.3 pg/ml (Amersham Corp.).

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. 8). 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 $1,25D_3$ 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

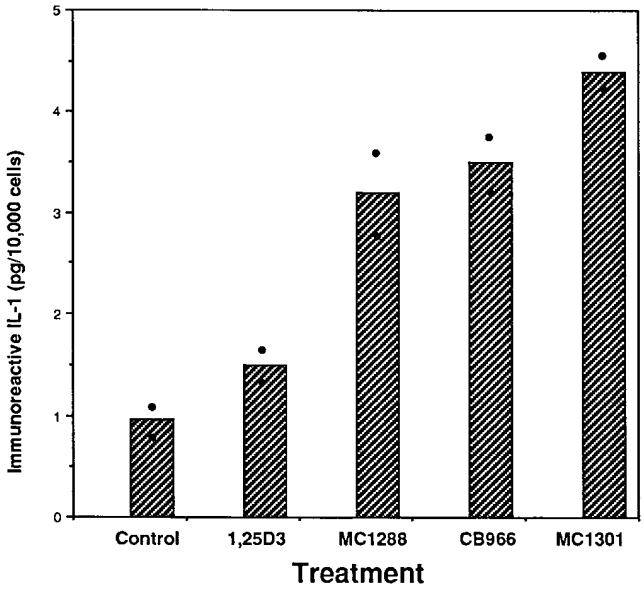


Figure 7. Quantification of immunoreactive IL-β production by OCIM2 cells. OCIM2 cells were plated in liquid culture at a density of 10^5 cells/ml, with or without 10 nM $1,25D_3$ and its analogues. Medium was collected after 1, 3, 6, 24, and 48 h, and cell extracts were prepared after 24 h. Immunoreactive IL-1β was quantified by an ELISA assay with 0.1 ml of medium or extract from 10^4 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-1β was undetectable in the medium at any of the time points and after any of the treatments indicated.

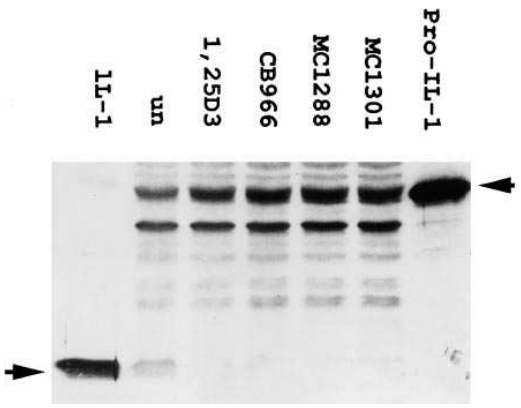


Figure 8. Western blot analysis of immunoreactive IL-1β in OCIM2 cells. OCIM2 cells were plated in liquid culture at a density of 2×10^5 cells/ml and treated for 24 h with vehicle only (*un*) or with 10 nM $1,25D_3$ or its analogues. Cellular proteins (500 μg/sample) were separated by SDS-PAGE and detected by Western blotting. The positions of pro–IL-1β and mature IL-1β in standard samples and cell extracts are indicated by arrows.

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 $1,25D_3$- 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 $1,25D_3$- 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 $1,25D_3$ 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 1,25D$_3$ and its analogues. We detected differences in the potency and mode of action of $1,25D_3$ 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 $1,25D_3$ 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 $1,25D_3$ 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 $1,25D_3$, and the growth-inhibitory action of $1,25D_3$ was reversed significantly by IL-1β in five patients. These results suggest that the growth-inhibitory effect of $1,25D_3$ is, at least in part, mediated by suppression of the IL-1β pathway. However, $1,25D_3$ 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

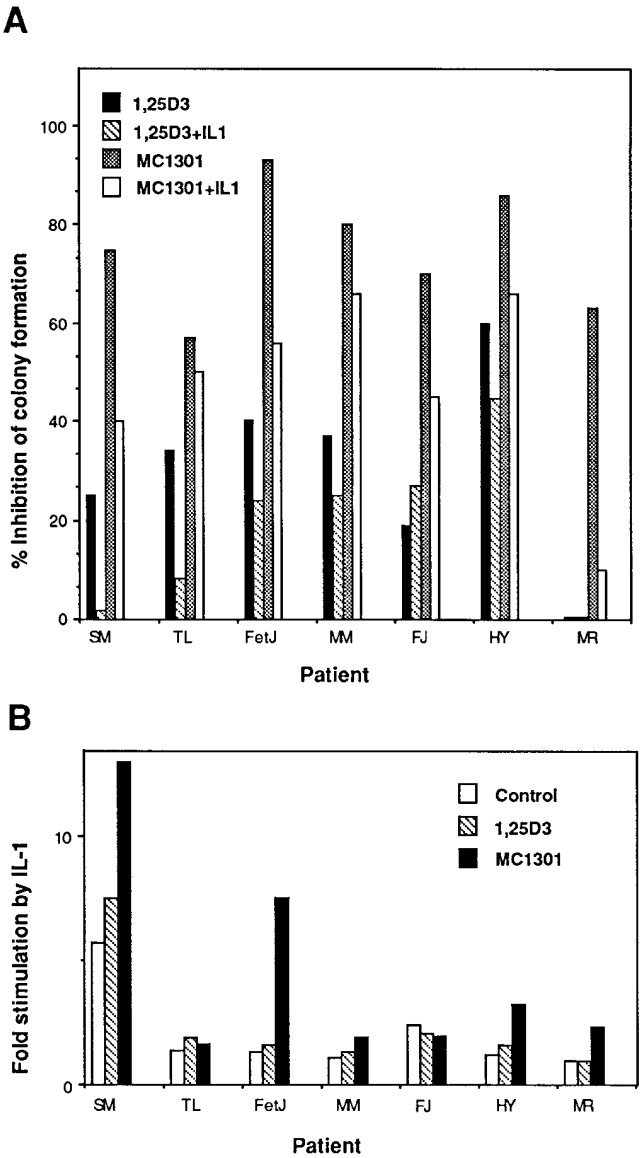


Figure 9. Growth-inhibitory effect of $1,25D_3$ and analogue MC1301 on hematopoietic blasts from AML patients. Blasts isolated from bone marrow samples of seven AML patients were plated in duplicate at a density of 10^5 cells/ml in methyl cellulose with or without 10 nM $1,25D_3$, MC1301, and bioactive (17.5 kD) recombinant human IL-1β (100 U/ml). Colonies containing > 20 cells were counted after 7 d of culture. (*A*) Percent inhibition of colony formation in the absence (*1,25D$_3$, MC1301*) or presence (*1,25D$_3$+IL-1, MC1301+IL-1*) of IL-1β. (*B*) Fold induction of colony formation by IL-1β in the absence of ligand (*control*), and in $1,25D_3$- and MC1301-treated cultures.

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. 9 *A*). 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. 9 *B*). 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 waf/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 analogs 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 concentration, 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 autocrine 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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References

1. Norman, A.W. 1979. Vitamin D: The Calcium Homeostatic Steroid Hormone. Academic Press, Inc., New York. 490 pp.

2. Abe, E., C. Miyaura, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki, and T. Suda. 1981. Differentiation of mouse myeloid leukemia cells induced by 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA.* 78:4990–4994.

3. Bar-Shavit, Z., S.L. Teitelbaum, P. Reitsma, A. Hall, L.E. Pegg, J. Trial, and A.J. Kahn. 1983. Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA.* 80:5907–5911.

4. Walters, M.R. 1992. Newly identified actions of the vitamin D endocrine system. *Endocr. Rev.* 13:719–764.

5. Hoang, T., A. Haman, O. Goncalves, and S.C. Clark. 1988. Interleukin-6 enhances growth-factor dependent proliferation of the blast cells of acute myeloblastic leukemia. *Blood.* 72:823–826.

6. Begley, C.G., D. Metcalf, and N.A. Nicola. 1987. Primary human myeloid leukemia cell: comparative responsiveness to proliferative stimulation by GM-CSF or G-CSF and membrane expression of CSF receptors. *Leukemia (Baltimore).* 2:382–387.

7. Miyauchi, J., C.A. Kelleher, G.G. Wong, Y.C. Yang, S.C. Clark, M.D. Minkin, and E.A. McCulloch. 1988. The effects of combinations of the recombinant growth factors GM-CSF, G-CSF, IL-3, and CSF-1 on leukemic blast cells in suspension culture. *Leukemia (Baltimore).* 2:382–387.

8. Young, D.C., and J.D. Griffin. 1986. Autocrine secretion of GM-CSF in acute myeloblastic leukemia. *Blood.* 68:1178–1181.

9. Souza, L.M., T.C. Boone, J. Gabrilove, P.H. Lai, K.M. Zsebo, D.C. Murdock, V.R. Chazin, J. Bruszewski, H. Lu, K.K. Chen, et al. 1986. Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. *Science (Wash. DC).* 232:61–65.

10. Rodriguez-Cimadevilla, J.C., V. Beauchemin, L. Villeneuve, F. Letendre, A. Shaw, and T. Hoang. 1990. Coordinate secretion of interleukin-1β and granulocyte-macrophage colony-stimulating factor by the blast cells of acute myeloblastic leukemia: role of interleukin-1 as an endogenous inducer. *Blood.* 76:1481–1489.

11. Segal, G.M., E. McCall, T. Stueve, and G.C. Bagby, Jr. 1987. Interleukin 1 stimulates endothelial cells to release multilineage human colony-stimulating activity. *J. Immunol.* 138:1772–1778.

12. Dinarello, C.A. 1996. Biologic basis for IL-1 in disease. *Blood.* 86:2095–2147.

13. Estrov, Z., R.A. Black, P.R. Sleath, D. Harris, Q. Van, R. LaPushin, E.H. Estey, and M. Talpaz. 1995. Effect of interleukin-1β converting enzyme inhibitor on acute myelogenous leukemia progenitor proliferation. *Blood.* 86:4594–4602.

14. Cozzolino, F., A. Rubartelli, D. Aldinucci, R. Sitia, M. Torcia, A. Shaw, and R. Di Guglielmo. 1989. Interleukin 1 as an autocrine growth factor for acute myeloid leukemia cells. *Proc. Natl. Acad. Sci. USA.* 86:2369–2373.

15. Bradbury, D., S. Rogers, I.A. Reilly, R. Kozlowski, N.H. Russell. 1992. Role of autocrine and paracrine production of granulocyte-macrophage colony-stimulating factor and interleukin-1 beta in the autonomous growth of acute myeloblastic leukaemia cells—studies using purified CD34-positive cells. *Leukemia (Baltimore).* 6:562–566.

16. Estrov, Z., R. Kurzrock, E. Estey, M. Wetzler, A. Ferrajoli, D. Harris, M. Blake, J.U. Gutterman, and M. Talpaz. 1992. Inhibition of acute myelogenous leukemia blast proliferation by IL-1 receptor antagonist and soluble IL-1 receptors. *Blood.* 79:1938–1945.

17. Rambaldi, A., M. Torcia, S. Bettoni, E. Vannier, T. Barbui, A.R. Shaw, C.A. Dinarello, and F. Cozzolino. 1991. Modulation of cell proliferation and cytokine production in acute myeloblastic leukemia by interleukin-1 receptor antagonist and lack of its expression by leukemic cells. *Blood.* 78:3248–3253.

18. Yin, M., V. Gopal, S. Banavali, P. Gartside, and H. Preisler. 1992. Effects of an IL-1 receptor antagonist on acute myeloid leukemic cells. *Leukemia (Baltimore).* 6:898–901.

19. Dinarello, C.A. 1991. Interleukin-1 and interleukin-1 antagonism. *Blood.* 77:1627–1652.

20. Miller, D.K., J.R. Calaycay, K.T. Chapman, A.D. Howard, M.J. Kostura, S.M. Molineaux, and N.A. Thornberry. 1993. The IL-1β converting enzyme as a therapeutic agent. *Ann. NY Acad. Sci.* 696:133–148.

21. Cerretti, D.P., C.J. Kozlosky, B. Mosley, N. Nelson, K. Van Ness, T.A. Greenstreet, C.J. March, S.R. Kronheim, T. Druck, L. Cammizzarro, et al. 1992. Molecular cloning of the interleukin-1β converting enzyme. *Science (Wash. DC).* 256:97–100.

22. Black, R.A., S.R. Kronheim, M. Cantrell, M.C. Defley, C.J. March, K.S. Pickett, J. Wignall, P.J. Conlon, D. Cosman, T.P. Hopp, and D.Y. Mochizuki. 1988. Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. *J. Biol. Chem.* 263:9437–9442.

23. Kostura, M.J., M.J. Tocci, G. Limjuxo, J. Chin, P. Comeron, A.G. Hillman, N.A. Chartrain, and J.A. Schmidt. 1989. Identification of a monocyte specific pre-interleukin 1 beta convertase activity. *Proc. Natl. Acad. Sci. USA.* 86:5227–5231.

24. Thornberry, N.A., H.G. Bull, J.R. Calaycay, K.T. Chapman, A.D. Howard, M.J. Kostura, D.K. Miller, S.M. Molineaux, J.R. Weidner, J. Aunins, et al. 1992. A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. *Nature (Lond.)* 356:768–774.

25. Papayannopoulou, T., B. Nakamoto, S. Kurachi, M. Tweeddale, and H. Messner. 1988. Surface antigenic profile and globin phenotype of two new human erythroleukemia lines: characterization and interpretations. *Blood.* 72:1029–1038.

26. Sone, T., S. Kerner, and J.W. Pike. 1991. Vitamin D receptor interaction with specific DNA: association as a 1,25-dihydroxyvitamin D3-modulated heterodimer. *J. Biol. Chem.* 266:23296–23305.

27. Peleg, S., R.V. Abruzzese, G.J. Cote, and R.F. Gagel. 1990. Transcription of the human calcitonin gene is mediated by a C-cell-specific enhancer containing E-box-like elements. *Mol. Endocrinol.* 4:1750–1757.

28. Gorman, C.M., L.F. Moffat, and B.H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044–1051.

29. Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

30. Estrov, Z., A. Cohen, E.W. Gelfand, and M.H. Freedman. 1988. Synergistic antiproliferative effects on HL-60 cells: deferoxamine enhances cytosine arabinoside, methotrexate, and daunorubicin cytotoxicity. *Am. J. Pediatr. Hematol. Oncol.* 10:288–291.

31. Gershoni, J.M., and G.E. Palade. 1983. Protein blotting: principles and applications. *Anal. Biochem.* 131:1–15.

32. Shaw, G., P.C. Levy, and A.F. LoBuglio. 1978. Human monocyte antibody-dependent cell-mediated cytotoxicity to tumor cells. *J. Clin. Invest.* 62:1172–1180.

33. Estrov, Z., T. Grunberger, H.S.L. Chan, and M.H. Freedman. 1986. Juvenile chronic myelogenous leukemia: characterization of the disease using cell cultures. *Blood.* 67:1382–1387.

34. Bertoncello, I., R. Bradley, S.M. Watt. 1991. An improved negative immunomagnetic selection strategy for the purification of primitive hemopoietic cells from normal bone marrow. *Exp. Hematol.* 19:95–100.

35. Buick, R.N., J.E. Till, and E.A. McCulloch. 1977. A colony assay for proliferative blast cells circulating in myeloblastic leukemia. *Lancet.* 1:862–864.

36. Minden, M.D., R.N. Buick, and E.A. McCulloch. 1979. Separation of blast cell and T-lymphocyte progenitors in the blood of patients with acute myeloblastic leukemia. *Blood.* 54:186–195.

37. Freedman, M.H., H.S.L. Chan, and L. Chang. 1986. Childhood erythroleukemia. Studies on pathogenesis using colony assays. *Am. J. Pediatr. Hematol. Oncol.* 8:2–9.

38. Haussler, M.R., C.A. Donaldson, S.L. Marion, E.A. Allegretto, and M.A. Kelly. 1986. Receptors for the vitamin D hormone: characterization and functional involvement in 1,25-dihydroxyvitamin D3 regulated events. *In* The Role of Receptors in Biology and Medicine. M. Gotto and B.W. O'Malley, editors. Raven Press, Ltd., New York. 91–104.

39. Mengelsdorf, D.J., H.P. Koeffler, C.A. Donaldson, J.W. Pike, and M.R. Haussler. 1984. 1,25-dihydroxyvitamin D3-induced differentiation in a human promyelocytic leukemic cell line (HL-60): receptor-mediated maturation to macrophage-like cells. *Endocrinology.* 98:391–398.

40. Peleg, S., Y.-Y. Liu, S. Reddy, R.L. Horst, and G.H. Posner. 1996. A 20-epi side chain restores growth inhibitory and transcriptional activity of an A ring-modified hybrid analog of 1α,25-dihydroxyvitamin D3 without increasing its affinity to the vitamin D receptor. *J. Cell. Biochem.* 63:149–161.

41. Liu, Y.-Y., E.D. Collins, A.W. Norman, and S. Peleg. 1997. Differential interaction of 1α,25-dihydroxyvitamin D3 analogues and their 20-epi homologues with the vitamin D receptor. *J. Biol. Chem.* 272:3336–3345.

42. Studzinski, G.P., J.A. McLane, and M.R. Uskokovic. 1993. Signaling pathways for vitamin D-induced differentiation: implications for therapy of proliferative and neoplastic disease. *Crit. Rev. Eukaryotic Gene Expr.* 3:279–

312.

43. Reitsma, P.H., P.G. Rothberg, S.M. Astrin, Z. Bar-Shavit, A. Hall, S.L. Teitelbaum, and A.J. Kahn. 1983. Regulation of *myc* gene expression in HL-60 leukemia cells by a vitamin D metabolite. *Nature (Lond.).* 306:492–494.

44. Boise, L.H., K.M. Gorse, and E.H. Westin. 1992. Multiple mechanisms of regulation of the human c-*myb* gene during myelomonocytic differentiation. *Oncogene.* 7:1817–1825.

45. Liu, M., M.-H. Lee, M. Cohen, M. Bommakanti, and L.P. Freedman. 1996. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev.* 10:142–153.

46. Nett, M.A., D.P. Cerretti, D.R. Berson, J. Seavitt, D.J. Gilbert, N.A. Jenkins, N.G. Copeland, R.A. Black, and D.D. Chaplin. 1992. Molecular cloning of the murine IL-1 beta converting enzyme cDNA. *J. Immunol.* 149:3254–3259.

47. Miossec, C., L.-D. Decoen, F. Fassy, and A. Diu-Hercend. 1996. Use of monoclonal antibodies to study interleukin-1β-converting enzyme expression: only precursor forms are detected in interleukin-1β-secreting cells. *Eur. J. Immunol.* 26:1032–1042.

48. Wang, S., M. Miura, Y.-K. Jung, H. Zhu, V. Gagliardini, L. Shi, A.H. Greenberg, and J. Yuan. 1996. Identification and characterization of Ich-3, a member of the interleukin-1β converting enzyme (ICE)/Ced-3 family and an upstream regulator of ICE. *J. Biol. Chem.* 272:20580–20587.

49. Komiyama, T., C.A. Ray, D.J. Pickup, A.D. Howard, N.A. Thornberry, E.P. Peterson, and G. Salvesen. 1994. Inhibition of interleukin-1β converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. *J. Biol. Chem.* 269:19331–19337.

50. Bar-Shavit, Z., A.J. Kahn, K.R. Stone, J. Trial, T. Hilliard, P.H. Reitsma, and S.L. Teitelbaum. 1986. Reversibility of vitamin D-induced human leukemia cell-line maturation. *Endocrinology.* 118:679–686.

51. Bouillon, R., W.H. Okamura, and A.W. Norman. 1995. Structure function relationships in the vitamin D endocrine system. *Endocr. Rev.* 16:200–257.