Granulocyte-macrophage colony-stimulating factor. Sensitive and receptor-mediated regulation by 1,25-dihydroxyvitamin D3 in normal human peripheral blood lymphocytes.

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Research Article

We show that 1,25-dihydroxyvitamin D3 (1,25[OH]2D3), the most hormonally active metabolite of vitamin D3, modulates sensitively and specifically both the protein and messenger RNA accumulation of the multilineage growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF). The regulation of GM-CSF expression is seen in both normal human mitogen-activated T lymphocytes and T lymphocytes from a line (S-LB1) transformed with human T cell lymphotropic virus 1 (HTLV-1). In contrast, cells from a HTLV-1 transformed T lymphocyte line (Ab-VDR) established from a patient with vitamin D-resistant rickets type II with undetectable 1,25(OH)2D3 cellular receptors are resistant to the action of 1,25(OH)2D3. Inhibition of GM-CSF expression by 1,25(OH)2D3 can occur independently of interleukin 2 regulation and is probably mediated through cellular 1,25(OH)2D3 receptors. We conclude that 1,25(OH)2D3 may be important in the physiology of hematopoiesis.

Granulocyte–Macrophage Colony-stimulating Factor

Sensitive and Receptor-mediated Regulation by 1,25-Dihydroxyvitamin D_3 in Normal Human Peripheral Blood Lymphocytes

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Abstract

We show that 1,25-dihydroxyvitamin D_3 (1,25[OH]$_2$D$_3$), the most hormonally active metabolite of vitamin D_3, modulates sensitively and specifically both the protein and messenger RNA accumulation of the multilineage growth factor granulocyte–macrophage colony-stimulating factor (GM-CSF). The regulation of GM-CSF expression is seen in both normal human mitogen–activated T lymphocytes and T lymphocytes from a line (S-LB1) transformed with human T cell lymphotropic virus 1 (HTLV-1). In contrast, cells from a HTLV-1 transformed T lymphocyte line (Ab-VDR) established from a patient with vitamin D–resistant rickets type II with undetectable 1,25(OH)$_2$D$_3$ cellular receptors are resistant to the action of 1,25(OH)$_2$D$_3$. Inhibition of GM-CSF expression by 1,25(OH)$_2$D$_3$ can occur independently of interleukin 2 regulation and is probably mediated through cellular 1,25(OH)$_2$D$_3$ receptors. We conclude that 1,25(OH)$_2$D$_3$ may be important in the physiology of hematopoiesis.

Introduction

Evidence is accumulating that the hormonally active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25[OH]$_2$D$_3$),[1] might play an important role as an immunohematopoietic regulatory hormone (reviewed in references 1, 2). Specific 1,25(OH)$_2$D$_3$ receptors are present in normal human mitogen–activated and malignant T and B lymphocytes and myeloid cells (3, 4). 1,25(OH)$_2$D$_3$ enhances the in vitro differentiation of normal and leukemic myeloid stem cells towards macrophages (5–7). 1,25(OH)$_2$D$_3$ inhibits proliferation and suppresses interleukin 2 (IL-2) synthesis of mitogen-activated T lymphocytes (8–11). Human macrophages activated by γ-interferon synthesize 1,25(OH)$_2$D$_3$ (12, 13).

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1. *Abbreviations used in this paper:* 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D_3; CM, conditioned medium; ED_{50}, dose effective in achieving half-maximal response; GM-CFC, granulocyte–macrophage colony-forming cells; GM-CSF, granulocyte–macrophage colony-stimulating factor; HTLV-1, human T cell lymphotropic virus 1; IL-2, interleukin 2; PBL, normal human peripheral blood lymphocytes; PHA, phytohemagglutinin.

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Survival, proliferation, and differentiation of hematopoietic cells are dependent on colony-stimulating factors (CSF). The granulocyte–macrophage CSF (GM-CSF), which is synthesized by activated T lymphocytes, has been cloned (14, 15). Recombinant and natural GM-CSF possess multilineage colony-stimulating activity (16, 17) and also enhances mature cell function of neutrophils and eosinophils (18, 19). We report here that 1,25(OH)$_2$D$_3$ sensitively and specifically inhibits the expression of GM-CSF in T lymphocytes.

Methods

Compounds. The vitamin D_3 metabolites (1,25[OH]$_2$D$_3$, 1,24,25-[OH]$_3$D$_3$, and 25[OH]D$_3$) were a generous gift from Dr. M. Uskokovic, Hoffmann-La Roche, Inc., Nutley, NJ. The compounds were dissolved in 100% ethanol to a stock concentration of 1×10^{-3} M and stored at −20°C. The various concentrations (10^{-11}–10^{-7} M) were obtained by diluting the stock solutions in phosphate-buffered saline (PBS). The concentration of ethanol did not exceed 0.1%. Recombinant human IL-2 was generously provided by Jürgen Besemer (Sandoz Ltd., Vienna, Austria). Phytohemagglutinin (PHA) (Wellcome Diagnostics, Datford, England) was used in a final concentration of 0.5%.

Cells and liquid culture assay. The human cell lines S-LB1 (20), Ab-VDR (H. P. Koeffler and A. W. Norman, unpublished observations), HL-60 (21), and KG-1 (22) were maintained in suspension culture T flask (Miles Laboratories Inc., Naperville, IL), containing alpha medium (Flow Laboratories, Inc., Rockville, MD) and 10% fetal calf serum (FCS) (Irvine Scientific, Santa Ana, CA) in a humidified atmosphere of 7% CO_2. The medium was changed two times a week.

Human peripheral blood lymphocytes (PBL) and human bone marrow cells were obtained from normal volunteers and isolated by Ficoll-Hypaque density gradient (1.077 g/ml) (Sigma Chemical Co., St. Louis, MO). Monocytes were removed from PBL by their ability to adhere to plastic for 6 h. The PBL and the human HTLV-1 immortalized T lymphocyte cell lines (S-LB1, Ab-VDR) were cultured for 2 d at 1×10^6 cells/ml in alpha medium–supplemented with 10% FCS in the presence of the various additives (vitamin D_3 compounds, PHA, IL-2) at 37°C in a humidified atmosphere of 7% CO_2. The conditioned media (CM) were stored at 4°C. Cell viability was not affected in the various experimental protocols, as determined by trypan blue exclusion.

Colony formation assay by two-layer agar techniques. The underlayer was plated in 1-ml portions in 35-mm petri dishes (Miles Laboratories Inc.) containing 0.5% agar, alpha medium, 16% FCS and CM from PBL as a source of CSF. CM obtained from the human T lymphocyte Mo cell line was used for control purpose at a final concentration of 1.5% (23). The overlayer contained the GM-CSF responder cells (bone marrow, KG-1), alpha medium, 16% FCS, penicillin, streptomycin, and 0.3% agar. The culture dishes were incubated at 37°C in a humidified atmosphere with 7% CO_2 for 10–13 d. Colonies (> 40 cells) were counted using an inverted microscope.

Complementary DNA (cDNA) probes, Northern blot technique. The IL-2 cDNA was a generous gift from S. Clarke, Genetics Institute, Boston, MA (24). The IL-2 receptor cDNA was kindly provided by T. Nikaido (25). The IL-2 and the IL-2 receptor probes were [^{32}P]-labeled by the

random priming method as described (26). The GM-CSF probes were [^{32}P]-labeled by nick translation (27).

For cytoplasmic RNA, freshly harvested cells were suspended in hypotonic buffer (10 mM Tris-HCl [pH 7.4], 1 mM KCl, 3 mM $MgCl_2$) and were lysed with Nonidet P-40 (0.3%). Nuclei were removed by centrifugation. Cytoplasmic RNA was extracted by the phenol/chlorofom method as essentially described (28) and quantified by absorbance at 260 nm. Samples were denatured at 65°C for 10 min, size-separated by an agarose formaldehyde gel (1% agarose [Bethesda Research Laboratories, Gaithersburg, MD], 50 mM Na acetate, 10 mM Na_2 EDTA, 200 mM MOPS, and 2.2 M formaldehyde), and transferred to a nylon membrane filter (ICN Biomedicals Inc., Irvine, CA). Filters were dried, baked at 80°C in vacuo for 2 h, and then prehybridized for 16–24 h. Hybridizations with [^{32}P]-labeled cDNA (1×10^6 cpm/ml) were performed at 42°C for 16–24 h in a solution containing 50% (vol/vol) formamide, 2× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate), 5× Denhardts (1× Denhardts is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 10% (vol/vol) dextran sulfate (500,000 mol wt) (Sigma Chemical Co.), and 100 μg/ml salmon sperm DNA (Sigma Chemical Co.). Filters were washed to a stringency of 0.1× SSC, 1% SDS at 65°C and exposed for 24–48 h at −70°C to XAR-5 film (Eastman Kodak Co., Rochester, NY).

Measurement of [^{14}C]uridine incorporation. S-LB1 cells (1×10^6/ml) were exposed in 24-well plates (Miles Laboratories Inc.) in quadruplicates for various durations (0, 6, 48 h) to $1,25(OH)_2D_3$ (10^{-8} M). Cells were pulsed with 0.5 μCi of [^{14}C]uridine (New England Nuclear, Boston, MA) for 2 h at 37°C, washed twice in PBS, precipitated in 5% trichloroacetic acid (TCA) for 10 min, washed twice in 5% TCA, and heated at 80°C for 60 min. 200 μl of each sample was counted by liquid scintillation.

Measurement of [^{3}H]thymidine incorporation. PBL (1×10^6/ml) were cultured in the presence of 0.5% PHA and $1,25(OH)_2D_3$ (10^{-7} M) in equal volumes (100 μl) in triplicates in 96-well plates (Corning Glass Works, Corning, NY). After 48 h, cells were pulsed with 1 μCi of [^{3}H]thymidine (ICN Biomedicals Inc.) for 5 h at 37°C and harvested into glass filter fiber with a Mash II cell harvester (Skatron, Inc., Sterling, VA) and counted by liquid scintillation.

Results

Modulation of GM-CSF protein levels by $1,25(OH)_2D_3$ and influence of IL-2 on GM-CSF expression in peripheral blood lymphocytes: dose response. The PBL (1×10^6/ml) were cultured in the presence of increasing concentrations of $1,25(OH)_2D_3$ (10^{-11}–10^{-7} M) and PHA (0.5%). After 2 d, CSF activity of the CM was assayed for stimulation of clonal growth of both normal human granulocyte–macrophage colony-forming cells (GM-CFC) and the human myeloblastic leukemic cell line KG-1 (Fig. 1 *A*). Clonal growth of both GM-CFC and KG-1 cells is dependent on CSF (22). We found that $1,25(OH)_2D_3$ decreased levels of GM-CSF protein in a dose-dependent fashion with a 50% reduction of CSF activity (ED_{50}) at $\sim 5 \times 10^{-10}$ M $1,25(OH)_2D_3$ when tested on clonal growth of GM-CFC and KG-1 cells. Control dishes containing no $1,25(OH)_2D_3$ formed 127±8 (±SD) GM-CFC and 62±10 (±SD) KG-1 colonies. No difference in colony formation could be observed when $1,25(OH)_2D_3$ (10^{-8} M) was added directly to the culture dishes containing CM of PHA-activated PBL compared with the control dishes containing CM of PHA-stimulated PBL only.

Prior studies showed that $1,25(OH)_2D_3$ decreased proliferation and IL-2 synthesis by PBL (8–11). We examined, therefore, whether IL-2 might affect the modulation of GM-CSF production by $1,25(OH)_2D_3$ in lymphocytes (Fig. 1 *A*). The PBL were cultured for 48 h in the presence of a high concentration of recombinant human IL-2 (200 ng/ml/10^6 cells), $1,25(OH)_2D_3$ (10^{-11}–10^{-7} M) and PHA (0.5%); the CM was assayed for stimulation of GM-CFC. We found that the addition of IL-2 to the cultures slightly blunted but did not reverse the inhibitory action of $1,25(OH)_2D_3$ on GM-CSF protein activity. The IL-2 alone

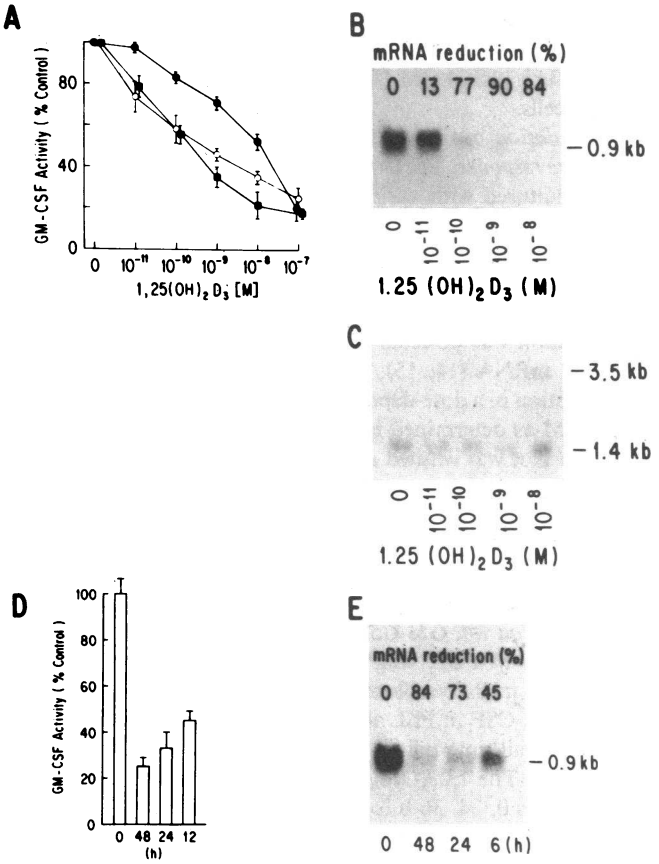

Figure 1. Modulation of GM-CSF expression by $1,25(OH)_2D_3$ in PBL. Dose-response studies (*A–C*): (*A*) Regulation of GM-CSF protein production. PBL were cultured for 48 h in the presence of PHA and $1,25(OH)_2D_3$. GM-CSF activity of the CM was tested by measuring clonal growth of both a normal human GM-CFC (○) and a leukemic myeloblastic cell line KG-1 (■). In a parellel series of experiments, recombinant IL-2 (200 ng/ml) was added to the cultures and the CM was assayed on GM-CFC (●). The IL-2 alone had no effect on clonal growth. (*B* and *C*) Regulation of GM-CSF mRNA levels (Northern blots). PBL were incubated for 48 hours with PHA and $1,25(OH)_2D_3$. When probed with GM-CSF cDNA, a single band was detected at 0.9 kb consistent with the known length of GM-CSF mRNA (*B*). The same Northern blot was reprobed with IL-2 receptor cDNA, as a control (*C*). Two bands were observed at 1.4 and 3.5 kb consistent with IL-2 receptor mRNA. Time-response studies (*D* and *E*): (*D*) Regulation of GM-CSF protein production. PBL were exposed to PHA and for various durations (12–48 h) to $1,25(OH)_2D_3$ (10^{-8} M). All time points were harvested after 48 h. GM-CSF activity was assayed on GM-CFC. (*E*) Regulation of GM-CSF mRNA levels (Northern blot). PHA-activated PBL were cultured for various durations (6–48 h) with $1,25(OH)_2D_3$ (10^{-8} M). PBL at all time points were harvested after 48 h. Each experiment in *A* and *D* represents the mean±SD from three independent experiments with PBL from three different donors. RNA was extracted as described in Methods. Each lane in *B, C,* and *E* contains 30 μg cytoplasmic RNA. Results are expressed as a percent reduction of mRNA accumulation by $1,25(OH)_2D_3$ as compared with the untreated control sample not exposed to $1,25(OH)_2D_3$ (determined by densitometry on different exposures of preflashed autoradiograms).

had no effect on clonal growth of GM-CFC. CM from control cultures (PHA 0.5%, no IL-2, no $1,25[OH]_2D_3$) stimulated a mean 243±8 (±SD) GM-CFC per 2×10^5 mononuclear bone marrow cells.

Modulation of GM-CSF mRNA levels by $1,25(OH)_2D_3$ in PBL: dose response. The modulation of GM-CSF mRNA levels of PBL cultured with various concentrations of $1,25(OH)_2D_3$ was determined by Northern blot analysis (Fig. 1 *B*). The PBL were pooled from seven different donors, exposed for 2 d to increasing concentrations of $1,25(OH)_2D_3$ (10^{-11}–10^{-8} M) and PHA (0.5%) and the mRNA was extracted. A single band of hybridization was detected at 0.9 kb, which is consistent with GM-CSF mRNA (14, 15). The $1,25(OH)_2D_3$ reduced mRNA accumulation in a dose-dependent manner with an ED_{50} of $\sim 5 \times 10^{-11}$ M as determined by densitometry reading. The same Northern blot was washed and reprobed with a human cDNA for the IL-2 receptor gene to exclude a nonspecific degradation of mRNA (Fig. 1 *C*). Two classes of IL-2 receptor mRNA could be observed at 1.4 and 3.5 kb, which is in accordance with previously reported data (25). No significant change of IL-2 receptor mRNA (1.4-kb band) could be detected.

Modulation of GM-CSF protein and mRNA levels by $1,25(OH)_2D_3$ in PBL: time response. We examined whether $1,25(OH)_2D_3$ might be able to down-regulate protein and mRNA levels of GM-CSF in PBL already activated by PHA (Fig. 1, *D* and *E*). For all time points, the PBL were exposed for 48 h to PHA (0.5%). The $1,25(OH)_2D_3$ (10^{-8} M) was added to the cultures at times 0, 24, 36 h for the protein studies, and 0, 24, 42 h for the mRNA studies. Control samples were stimulated by PHA alone. We found that a 12-h exposure to $1,25(OH)_2D_3$ inhibited GM-CSF protein production by $\sim$ 50% in PBL previously activated with PHA for 36 h (Fig. 1 *D*); and a 6-h exposure to $1,25(OH)_2D_3$ was sufficient to reduce mRNA levels of GM-CSF by $\sim$ 50% in PBL previously activated for 42 h (Fig. 1 *E*).

Regulation of GM-CSF mRNA levels by $1,25(OH)_2D_3$ in a HTLV-1 immortalized T lymphocyte line (S-LB1). We performed a parallel series of experiments using a human T lymphocyte line (S-LB1) established by infection of normal human T lymphocytes with HTLV-1 (20). These cells constitutively synthesize GM-CSF and express high levels of IL-2 receptors (29, 30). S-LB1 cells were exposed for 48 h to increasing concentrations of $1,25(OH)_2D_3$ (10^{-11}–10^{-7} M) (Figs. 2 *A*). The $1,25(OH)_2D_3$ decreased GM-CSF mRNA levels in a dose-dependent fashion as shown on the Northern blot, and a 50% reduction of mRNA occurred at $\sim 2.5 \times 10^{-11}$ M. Time-response experiments showed that a 50% decrease of GM-CSF mRNA accumulation occurred within 6 h of exposure of S-LB1 cells to $1,25(OH)_2D_3$ (10^{-8} M) (Fig. 2 *B*). For control purpose, the same samples were reprobed with IL-2 receptor cDNA (Fig. 2 *C*). Only a slight change of the IL-2 receptor mRNA levels (1.4-kb band) could be detected when S-LB1 cells were exposed to $1,25(OH)_2D_3$ (10^{-8} M), as compared to the marked decrease of GM-CSF mRNA levels.

Receptor-mediated modulation of GM-CSF expression by vitamin D_3 seco-steroids. We first studied the ability of other metabolites of vitamin D_3 to regulate the accumulation of GM-CSF mRNA by S-LB1 cells (Fig. 3 *A*). The S-LB1 cells were cultured for 48 h with different vitamin D_3 metabolites (10^{-8} M). The rank order of potency ($1,25[OH]_2D_3 > 1,24,25[OH]_3D_3 > 25[OH]D_3$) of the metabolites to affect accumulation of GM-CSF mRNA paralleled the affinity of the metabolites to bind to the $1,25(OH)_2D_3$ cellular receptors in other tissues (31). Secondly,

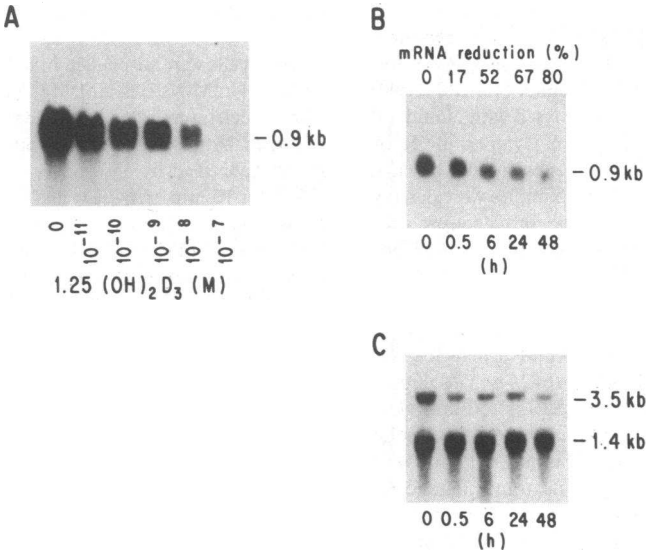

Figure 2. Modulation of GM-CSF mRNA levels by $1,25(OH)_2D_3$ in HTLV-1–immortalized T lymphocytes derived from a normal individual (S-LB1). (*A*) Dose-response experiments. S-LB1 cells were exposed for 48 h to $1,25(OH)_2D_3$. Analysis was performed by Northern blot technique with 30 μg cytoplasmic RNA per lane. (*B* and *C*) Time-course experiments (Northern blot). S-LB1 cells were cultured for various durations (0.5–48 h) with $1,25(OH)_2D_3$ (10^{-8} M). Each lane contains 20 μg RNA. As a control, the same samples were reprobed with IL-2 receptor cDNA (*C*). Two bands were observed at 1.4 and 3.5 kb consistent with IL-2 receptor mRNA. Analysis was performed as described in Fig. 1. A single band at 0.9 kb (*A* and *C*) was detected when probed with GM-CSF cDNA.

we established a HTLV-1 immortalized T lymphocyte cell line derived from a patient with vitamin D–resistant rickets type II (Ab-VDR). Vitamin D–resistant rickets II is a heterogenous group of syndromes characterized by a decreased or absent ability of $1,25(OH)_2D_3$ to bind to its cellular receptor (1, 32). Exposure of the Ab-VDR cells to $1,25(OH)_2D_3$ (10^{-8} M, 10^{-10} M) for 48 h had little effect on GM-CSF mRNA accumulation (reduction of mRNA of $\sim$ 15% compared with control samples, as determined by densitometry) (Fig. 3 *B*). These results are consistent with Ab-VDR cells having undetectable $1,25(OH)_2D_3$ cellular receptors, as determined by sucrose density gradient and DNA chromatography analyses (H. P. Koeffler and A. W. Norman, unpublished observations).

Influence of IL-2 on the expression of GM-CSF by S-LB1 cells. Previous studies suggested that most HTLV-1–transformed T lymphocytes do not synthesize IL-2 (33). We probed S-LB1 mRNA with a human cDNA for IL-2 (Fig. 3 *C*). The PHA-stimulated PBL were used as a positive control and the human promyelocytic cell line HL-60 as a negative control. A single band could be detected at 0.9 kb in PHA-activated lymphocytes consistent with IL-2 mRNA (24). However, no IL-2 mRNA could be detected in S-LB1 cells and yet cells from the same passage were regulated very sensitively by $1,25(OH)_2D_3$ (Fig. 2 *A*).

Total RNA synthesis by S-LB1 cells in the presence of $1,25(OH)_2D_3$ ($[^{14}C]$uridine incorporation). To further examine whether $1,25(OH)_2D_3$ influences total RNA synthesis in lymphocytes, we exposed S-LB1 cells (1×10^6/ml) for 0, 6 and 24 h to $1,25(OH)_2D_3$ (10^{-8} M) and measured [^{14}C]uridine incorporation into TCA precipitable counts (Table I). A 6-h exposure

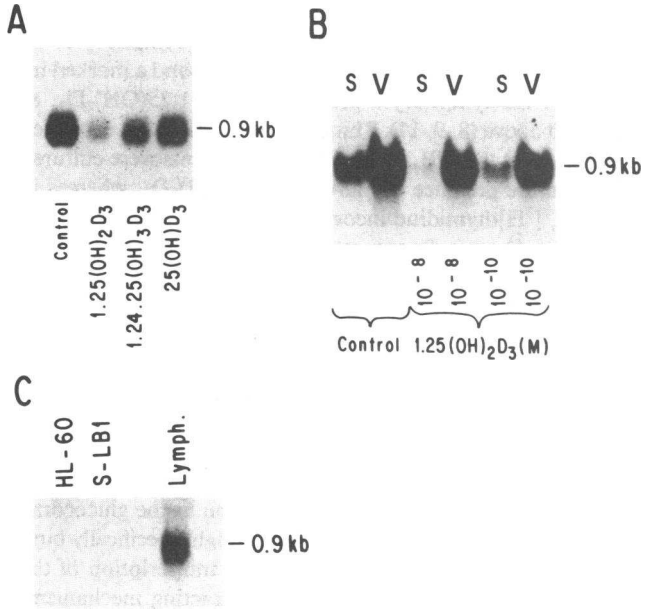

Figure 3. (*A*) Regulation of GM-CSF mRNA by different vitamin D metabolites in S-LB1 cells (Northern blot). S-LB1 cells were exposed for 48 h to different vitamin D metabolites (10^{-8} M). (*B*) Regulation of GM-CSF mRNA by $1,25(OH)_2D_3$ in the HTLV-1–immortalized T lymphocyte line Ab-VDR which was derived from a patient with vitamin D–resistant rickets, type II (Northern blot). S-LB1 cells (*S*) and Ab-VDR cells (*V*) were cultured for 48 h in the presence of $1,25(OH)_2D_3$ (10^{-8} and 10^{-10} M). (*C*) Analysis of IL-2 mRNA in S-LB1 cells (Northern blot). Using an IL-2 cDNA probe, a single band could be detected from RNA of PHA-activated PBL at 0.9 kb (*Lymph,* positive control) consistent with IL-2 mRNA; RNA from HL-60 promyelocytic leukemic cells was used as a negative control. Analysis was performed as described in Fig. 1 and Methods. Each lane in *A–C* contains 30 μg RNA. A single band at 0.9 kb (*A* and *B*) was detected when probed with GM-CSF cDNA.

to $1,25(OH)_2D_3$ had almost no effect on RNA synthesis. A 24-h exposure to $1,25(OH)_2D_3$ decreased the incorporation of [^{14}C]uridine TCA precipitable counts by ~ 33% as compared with the control sample not treated with $1,25(OH)_2D_3$.

Table I. [^{14}C]*Uridine Incorporation in S-LB1 Cells Exposed for Various Durations to* $1,25(OH)_2D_3$

Time exposed to $1,25(OH)_2D_3$	Experiments		Mean percent inhibition, nos. 1 and 2
	No. 1	No. 2	
h	*cpm × 10⁴ (%)*	*cpm × 10⁴ (%)*	
0 (control)	9.8±0.17 (0)	1.26±0.03 (0)	0
6	9.6±0.04 (2)	1.17±0.03 (6)	4
24	6.4±0.04 (33)	0.85±0.03 (32)	33

[^{14}C]Uridine incorporation as a measurement of RNA metabolism. S-LB1 cells (1×10^6/ml) were cultured in the presence of either ethanol control (≤ 0.01% ethanol) or $1,25(OH)_2D_3$ (10^{-8} M). Results represent mean±SD of quadruplicate cultures of two independent experiments (Nos. 1 and 2). Numbers in parentheses denote percentage of inhibition as compared with control sample treated with no $1,25(OH)_2D_3$. Analysis was performed as described in Methods.

Proliferation studies ([^{3}H]thymidine incorporation). We measured the effect of $1,25(OH)_2D_3$ (10^{-7} M) on PHA-activated PBL by measuring [^{3}H]thymidine incorporation. After 48 h in culture, PHA-activated PBL incorporated 11,911±4,446 cpm/10^6 cells. Treatment with $1,25(OH)_2D_3$ (10^{-7} M) for 48 h resulted in a statistically insignificant ($P > 0.5$) decrease of [^{3}H]thymidine incorporation into PBL (8,728±2,097 cpm/10^6 cells), representing a 16.7% inhibition as compared with the control samples not treated with $1,25(OH)_2D_3$. The results represent the mean±SD for nine incubations.

Discussion

We report in the present in vitro study that $1,25(OH)_2D_3$, the most active metabolite of vitamin D_3, is a potent inhibitor of the expression of the hematopoietic growth factor GM-CSF in PHA-activated normal human peripheral blood lymphocytes (PBL). The $1,25(OH)_2D_3$ regulated both protein and mRNA accumulation of GM-CSF; and this down-regulation occurred at concentrations close to those in vivo (Fig. 1). The recent reports that activated macrophages synthesize $1,25(OH)_2D_3$ (12, 13) further indicate that $1,25(OH)_2D_3$ might interact with GM-CSF production at a local level. Our time-response studies indicate that $1,25(OH)_2D_3$ can influence GM-CSF expression by PBL also in later stages of mitogenesis (Fig. 1 *E*). The concentrations of $1,25(OH)_2D_3$ achieving half-maximal response (ED_{50}) were 5×10^{-11} M and 5×10^{-10} M for GM-CSF mRNA levels and GM-CSF protein activity, respectively. These differences in the ED_{50} can be explained by the greater sensitivity and specificity of the RNA blot technique compared with the clonogenic assay in soft agar. In the clonogenic assay, CM from PHA-stimulated lymphocytes was used which, besides GM-CSF, also contains an admixture of other growth factors, including interleukin 3 and other lymphokines. These additional factors may have decreased both the sensitivity and specificity of the clonogenic assay. Similar to PBL, the $1,25(OH)_2D_3$ sensitively inhibited mRNA accumulation of GM-CSF by the HTLV-1 immortalized T lymphocytes (S-LB1) derived from a normal individual (19) (Fig. 2 *A*).

The $1,25(OH)_2D_3$ did not markedly affect mRNA levels for the IL-2 receptor in either the PHA-activated PBL or S-LB1 cells which constitutively produce high levels of IL-2 receptors (Figs. 1 *C*, 2 *C*). This suggests that $1,25(OH)_2D_3$ does not non-specifically decrease levels of mRNA in lymphocytes. The $1,25(OH)_2D_3$ inhibited total cellular RNA synthesis in S-LB1 cells as determined by incorporation of [^{14}C]uridine into TCA precipitable counts (Table I). This inhibition of total RNA, however, was much less pronounced and less rapid as compared with the decrease in GM-CSF mRNA in the same cells (4 and 52% inhibition, respectively, after exposure for 6 h to $1,25[OH]_2D_3$ [Fig. 2 *B*, Table I]).

Several recent reports suggested that $1,25(OH)_2D_3$ may be capable of immunoregulation similar to the glucocorticoids. Tsoukas et al. showed that $1,25(OH)_2D_3$ inhibited the lymphocyte growth–promoting factor IL-2 by PBL (10). Studies using different vitamin D_3 metabolites suggested that the effect of $1,25(OH)_2D_3$ was probably mediated by a specific receptor. Likewise, Rigby et al. demonstrated that $1,25(OH)_2D_3$ was a potent inhibitor of PHA-induced lymphocyte proliferation, achieving a 70% inhibition of [^{3}H]thymidine incorporation after 72 h in culture (9). Another study also showed that $1,25(OH)_2D_3$

inhibited the proliferation and IL-2 protein production by helper T lymphocytes (8). Similarly, $1,25(OH)_2D_3$ decreased the antigen-induced proliferation and IL-2 protein synthesis by cloned Ia-restricted T cell hybridomas after 24 h in culture (11). Taken together, these studies revealed a possible immunoregulatory role for $1,25(OH)_2D_3$ that has not previously been appreciated.

The $1,25(OH)_2D_3$ receptors are not present in resting T lymphocytes, but do appear 24 h after mitogenic stimulation (4). The HTLV-1–immortalized T-cell lines constitutively express $1,25(OH)_2D_3$ receptors. Our experiments using different vitamin D_3 metabolites suggest that the inhibition of the GM-CSF expression by $1,25(OH)_2D_3$ is most likely mediated through specific cellular receptors (Fig. 3 *A*). The potency of these compounds to reduce mRNA levels of GM-CSF paralleled their known affinity to bind to the specific cellular receptor in other tissues (31). Furthermore, our results are in accordance with the reported dissociation constant of 3.8×10^{-10} M for $1,25(OH)_2D_3$ by PHA-activated PBL (3). A more direct evidence for this assumption of a receptor-mediated mechanism relies on our study using a HTLV-1–immortalized T lymphocyte line (Ab-VDR) that was derived from a patient with vitamin D–resistant rickets type II. Exposure of Ab-VDR cells to $1,25(OH)_2D_3$ resulted in only a small decrease of GM-CSF mRNA levels compared with the marked decrease in S-LB1 cells (Fig. 3 *B*). These results are consistent with our observation that Ab-VDR cells have undetectable $1,25(OH)_2D_3$ receptors, whereas the $1,25(OH)_2D_3$-sensitive S-LB1 cells display 13 fmol $1,25(OH)_2D_3$ receptors/10^6 cells, as determined by sucrose density gradient and DNA chromatography analyses (H. P. Koeffler and A. W. Norman, unpublished observations). The fact that Ab-VDR cells responded at all to $1,25(OH)_2D_3$ suggests that either these cells have a low number of receptors not detectable by the present techniques or very high concentrations of $1,25(OH)_2D_3$ can affect all cells independent of expression of $1,25(OH)_2D_3$ receptors. Studies are currently under way to analyze the $1,25(OH)_2D_3$ receptors in these cells by using a specific cDNA probe for this hormone receptor. Koren et al. recently reported that peripheral blood mononuclear cells from patients with vitamin D–resistant rickets type II do not express $1,25(OH)_2D_3$ receptors, even after mitogenic stimulation (32). Moreover, proliferation of these lymphocytes could not be inhibited after a 72-h exposure to $1,25(OH)_2D_3$, in contrast to the normal cells.

Because prior studies showed that $1,25(OH)_2D_3$ inhibited the production of IL-2 (9, 10, 34), we questioned whether $1,25(OH)_2D_3$ might mediate its inhibitory effect on the expression of GM-CSF indirectly through a down-regulation of IL-2 production. Addition of very high concentrations of IL-2 (200 ng/ml) slightly abrogated but did not reverse the inhibition of GM-CSF protein production by $1,25(OH)_2D_3$ in PBL (Fig. 1 *A*). Furthermore, we observed that S-LB1 cells did not express IL-2 mRNA (Fig. 3 *C*). This T-cell line, therefore, provided an unusual tool to study our assumption of a possible IL-2–independent regulation of GM-CSF expression by $1,25(OH)_2D_3$. Our dose-response and time-course experiments clearly showed that $1,25(OH)_2D_3$ sensitively inhibited GM-CSF mRNA levels by S-LB1 cells in an IL-2–free system (Fig. 2, *A* and *B*). Taken together, our experiments suggest that $1,25(OH)_2D_3$ can regulate independently of IL-2 the expression of a lymphokine in transformed T lymphocytes. The effect of $1,25(OH)_2D_3$ on normal human PBL seems to be mostly but not totally independent from IL-2.

Our study using [^{3}H]thymidine showed no significant inhibition of the proliferation of PHA-activated lymphocytes by $1,25(OH)_2D_3$. Other investigators, however, found a marked inhibition of the lymphocyte proliferation by $1,25(OH)_2D_3$, as mentioned above (8, 9, 11). This discrepancy might be explained by the fact that the PBL in the other experiments were cultured for 72 h in the presence of PHA and $1,25(OH)_2D_3$, whereas in our study, [^{3}H]thymidine incorporation was determined at 48 h of culture. Our results suggest that the inhibition of GM-CSF production by $1,25(OH)_2D_3$ is not necessarily linked to its reported antiproliferative properties.

How does $1,25(OH)_2D_3$ regulate the expression of GM-CSF? In the case of glucocorticoids, studies showed that mouse mammary tumor virus DNA contains regions to which the glucocorticoid-receptor complex can specifically bind. Transcription is directly stimulated by interaction of the glucocorticoid-receptor complex with DNA sequences adjacent to the promoter (reviewed in reference 35). In a parallel fashion to the glucocorticoids, the $1,25(OH)_2D_3$ receptor complex might specifically bind to certain regions of DNA and inhibit the transcription of the GM-CSF gene either directly or via a transacting mechanism. Vanice et al., however, recently reported that dexamethasone regulated the expression of the alpha-1-acid glycoprotein at the posttranscriptional level (36). Preliminary experiments by us using in vitro nuclear run-on assays suggest that $1,25(OH)_2D_3$ affects the stability of GM-CSF mRNA.

In summary, our study shows that $1,25(OH)_2D_3$ inhibits sensitively the expression of GM-CSF by PBL and a HTLV-1 immortalized T lymphocyte line at the mRNA level. This down-regulation is mediated by a specific $1,25(OH)_2D_3$ receptor and can further occur independently of IL-2. We conclude that $1,25(OH)_2D_3$ might be important as a regulatory hormone in hematopoiesis.

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