1,25-Dihydroxyvitamin D3-regulated Expression of Genes Involved in Human T-Lymphocyte Proliferation and Differentiation

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ABSTRACT

1,25-Dihydroxyvitamin D₃ [1,25-(OH)₂D₃] inhibited the secretion of γ-interferon from human T-lymphocytes activated by the calcium ionophore, A23187, or phytohemagglutinin with or without 12-O-tetradecanoylphorbol-13-acetate. The agent also inhibited cell proliferation and interleukin 2 secretion by these cells. The inhibition of γ-interferon secretion was time and dose dependent and partially abolished by the addition of exogenous human recombinant interleukin 2. To elucidate the molecular events by which 1,25-(OH)₂D₃ inhibits cell proliferation and lymphokine secretion, complementary DNA probes were used to follow the expression of genes involved in human T-lymphocyte proliferation and differentiation. 1,25-(OH)₂D₃ inhibited the expression of interleukin 2 and γ-interferon messenger RNA in human lymphocytes activated by phytohemagglutinin and 12-O-tetradecanoylphorbol-13-acetate. It also inhibited the accumulation of c-myc protooncogene messenger RNA and, to a lesser extent, interleukin 2 receptor messenger RNA in these cells. However, it did not affect the expression of the HLA-DR gene. These results suggest that 1,25-(OH)₂D₃ selectively regulates T-lymphocyte activation-related genes at the level of messenger RNA.

INTRODUCTION

The discovery of high-affinity receptors specific for 1,25-(OH)₂D₃ in normal and cancer cells not derived from the conventional targets for this hormone, such as the small intestine, bone, and kidney, has widened our knowledge of the physiological roles of 1,25-(OH)₂D₃ apart from the maintenance of extracellular calcium homeostasis (1, 2). Recent studies have demonstrated that 1,25-(OH)₂D₃ is the most active and physiological metabolite of vitamin D₃ involved in differentiation and proliferation of various cancer cell lines in vitro (3–6).

In hematopoietic and immune systems, normal human peripheral monocytes, activated T- and B-lymphocytes, and a variety of leukemic cells possess 1,25-(OH)₂D₃ receptors (7, 8). Since 1,25-(OH)₂D₃ induces differentiation of human leukemic cell lines in vitro, vitamin D₃ analogues have attracted considerable interest as compounds that may prevent the development of cancers in vivo (9). Moreover, recent studies suggest that 1,25-(OH)₂D₃ modulates chemical carcinogenesis and tumor promotion in vivo (10–12). However, the stimulatory effect of 1,25-(OH)₂D₃ on cell transformation in vitro (13, 14) is not always consistent with the in vivo findings. The effects were paradoxical among different experimental models of tumor development in vivo. Therefore, the capacity of vitamin D₃ analogues to modulate tumor growth in vivo might be attributed in part to the ability of 1,25-(OH)₂D₃ to regulate the immunenetwork, because this hormone also exerts an immunoregulatory effect in vitro; 1,25-(OH)₂D₃ has an antiproliferative effect on human T-lymphocytes (15–18), potentiates interleukin 1 production (19) and HLA antigen expression of monocytes (20), and inhibits immunoglobulin secretion from B-lymphocytes (21). Excess 1,25-(OH)₂D₃ synthesis in Hodgkin’s lymphoma and 1,25-(OH)₂D₃ autocrine of normal human macrophages stimulated by IFN-γ were also reported by Davies et al. (22) and Koeffler et al. (23), respectively. Moreover, we found synergism of 1,25-(OH)₂D₃ and IFN-γ in differentiation-induction and c-myc reduction of a human myeloid leukemic cell line (24). It is therefore important to examine how 1,25-(OH)₂D₃ regulates IFN-γ secretion, which provides growth inhibition and differentiation signals for cancer cells (25). The antiproliferative effect of 1,25-(OH)₂D₃ on T-lymphocytes is mediated at least in part by inhibition of IL-2 secretion, which is the principal stimulus causing proliferation of activated T-lymphocytes, without the inhibition of IL-2 receptor expression (18, 21). However, the precise mechanism by which 1,25-(OH)₂D₃ suppresses T-lymphocyte proliferation remains unknown. We report here how this hormone regulates IFN-γ secretion, another major lymphokine produced by T-lymphocytes, and the specificity of 1,25-(OH)₂D₃ to regulate the gene expression inducible in human activated T-lymphocytes (IL-2, IFN-γ, IL-2 receptor, and c-myc) at the mRNA level.

MATERIALS AND METHODS

Chemicals. Recombinant human IL-2 was obtained from Genzyme (Boston, MA). Calcium ionophore A23187 was purchased from Sigma Chemical Co. (St. Louis, MO). TPA was obtained from P-L Biochemicals, Inc. (Milwaukee, WI). 1,25-(OH)₂D₃ was a gift of Teijin Institute for Bio-medical Research (Tokyo, Japan).

Cells and Treatment Protocols. Peripheral blood mononuclear cells were isolated from the buffy coat of normal human donors by sedimentation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). Human tonsils from otherwise healthy donors were disrupted mechanically and prepared as single cell suspensions. These cells were cultured in RPMI 1640 medium (Flow Laboratories, Rockville, MD) supplemented with 5% heat-inactivated fetal calf serum, 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, penicillin (50 units/ml), streptomycin (50 μg/ml), and 5 × 10⁻⁴ M 2-mercaptoethanol in a humidified atmosphere of 5% CO₂ in air. For T-lymphocyte enrichment, the cells were allowed to adhere for 1 h, and the nonadherent cells were passed through a nylon wool column as described previously (19). Cells were seeded at a density of 2 × 10⁶ cells/ml in culture flasks (Corning Glass Works, Corning, NY). For mitogenic activation, PHA (1 μg/ml; Difco, Detroit, MI) or A23187, with or without TPA, was added to the cells. After 24 h, 1 μCi of [methyl-³H]thymidine per ml (6.7 Ci/mmol; New England
Nuclear, Boston, MA) was added for a further 4 h. Cells were harvested by centrifugation and precipitated with 5% trichloroacetic acid, and the radioactivity of NCS (Amersham-Searle, Evanston, IL)-solubilized pellets was counted in a liquid scintillation counter (Packard Instruments). IL-2 titers were expressed as the reciprocal of the supernatant dilution that induced 50% maximal incorporation, with reference to a human recombinant IL-2 (Genzyme) as a standard.

IFN-γ Assay. An immunoradiometric assay using two mouse monoclonal antibodies (B1 and B3), which are specific for natural and recombinant human IFN-γ, was used to determine IFN-γ activity. The B1 antibody-coated polystyrene beads were incubated with 200 μl of supernatant in an assay tray at room temperature for 2 h. The beads were then washed with water and incubated with 200 μl of 125I-B3 antibody for 2 h. After washing, the beads were assayed for 125I in a gamma counter, according to the assay procedure of the IMRx INTERFERON-GAMMA RIA (Centocor, Inc., Malvern, PA).

RNA Extraction. Total RNA was isolated from human tonsillar lymphocytes by the method of Chirgwin et al. utilizing the CsCl gradient modification (27), and poly(A)+RNA was selected by oligo(dT)-cellulose chromatography (28).

Hybridization. For the Northern blot hybridization, mRNA was denatured by incubating RNA in capped plastic tubes for 1 h at 50°C in a buffer containing 1.0 ml glyoxal, 50% (vol/vol) dimethyl sulfoxide, and 10 mM NaH2PO4/Na2HPO4 (pH 7.0) and was electrophoresed (20 μg of mRNA per lane) in a 1.5% agarose slab gel (23). The mRNA in the gel was transferred to a nylon membrane filter (BIOBYDNE; Pall Ultrafine Filtration Corp., NY), and the membrane was baked at 80°C for 1 h in a vacuum. 28S and 18S rRNAs were used as molecular size markers. Hybridization with 32P-labeled cloned probes was performed at 42°C for 48 h in a mixture containing 50% formamide, 5× standard saline citrate (0.75 M NaCl-75 mM sodium citrate [pH 7.0]), 5× Denhardt’s solution (0.1% Ficoll-0.1% polyvinylpyrrolidone-0.1% bovine serum albumin), denatured salmon sperm DNA (250 μg/ml), NaCl (43.5 g/liter)-Na2HPO4-H2O (6.9 g/liter)-EDTA (1.85 g/liter) (pH 7.4), and 0.3% sodium dodecyl sulfate. The membrane was subsequently washed 3 times with 0.1× standard saline citrate-0.1% sodium dodecyl sulfate at 50°C for 15 min and exposed to a Fuji RX X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan) using an intensifying screen at −80°C.

cDNA Probes. Plasmid pBR322 DNA, containing complementary DNA specific for human IL-2 and IFN-γ, was the kind gift from Dr. T. Taniguchi (Osaka University, Osaka, Japan) (29). The inserts were purified by Per1 digestion, preparative agarose electrophoresis, and electroelution. The cDNA probes for IL-2 receptor (Tac-2 clone) were obtained as a generous gift of Dr. R. C. Gallo, National Cancer Institute (31) and clone pDRE11 (a generous gift of Dr. H. Inoko, Tokai University, Isehara, Japan) (32), respectively. For hybridization, cDNA probes were nick-translation with [32P]dCTP (3000 Ci/mmol) to specific activities of 1 to 2 × 108 cpm/μg.

RESULTS

Effect of 1,25-(OH)2D3 on the Secretion of Lymphokines by Human Peripheral Lymphocytes. Fig. 1 shows the kinetics of IL-2 and IFN-γ activities during PHA-induced activation. 1,25-(OH)2D3 inhibited IL-2 secretion. IFN-γ release was also inhibited by 1,25-(OH)2D3 in a time-dependent manner. This effect was apparent at 0.01 nM 1,25-(OH)2D3 and dose dependent (Fig. 2). Half-maximal inhibition was observed at 0.1 nM 1,25-(OH)2D3.

Effect of Exogenous IL-2 on the 1,25-(OH)2D3-induced Inhibition of IFN-γ Secretion. rIL-2 (100 units/ml) was added simultaneously in the above experiment (Fig. 2). 1,25-(OH)2D3 at doses of more than 0.1 nM inhibited IFN-γ secretion in the presence of rIL-2 (100 units/ml), which markedly increased IFN-γ production. However, the inhibition of IFN-γ secretion by 0.01 nM 1,25-(OH)2D3 was abolished. Moreover, the IFN-γ secretion in the presence of 0.1 nM 1,25-(OH)2D3 and rIL-2 (100 units/ml) was restored to the level of control culture without rIL-2. As shown in Fig. 2A, the addition of rIL-2 (100 units/ml) attenuated the inhibition of IL-2 activity by 1,25-(OH)2D3 to the level of a 10-fold higher dose of 1,25-(OH)2D3. IL-2 activity was inhibited 50% by 0.1 nM 1,25-(OH)2D3 in the absence of rIL-2, whereas 1.0 nM 1,25-(OH)2D3 was required in the presence of rIL-2 (100 units/ml) to obtain 50% inhibition.

Inhibition of TPA-induced IFN-γ Secretion by 1,25-(OH)2D3. The inhibition of IFN-γ secretion by 1,25-(OH)2D3 was also seen in human tonsillar lymphocytes (Fig. 3). In the absence of PHA, TPA did not induce IFN-γ release from human lymphocytes. TPA, however, synergistically augmented IFN-γ secretion from PHA-stimulated lymphocytes, but the secretion was inhibited by 1,25-(OH)2D3. The augmentation by TPA occurred in a dose-dependent manner, and the inhibition by 10 nM 1,25-(OH)2D3 was constant as shown in Fig. 4.

Essentially similar results were obtained when A23187 was used instead of PHA. TPA acts as a potent comitogen with calcium ionophore, A23187, for human lymphocytes. This observation was confirmed with respect to IFN-γ secretion from human T-lymphocytes (Fig. 5). Submaximal concentrations of A23187 acted synergistically with TPA to produce IFN-γ, although A23187 alone stimulated IFN-γ release from human lymphocytes. 1,25-(OH)2D3 also inhibited the A23187-induced IFN-γ secretion in the presence or absence of TPA. The inhibition by 1,25-(OH)2D3 was also dose dependent (data not shown).

Effect of 1,25-(OH)2D3 on the Expression of Genes Involved in T-Lymphocyte Activation. To examine whether the effects of
from these cultures was analyzed for IL-2-specific mRNA by Northern blot analysis (Fig. 6). Tonsillar lymphocytes were incubated with PHA (O, •), PHA plus TPA (10 ng/ml) (□, △) or A23187 plus TPA (10 ng/ml) (×, ○) for 48 h. 1,25-(OH)2D3 (10 nm) (closed) or 0.1% ethanol (open) was added at time 0. Points, mean of duplicate samples; bars, SD.

**Fig. 4.** Dose-response for TPA-induced IFN-γ secretion of PHA-activated tonsillar lymphocytes and the inhibition by 1,25-(OH)2D3. Human tonsillar lymphocytes were stimulated with PHA plus various concentrations of TPA in the absence (□) or presence (○) of 10 nM 1,25-(OH)2D3. Points, mean of duplicate samples; bars, SD.

**Fig. 5.** Effect of 1,25-(OH)2D3 on A23187-induced IFN-γ secretion from human tonsillar T-lymphocytes. Human tonsillar lymphocytes were enriched for T-cells by passage through a nylon wool column and were stimulated with A23187 (□, △) or A23187 plus TPA (10 ng/ml) (×, ○) for 48 h. 1,25-(OH)2D3 (10 nm) (triangle) or 0.1% ethanol (circle) was added at 0 time. Points, mean of duplicate samples; bars, SD.

1,25-(OH)2D3, on the release of IL-2 and IFN-γ in activated lymphocytes is accompanied by the decrease of the lymphokine mRNAs, the amounts of IL-2 and IFN-γ mRNA extracted from stimulated tonsillar lymphocytes were analyzed by Northern blot analysis (Fig. 6). Tonsillar lymphocytes were incubated for 20 h with PHA and TPA (10 ng/ml) in the presence or absence of 10 nM 1,25-(OH)2D3. The poly(A)+-selected RNA from these cultures was analyzed for IL-2-specific mRNA by using labeled cloned IL-2 DNA as a probe. PHA and TPA induced marked levels of IL-2 mRNA in these cells, and this induction was substantially inhibited by 1,25-(OH)2D3. The filter was rehybridized with the cloned IFN-γ DNA probe. IFN-γ mRNA was also decreased by 1,25-(OH)2D3 treatment. To examine whether 1,25-(OH)2D3 treatment induced generalized inhibition or degradation of mRNA, the same filter was sequentially hybridized with the HLA-DR cDNA probe. 1,25-(OH)2D3-treated cells contained the same degree of HLA-DR-specific mRNA as untreated cells. 1,25-(OH)2D3 suppressed not only c-myc expression but also the mRNA of IL-2 receptor. However, the suppression of IL-2 receptor mRNA was less than that of IL-2, IFN-γ, or c-myc, quantified by the analysis using a densitometer.

**DISCUSSION**

The functional responses to 1,25-(OH)2D3 include induction of monocyte differentiation, regulation of protooncogene expression, inhibition of cell proliferation (5, 6, 24), and induction of HLA antigens (20), and these effects resemble those to IFN-γ (20, 24, 25). Moreover, Koehler et al. (23) demonstrated that IFN-γ stimulated production of 1,25-(OH)2D3 by normal human macrophages. These findings suggest that immunoregulator cells, monocytes, and lymphocytes can interact through both 1,25-(OH)2D3 and IFN-γ (19, 24). In this study, we demonstrated that 1,25-(OH)2D3 inhibits the production of IFN-γ in activated human lymphocytes as well as the level of IFN-γ mRNA.

As demonstrated by some investigators and us (15, 17, 18), 1,25-(OH)2D3 inhibits IL-2 production and therefore T-lymphocyte proliferation. The antiproliferative effect can be reversed, if exogenous IL-2 is added to the culture (17, 18). Exogenous rIL-2 (100 units/ml) increased the IL-2 activity of the supernatant from lymphocytes cultured in the presence of 1,25-(OH)2D3 for 48 h to the level of that cultured in the absence of one-tenth concentrations of 1,25-(OH)2D3 and IFN-γ (Fig. 2). This dose of rIL-2 abolished the inhibition of IFN-γ release by 0.01 nM 1,25-(OH)2D3 and reduced the inhibition by 0.1 nM 1,25-(OH)2D3 up to the level of control culture without rIL-2. These results suggest that rIL-2 (100 units/ml) sufficiently compensated for the total IL-2 production inhibited by 0.01 nM or 0.1 nM 1,25-(OH)2D3 and that this dose of rIL-2 restored the IFN-γ secretion completely. These findings are consistent with those by others (33, 34), although it is difficult to determine how much the IL-2 inhibition contributes to the inhibition of IFN-γ production (35, 36).

T-lymphocyte activation initiated by antigens occurs through a complex series of molecular interactions, such as Ca2+ mobilization and activation of protein kinase C (37–39). Both suboptimal doses of Ca2+ ionophore and TPA synergistically act to induce IFN-γ secretion from human T-lymphocytes (Fig. 5). Bhalla et al. suggested that 1,25-(OH)2D3 interferes with the early events of antigen-induced T-lymphocyte activation by hindering T-lymphocyte recognition of antigen (14). However, calcium ionophore could not overcome the inhibition by 1,25-(OH)2D3, and 1,25-(OH)2D3 also inhibited the TPA-induced increase of IFN-γ. These results suggest that 1,25-(OH)2D3 does not interfere simply with events occurring at the cell membrane, but does interfere with events occurring after the increase of cytoplasmic Ca2+ and activation of protein kinase C and before gene translation.

Lymphokines were selectively inhibited by 1,25-(OH)2D3, at the level of transcripts in human activated T-lymphocytes. Thus, the antiproliferative effects of 1,25-(OH)2D3, for T-lymph-
phocytes are at least partly due to the decrease of IL-2 mRNA. The dual inhibition of IL-2 and IFN-γ may explain some of the immunosuppressive effects of 1,25-(OH)2D3, such as the suppression of immunoglobulin synthesis demonstrated by Lemire et al. (21). 1,25-(OH)2D3 may interact with regulatory elements of these lymphokine genes to reduce their transcription or specifically cause the degradation of mRNA. T-lymphocyte activation has been reported to result in the early expression of the c-myc protooncogene (40, 41). 1,25-(OH)2D3 can down-regulate c-myc expression of human promyelocytic leukemia cells as well as human lymphocytes and inhibit the proliferation (5, 24). c-myc expression of activated lymphocytes has been shown to be regulated in a cell cycle-dependent manner (40-42). It is important to determine whether 1,25-(OH)2D3 regulates the transcription of c-myc and lymphokines in such a cell cycle-dependent manner or not.

In contrast to the genes discussed above, 1,25-(OH)2D3 did not reduce the levels of the HLA-DR gene transcript of human mononuclear cells or the cell surface HLA-DR antigen (18). Koeffler et al. found that 1,25-(OH)2D3 induces expression of HLA-D antigens on human leukemic myeloid cells as does IFN-γ (20). The effect of 1,25-(OH)2D3 on the cell surface level of HLA-DR molecules might not always correspond to the enhancement of HLA-DR mRNA (43). Or, the enhancement of this mRNA on monocytes might be counteracted, indirectly, by the reduction of IFN-γ secretion.

The cell surface Tac antigens (IL-2 receptors) and the transcript of IL-2 receptor were differently influenced by 1,25-(OH)2D3 during the antiproliferative effect on human T-lymphocytes (18). The effect of IL-2 on the transcription of IL-2 receptor or IFN-γ has shown that these are not always coupled in early steps of lymphocyte activation (35), although several reports have demonstrated that IL-2 regulates IL-2 receptors and IFN-γ secretion (34, 44, 45). Detailed quantitative studies, particularly on the kinetics of induction of these genes and on inhibition by 1,25-(OH)2D3 as well as other T-lymphocyte inhibitors such as corticosteroids and cyclosporin A, are needed to clarify the interaction of these genes (46, 47). 1,25-(OH)2D3 may be useful in exploring the molecular mechanism for T-lymphocyte proliferation and differentiation.

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REFERENCES


of 1.25-(OH)2D3 REGULATION OF ACTIVATED T-CELL GENES


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