Induction of Differentiation of the Human Histiocytic Lymphoma Cell Line U-937 by 1α,25-Dihydroxycholecalciferol

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ABSTRACT

Some clones of the human histiocytic lymphoma line, U-937, were induced to differentiate into monocyte-like cells with loss of plating efficiency in agar by incubation with 0.1 to 10 nm 1α,25-dihydroxycholecalciferol [1,25(OH)₂D₃]. At 1 nm, 40% of the cells of one sensitive clone exhibited differentiation after 2 days of incubation judging from assays for phagocytosis and capacity to reduce nitroblue tetrazolium. Induction appeared to occur by binding of the cholecalciferol to a specific cytoplasmic and/or nuclear receptor for 1,25(OH)₂D₃. However, the presence of this receptor was not sufficient for differentiation, since one clone which contained the receptor did not respond with differentiation upon addition of 1,25(OH)₂D₃. Differentiation induction did not require DNA synthesis but was blocked by agents which inhibit RNA or protein synthesis. It was also blocked by the calcium ionophore A 23187. A synergistic inducing effect was seen between 1,25(OH)₂D₃ and retinoic acid. In addition, the U-937 cells could be primed by a short incubation with 1,25(OH)₂D₃ to respond, with maturation, to the addition of agents which increase the intracellular level of cyclic adenosine 3':5'-monophosphate, such as prostaglandin E₂, cholera toxin, and N⁶,O⁴′-dibutyryl adenosine 3':5'-monophosphate and which alone did not induce differentiation. Priming does not depend on the normal rate of RNA or protein synthesis, since it was not significantly inhibited by actinomycin D, cordycepin, or cycloheximide. It remains to be determined if unoccupied receptors for 1,25(OH)₂D₃ are present in fresh leukemia cells and if such cells can sometimes be induced to differentiate upon addition of cholecalciferol.

INTRODUCTION

Cells from patients with acute myeloid leukemia have an apparent inability to differentiate and mature normally in vivo. Unlike normal progenitor cells, leukemic cells display an interruption of the alleged coupling between proliferation and differentiation (10). Myeloid leukemic cell lines, which proliferate continuously and/or nuclear receptor for 1,25(OH)₂D₃, differentiated into morphologically mature macrophage-like cells after treatment with various agents, manuscript in preparation.

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4. The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; RA, 5α,6α,12α,14α-tetrahydroxycholecalciferol; 1α,25-dihydroxycholecalciferol; 25(OH)D₃; 25(R,S)-26(OH)₂D₃; 25(R)-26(OH)₂D₃; 1α,25(OH)₂D₃; PGE₂, prostaglandin E₂; dibutyryl cAMP; TPA, NBT, cycloheximide, cordycepin, and actinomycin D were from Sigma Chemical Co., St. Louis, Mo. ara-C was from The Upjohn Co., Kalamazoo, Mich. The calcium ionophore A 23187 was from Calbiochem AG, Lucerne, Switzerland. The cholecalciferol derivatives RA and PGE₂ were dissolved in 100% ethanol and diluted at least 1000-fold into the growth medium, so that the final concentration of ethanol was no higher than 0.1%. Control cultures were treated with the same concentration of ethanol. Cholera toxin, dibutyryl cAMP, cycloheximide, actinomycin D, cordycepin, and ara-C were dissolved in phosphate-buffered saline (136.9 mM NaCl-2.7 mM KCl-8.1 mM Na₂HPO₄). TPA was stored at −20°C in acetone at a concentration of 200 μg/ml. The calcium ionophore A 23187 was dissolved in DMSO and diluted at least 1000-fold into the growth medium. Control cultures were treated with the same volume of DMSO. Hydroxylation (Bio-Gel HTP) was from Bio-Rad Laboratories, Richmond, Calif.

Cells. The U-937 line and subclones obtained from cloning in agar were maintained in suspension culture in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, penicillin (100 μg/ml) and streptomycin (100 μg/ml). A survey of various subclones of U-937 indicated that 3 of the U-937 represents a histiocytic lymphoma line (13) with monoblast-like characteristics (6, 7). It can be induced to mature into morphologically mature macrophage-like cells after treatment with agents such as TPA (7), supernatants from mixed leukocyte cultures (4, 5, 7), or RA (8). Cells from the human promyelocytic leukemia cell line HL-60 (3) respond with maturation to the exposure of 1,25(OH)₂D₃ (14) which is the active form of cholecalciferol; the induced cells develop α-naphthylacetate esterase, indicating monocyte-macrophage differentiation. Therefore, it was of interest to investigate the effect of the addition of 1,25(OH)₂D₃ to the monoblast-like cell line U-937. The results obtained showed that some clones of U-937 are highly sensitive to a differentiation-inducing effect of 1,25(OH)₂D₃ mediated through a cellular receptor to this form of cholecalciferol. Although differentiation is mediated by a receptor, the presence of this receptor alone is not sufficient for differentiation, since one clone of U-937, which contained the receptor, did not respond with differentiation upon addition of 1,25(OH)₂D₃.

MATERIALS AND METHODS

Materials. 25(OH)D₃, 1,25(OH)₂D₃, and 25(R,S), 26(OH)₂D₃ were generous gifts from Roche, Basel, Switzerland. 1,25(OH)₂D₃, [methyl-³H]thymidine (15 Ci/mmol), [G-³H]uridine (7.1 Ci/mmol), and [³S]methionine (105 Ci/mmol) were from New England Nuclear, Dreieich, West Germany. RA, PGE₂, cholera toxin, dibutyryl cAMP, TPA, NBT, cycloheximide, cordycepin, and actinomycin D were from Sigma Chemical Co., St. Louis, Mo. ara-C was from The Upjohn Co., Kalamazoo, Mich. The calcium ionophore A 23187 was from Calbiochem AG, Lucerne, Switzerland. The cholecalciferol derivatives RA and PGE₂ were dissolved in 100% ethanol and diluted at least 1000-fold into the growth medium, so that the final concentration of ethanol was no higher than 0.1%. Control cultures were treated with the same concentration of ethanol. Cholera toxin, dibutyryl cAMP, cycloheximide, actinomycin D, cordycepin, and ara-C were dissolved in phosphate-buffered saline (136.9 mM NaCl-2.7 mM KCl-8.1 mM Na₂HPO₄-1.5 mM KH₂PO₄). TPA was stored at −20°C in acetone at a concentration of 200 μg/ml. The calcium ionophore A 23187 was dissolved in DMSO and diluted at least 1000-fold into the growth medium. Control cultures were treated with the same volume of DMSO. Hydroxylation (Bio-Gel HTP) was from Bio-Rad Laboratories, Richmond, Calif.

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20 were sensitive to induction of differentiation with 1,25(OH)2D3. Unless otherwise stated, the experiments described were carried out using a 1,25(OH)2D3-sensitive clone (clone 4) referred to as U-937-4. In some experiments, a 1,25(OH)2D3-resistant clone (clone 3) was used, U-937-3. All studies were made with exponentially growing cells. Cell counts were performed with a Model ZF Coulter Counter (Coulter Electronics, Harpenden, England). Viability was assessed by trypan blue exclusion. Cytospin slide preparations were stained with Wright-Giemsa for morphological evaluation.

Induction and Assessment of Differentiation. Cells (2 × 10^5/ml) were treated with cholecalciferol derivatives and other agents for 1 to 4 days. Maturation was assessed by the ability of the cells to reduce NBT.

Some experiments (priming experiments) consisted of treating cells (3 to 4 × 10^5/ml) with 1,25(OH)2D3 alone or together with cycloheximide, cordycepin, A 23187, or actinomycin D for various time intervals; harvesting and washing the cells; and then resuspending them (2 × 10^5/ml) in fresh medium containing various modulators of differentiation such as PGE2, cholea toxin, and dibutyryl cAMP. Priming of cells at 2 × 10^5/ml gave identical results compared to the standard concentration of 3 to 4 × 10^5/ml used in this study. The number of mature cells was determined by the NBT reduction method 2 days after addition of the modulators.

NBT reduction was carried out as described previously (2). Approximately 2 × 10^5 cells/ml of RPMI 1640 with 20% FCS were incubated for 25 min at 37° with an equal volume of 0.2% NBT dissolved in phosphate-buffered saline (136.9 mM NaCl-2.7 mM KCl-8.1 mM Na2HPO4-1.5 mM KH2PO4 containing 200 ng of freshly diluted TPA per ml. Cytospin slides were prepared and stained with Wright-Giemsa, and the number of cells containing formazan deposits were counted. The phagocytic capacity was determined by mixing cells at 10^6/ml in glass medium containing 10% fresh human serum with 1 × 10^5 heat-inactivated yeast cells per ml. The percentage of phagocytic cells containing at least 3 yeast organisms was determined on stained smears after 20 min of incubation at 37°.

Agar Culture. U-937-4 cells (5,000) in 1 ml of 0.3% agar were cultured in a humidified atmosphere with 4% CO2 for 7 days on top of a layer of 1 ml 0.5% agar. The growth medium was RPMI 1640 with 15% FCS. Small clusters (3 to 20 cells), large clusters (21 to 40 cells), and colonies (more than 40 cells) were counted. For determination of plating efficiency, both colonies and clusters were counted together.

Assessment of DNA, RNA and Protein Synthesis. Cells, 4 × 10^5/ml, were preexposed for 3 hr to the drug to be tested (ara-C, actinomycin D, cordycepin, or cycloheximide). For assay of DNA synthesis, [3H]thymidine was then added to each culture at a final concentration of 1 μCi/ml. At 0 hr and every 30 min thereafter for 2 hr, 100 μl of cells were removed, and [3H] incorporation into trichloroacetic acid-precipitable material was determined. RNA and protein synthesis were determined similarly using [3H]uridine (50 μCi/ml) and [35S]methionine (20 μCi/ml), respectively.

Preparation of Cytosol and Chromatin Fractions. Washed U-937 cells (approximatively, 2 × 10^6 cells) were homogenized in a Dounce glass homogenizer in HED buffer (10% w/v) with 10 strokes. The homogenate was diluted to 4 ml; after a low-speed spin (800 × g for 10 min) there after for 2 hr. 100 μl of cells were removed, and centrifugation was at 20,000 × g for 15 min. Chromatin extracts were prepared by treating the crude chromatin with 5 ml KHED buffer at 4° for 45 min with frequent mixing. The supernatant (extract) was cleaned by centrifugation at 200,000 × g for 2 hr.

In some experiments, approximately 2 × 10^6 cells were extracted by homogenization in 4 ml KHED buffer followed by centrifugation at 200,000 × g for 2 hr to clean the extract.

Assay for 1,25(OH)2D3 Receptor. This assay was carried out essentially according to the method of Walters et al. (15). KHED extracts of whole cells or chromatin and HED cytosol, 200 μl, were incubated with 1.2 nM [3H]-1,25(OH)2D3 in the presence and absence of 1000-fold excess unlabeled 1,25(OH)2D3 at 4° for 2 or 18 to 24 hr. Incubations were terminated by the addition of 500 μl of 50% (v/v) hydroxylapatite in KHED and further incubation at 4° for 15 min with frequent blending on a Vortex mixer. Bound and free hormones were separated by washing the pellets 3 times with KHED plus 0.5% Triton X-100 at 5000 × g. Radioactivity was then extracted with 1.0 ml of 100% ethanalysis at 30° for 30 min with blending on a Vortex mixer. After centrifugation, an aliquot was placed in a scintillation counter with 5 ml Instagel. Specific [3H]-1,25(OH)2D3 binding was calculated by subtracting nonspecific binding observed with excess 1,25(OH)2D3.

Gei Filtration Chromatography of 1,25(OH)2D3 Binding Components. A column (58 x 1 cm) was packed with Sephacryl S-200 in KHED at 4°. Aliquots of 500 μl KHED extracts were applied to the column after incubation with 1.2 nM [3H]1,25(OH)2D3 alone or with a 1000-fold excess of 1,25(OH)2D3 at 4° for 2 hr. The column was eluted with KHED at a flow rate of 5 ml/hr; 2-ml fractions were collected for scintillation counting.

Presentation of Results. Data for differentiation are given as the percentage of cells reducing NBT. The various chemical compounds used to modify the inducing effect of 1,25(OH)2D3 inhibited cell growth to a variable degree. However, the percentage of viable cells as judged by trypan blue exclusion was always in excess of 90%. Therefore, differentiation can reliably be expressed as the percentage of cells reducing NBT.

RESULTS

Induction of Differentiation by 1,25(OH)2D3. After the addition of 10 nm 1,25(OH)2D3 to U-937-4, there was a decrease in the rate of cell growth (Chart 1) which corresponded to morphological changes. After 4 days, cells no longer proliferated. They became larger and the cytoplasmic granulation disappeared. Irregular nuclei with a tendency for lobulation together with a pale, somewhat vacuolated cytoplasm with villous membranes suggested differentiation into monocyte-like cells. A tendency for adherence developed, but the cells could be suspended by vigorous pipeting.

Simultaneously with alterations in cell growth and morphology,
cells occurred that reduced NBT when stimulated with TPA (Chart 1). Thus, 1,25(OH)2D3 induces the oxidase system necessary for superoxide formation, a functional characteristic of mature monocytes. After 4 days of incubation with 10 nM 1,25(OH)2D3, approximately 65% of the cells reduced NBT. Furthermore, Chart 1 shows that there was a concomitant time-dependent increase also in phagocytic capacity.

Chart 2 shows the dose-response effects of various derivatives of cholecalciferol on U-937-4. 1,25(OH)2D3 was the most potent inducer; a 1 nM concentration induced approximately 40% of the cells, and maximal induction was obtained with less than 10 nM. 25(OH)D3, 24R, 25(OH)2D3 and 25(RS), 26(OH)2D3 also induced some differentiation, but 500-fold higher concentrations on a molar basis were needed.

Another clone of U-937, U-937-3, was resistant to induction with 1,25(OH)2D3 (data not recorded).

Pharmacological Modulation of 1,25(OH)2D3-mediated Differentiation. In the experiments depicted in Chart 3, cells were incubated for 2 days with various concentrations of 1,25(OH)2D3 with or without different pharmacological agents added. The reversibility of the growth inhibition by the drugs used was tested in separate experiments with incubation for 2 days without added 1,25(OH)2D3, washing, and reseeding in fresh medium without added drugs to follow the growth rate. ara-C, 0.5 μM, produced an almost complete inhibition of cell growth, which was partly reversible upon reseeding of the cells in fresh medium. Actinomycin D, 30 ng/ml, caused complete growth inhibition, which was almost irreversible upon reseeding in fresh medium. Cordycepin, 5 μg/ml, also produced profound growth inhibition with a very low growth rate upon reseeding in fresh medium. Cycloheximide, 1 μg/ml, caused a reduced growth rate, which was, however, completely reversible upon reseeding in fresh medium. A 23187, 100 ng/ml, reduced the growth rate somewhat but in a fully reversible fashion.

The addition of 0.5 μM ara-C resulted in 80% inhibition of incorporation of [3H]thymidine into DNA. However, the differentiation induction by 1,25(OH)2D3 was not affected (Chart 3). The addition of actinomycin D (30 ng/ml), which inhibited 72% of the [3H]uridine incorporation into RNA, abolished differentiation induction completely. Also, cordycepin (5 μg/ml), resulting in 70% inhibition of RNA synthesis, abolished differentiation induction completely. This was also seen with cycloheximide, 1 μg/ml, which inhibited 40% of the protein synthesis. In addition, we tested the effect of a calcium ionophore, A 23187, which was found to inhibit differentiation induction by 1,25(OH)2D3 in a dose-dependent fashion. In all these experiments, cell viability was always higher than 90% judging from trypan blue exclusion.

Synergistic Effects between 1,25(OH)2D3 and RA. RA alone at 0.1 to 1 μM induces some differentiation of U-937 (Chart 4). However, 1 to 10 nM RA, by itself inactive, clearly potentiates the inducing effect of 1,25(OH)2D3 (Chart 4).

Priming with 1,25(OH)2D3 for Differentiation Effects of cAMP-inducing Agents. U-937-4 cells were pretreated (primed)
for 20 hr with 10 nM 1,25(OH)2D3; washed; resuspended in fresh 1,25(OH)2D3-free medium containing PGE2 (10 nM), cholera toxin (1 nM), or dibutyryl cAMP (0.1 mM); and incubated for 2 days. These agents did not induce any differentiation at all of unprimed cells. Treatment with 10 nM 1,25(OH)2D3 for 20 hr resulted in a slight production of mature cells (approximately, 12%) 2 days later. The primed cells, however, responded with maturation to the modulators (Chart 5). The cell concentrations were 5.0 × 10^5, 4.5 × 10^5, 4.6 × 10^5, and 5.0 × 10^5, respectively, per ml for primed cells incubated for 2 days with PGE2, cholera toxin, dibutyryl cAMP, or no modulator. The initial cell concentration was 3 × 10^6/ml. These data show that the modulators had little effect on growth during a 2-day incubation of 1,25(OH)2D3-primed cells. The percentage of viable cells was always in excess of 90%.

To evaluate the mechanisms for the priming effect by 1,25(OH)2D3, various drugs were added during the priming period with 1,25(OH)2D3 to inhibit RNA synthesis (actinomycin D and cordycepin) or protein synthesis (cycloheximide). Furthermore, the effect on priming by a Ca²⁺ ionophore (A 23187) was tested. Incubations were carried out for 20 hr with 10 nM 1,25(OH)2D3 with or without the various drugs mentioned followed by washing the cells and by the addition of prostaglandin, cholera toxin, or dibutyryl cAMP for 2 days. Cell growth with these modulators added was slightly decreased with cells primed in the presence of actinomycin D, cordycepin, or cycloheximide. Priming in the presence of actinomycin D, 5 ng/ml, which inhibited 60% of [³H]uridine incorporation into RNA, did not affect the response to the modulators (Chart 5). Cordycepin, 5 μg/ml, which inhibited 70% of [³H]uridine incorporation into RNA, did not significantly inhibit priming for the effect of the modulators; neither did cycloheximide affect priming with 1,25(OH)2D3 significantly at concentrations (1 μg/ml) which inhibited 40% of protein synthesis. The Ca²⁺ ionophore A 23187, however, inhibited the priming with 1,25(OH)2D3, since the response to the modulators by primed cells was profoundly decreased. Viability in all these experiments was in excess of 90% judging from trypan blue exclusion.

The time course for the priming by 1,25(OH)2D3 for the effects of PGE2, cholera toxin, and dibutyryl cAMP was maximal within less than 20 hr (Chart 6). With high concentrations of dibutyryl cAMP, some priming effect was seen already after 1 hr of exposure for 1,25(OH)2D3.
for induction of differentiation by this form of cholecalciferol. Thus, one clone (U-937-3), was resistant to the inducing effect of 1,25(OH)_2D_3 despite the fact that it contained specific binding components. Such clones will be useful for further dissection of the mechanisms of induction.

The mechanism for 1,25(OH)_2D_3-induced differentiation was evaluated by pharmacological modulation under conditions where cellular cytotoxicity was minimal. Treatment with ara-C, an inhibitor of DNA synthesis, did not affect induction. These experiments suggest that induction does not require DNA synthesis. On the other hand, agents like actinomycin D and cordycepin, which inhibited RNA synthesis substantially, inhibited induction by 1,25(OH)_2D_3. Thus, RNA synthesis is needed for induction. This is true also for protein synthesis, since low concentrations of cycloheximide, which produced only partial inhibition of protein synthesis, blocked the induction process completely. The calcium ionophore A 23187 also blocked this process for unknown reasons.

RA induces differentiation of U-937 by unknown mechanisms (8). The present work showed a synergistic inducing effect between RA and 1,25(OH)_2D_3, which suggests that the mechanism of induction is different for these 2 agents.

It has been demonstrated that U-937 can be primed for differentiation by treatment for approximately 1 day with RA so that it becomes susceptible to exposure for a cAMP-inducing agent while the reverse sequence is ineffective (9). The present work demonstrated that it was also possible to prime with 1,25(OH)_2D_3 for the effect of cAMP-inducing agents such as PGE_2, cholera toxin, and dibutyryl cAMP. U-937 is not susceptible to the cAMP-inducing agents alone. The priming effect occurred also in the presence of RNA and protein synthesis inhibitors. Thus, priming with 1,25(OH)_2D_3 does not depend on a normal rate of RNA and protein synthesis. However, the expression of the differentiated phenotype is, as expected, dependent on RNA and protein synthesis. The finding that the calcium ionophore A 23187 blocked priming by 1,25(OH)_2D_3 cannot be explained at the present time. These data show that agents which act by increasing the intracellular level of cAMP, are potent modulators of cholecalciferol-induced differentiation. Therefore, it is possible that cAMP-dependent phosphorylation reactions may modulate the differentiation response to 1,25(OH)_2D_3.

It remains to be tested if fresh cells from patients with acute leukemia bearing unoccupied receptors may sometimes respond to 1,25(OH)_2D_3. The role of 1,25(OH)_2D_3 in normal hemopoiesis, in particular for monocyte production, also remains to be established.

### DISCUSSION

This study clearly demonstrates that 0.1 to 10 nM 1,25(OH)_2D_3 induces the histiocytic cell line U-937 to mature into monocyte-like cells with loss of clonogenicity. The maturation was determined by phagocytosis and NBT reduction. NBT reduction has been shown previously to be a reliable measure of RA-induced maturation of U-937 inasmuch as RA induces monocyte-like cells with a concomitant development of functional characteristics, including phagocytosis, hexose monophosphate shunt activity, and the capacity to reduce NBT (8). The present study on 1,25(OH)_2D_3-induced differentiation showed a striking correlation between induction of phagocytic capacity and the capacity to reduce NBT (Chart 1). Thus, some important functional characteristics of mature monocytes developed. In addition, self-renewal capacity disappeared judging from clonogenicity in agar. It is, however, not possible to decide from these data if complete differentiation occurred with the appearance of all differentiation-specific markers. The induced cells possess some monocyte-like properties but may not meet other important functional criteria of monocyte identity.

The mechanisms by which various inducers such as DMSO (3), TPA (11), RA (1), and lymphocyte-derived differentiation-inducing factors (10) act to induce differentiation of myeloid leukemic cells are poorly understood. However, the induction by 1,25(OH)_2D_3 appears to occur by binding to specific cytoplasmic and/or nuclear receptors. Such receptors have also been detected in the promyelocytic HL-60 cell line (14) which matures into macrophage-like cells with 1,25(OH)_2D_3. Our data indicate that approximately 50% of the receptor for 1,25(OH)_2D_3 in U-937 was associated with nuclear components. Chromatin-associated unoccupied receptors for 1,25(OH)_2D_3 have been found in several target tissues (15). The presence of specific binding components for 1,25(OH)_2D_3 in U-937 is, however, not the only prerequisite for induction of differentiation by this form of cholecalciferol. Thus, one clone (U-937-3), was resistant to the inducing effect of 1,25(OH)_2D_3 with the appearance of all differentiation-like properties but may not meet other important functional criteria of monocyte identity.

#### Clonogenicity in Agar of U-937-4

Uninduced U-937-4 showed a plating efficiency of 20% when grown in agar. The plating efficiency decreased to 15, 2, 0, and 0%, respectively, in the presence of 0.1, 1, 10, or 100 nM 1,25(OH)_2D_3.

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