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Vitamin D-dependent rickets type II. Defective induction of 25-hydroxyvitamin D3-24-hydroxylase by 1,25-dihydroxyvitamin D3 in cultured skin fibroblasts.

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Research Article

1,25(OH)2D3 induces 25(OH)D3-24-hydroxylase (24-OHase) in cultured skin fibroblasts from normal subjects. We evaluated 24-OHase induction by 1,25(OH)2D3 in skin fibroblasts from 10 normal subjects and from four unrelated patients with hereditary resistance to 1,25(OH)2D or vitamin D-dependent rickets type II (DD II). Fibroblasts were preincubated with varying concentrations of 1,25(OH)2D3 for 15 h and were then incubated with 0.5 microM [3H]25(OH)D3 at 37 degrees C for 30 min; lipid extracts of the cells were analyzed for [3H]24,25(OH)2D3 by high performance liquid chromatography and periodate oxidation. Apparent maximal [3H]24,25(OH)2D3 production in normal cell lines was 9 pmol/10(6) cells per 30 min and occurred after induction with 10(-8) M 1,25(OH)2D3. 24-OHase induction was detectable in normal fibroblasts at approximately 3 X 10(-10) M 1,25(OH)2D3. [3H]24,25(OH)2D3 formation after exposure to 1,25(OH)2D3 was abnormal in fibroblasts from all four patients with DD II. In fibroblasts from two patients with DD II, [3H]24,25(OH)2D3 formation was unmeasurable (below 0.2 pmol/10(6) cells per 30 min) at 1,25(OH)2D3 concentrations up to 10(-6) M. Fibroblasts from the other two patients with DD II required far higher than normal concentrations of 1,25(OH)2D3 for detectable [3H]24,25(OH)2D3 induction. In one, [3H]24,25(OH)2D3 production reached 2.9 pmol/10(6) cells per 30 min at 10(-6) M 1,25(OH)2D3 (30% normal maximum at 10(-6) M 1,25(OH)2D3). In the other, [3H]24,25(OH)2D3 production achieved normal levels, 7.3 pmol/10(6) cells per […]

Vitamin D-dependent Rickets Type II

Defective Induction of 25-Hydroxyvitamin D_3-24-Hydroxylase by 1,25-Dihydroxyvitamin D_3 in Cultured Skin Fibroblasts

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Abstract

$1,25(OH)_2D_3$ induces $25(OH)D_3$-24-hydroxylase (24-OHase) in cultured skin fibroblasts from normal subjects. We evaluated 24-OHase induction by $1,25(OH)_2D_3$ in skin fibroblasts from 10 normal subjects and from four unrelated patients with hereditary resistance to $1,25(OH)_2D$ or vitamin D-dependent rickets type II (DD II). Fibroblasts were preincubated with varying concentrations of $1,25(OH)_2D_3$ for 15 h and were then incubated with 0.5 μM [^{3}H]$25(OH)D_3$ at 37°C for 30 min; lipid extracts of the cells were analyzed for [^{3}H]$24,25(OH)_2D_3$ by high performance liquid chromatography and periodate oxidation. Apparent maximal [^{3}H]$24,25(OH)_2D_3$ production in normal cell lines was 9 pmol/10^6 cells per 30 min and occurred after induction with 10^{-8} M $1,25(OH)_2D_3$. 24-OHase induction was detectable in normal fibroblasts at $\sim 3 \times 10^{-10}$ M $1,25(OH)_2D_3$. [^{3}H]$24,25(OH)_2D_3$ formation after exposure to $1,25(OH)_2D_3$ was abnormal in fibroblasts from all four patients with DD II. In fibroblasts from two patients with DD II, [^{3}H]$24,25(OH)_2D_3$ formation was unmeasurable (below 0.2 pmol/10^6 cells per 30 min) at $1,25(OH)_2D_3$ concentrations up to 10^{-6} M. Fibroblasts from the other two patients with DD II required far higher than normal concentrations of $1,25(OH)_2D_3$ for detectable [^{3}H]$24,25(OH)_2D_3$ induction. In one, [^{3}H]$24,25(OH)_2D_3$ production reached 2.9 pmol/10^6 cells per 30 min at 10^{-6} M $1,25(OH)_2D_3$ (30% normal maximum at 10^{-6} M $1,25(OH)_2D_3$). In the other, [^{3}H]$24,25(OH)_2D_3$ production achieved normal levels, 7.3 pmol/10^6 cells per 30 min after 10^{-6} M $1,25(OH)_2D_3$.

The two patients whose cells had a detectable 24-OHase induction by $1,25(OH)_2D_3$ showed a calcemic response to high doses of calciferols in vivo. Our current observations correlate with these two patients' responsiveness to calciferols in vivo and suggest that their target organ defects can be partially or completely overcome with extremely high concentrations of $1,25(OH)_2D$. The two patients whose cells showed no detectable 24-OHase induction in vitro failed to show a calcemic response to high doses of calciferols in vivo.

In conclusion: (*a*) The measurement of 24-OHase induction by $1,25(OH)_2D_3$ in cultured skin fibroblasts is a sensitive in vitro test for defective genes in the $1,25(OH)_2D$ effector pathway. (*b*) This assay provides a useful tool for characterizing the target tissue defects in DD II and predicting response to calciferol therapy.

Introduction

Vitamin D-dependent rickets is a disorder characterized by hypocalcemia, early onset rickets, and normal vitamin D intake (1). In vitamin D-dependent rickets type I there is defective 1α-hydroxylation of 25(OH)D and hence low serum levels of $1,25(OH)_2D$ (2). Vitamin D-dependent rickets type II (DD II)[1] is associated with normal or elevated circulating levels of $1,25(OH)_2D$ and is thought to result from target tissue resistance to the action of $1,25(OH)_2D$ (3–15).

Recent studies from several centers have demonstrated heterogeneity of hereditary DD II in clinical manifestations and in associated cellular defects in cultured skin fibroblasts (13–20). Clinical diversity has been evidenced by a variable calcemic response to prolonged treatment with high doses of calciferol analogues and by the presence or absence of alopecia (21). In five families wherein multiple affected members have been studied, clinical features have been consistent among affected members of the family (4, 5, 16, 19).

Human skin fibroblasts have recently been shown to possess putative receptors for $1,25(OH)_2D$ (22,23) with binding properties similar to those in target tissues intestine (24), bone (25), and kidney (26). Cultured fibroblasts from patients with hereditary DD II frequently exhibit deficiencies in receptor binding or nuclear uptake of [^{3}H]$1,25(OH)_2D_3$ (13, 16–20) which, in one patient studied, were indistinguishable from these deficiencies present in cells cultured from bone (27). Four patterns of interaction with [^{3}H]$1,25(OH)_2D_3$ have been identified in cultured skin fibroblasts from these patients (17): (*a*) unmeasurable receptor binding and unmeasurable nuclear uptake; (*b*) decreased capacity but normal affinity for both receptor binding and nuclear uptake; (*c*) normal or near-normal receptor binding but no detectable nuclear uptake; and (*d*) normal receptor binding and normal nuclear uptake. While the recognition of heterogeneous receptor defects in fibroblasts cultured

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The opinions expressed herein are those of the authors and are not to be construed as reflecting the views of the Navy Department, of the Naval Service at large, or of the Department of Defense.

Portions of this work were presented at the Annual Meeting of the American Society for Bone and Mineral Research in San Antonio, Texas, 1983, and at the Eastern Sectional and Annual Meetings of the American Federation for Clinical Research in Boston, MA, 1983, and in Washington, DC, 1984, and have been published in part in abstract form in 1983, *Clin. Res.* 31:693a, and in 1984, 32:395a.

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1. *Abbreviations used in this paper:* HPLC, high performance liquid chromatography; 24-OHase, 24-hydroxylase; DD II, vitamin D-dependent rickets type II.

from affected patients has underscored the diversity of molecular abnormalities in this disorder, these receptor abnormalities per se have not accounted for the heterogeneity of clinical features such as alopecia and variable responsiveness[2] to treatment with high doses of calciferol analogues.

Renal (28–35) as well as a variety of extrarenal tissues, including intestine (36), bone cells (37, 38), and skin fibroblasts (13, 14), have been shown to possess the enzyme $25(OH)D_3$-24-hydroxylase (24-OHase), which can be induced by $1,25(OH)_2D$; this induction appears to be mediated through the receptor for $1,25(OH)_2D$ via a classical steroid hormone mechanism (34, 35). Cultured skin fibroblasts from seven patients representing six kindreds with DD II have been evaluated and showed deficient 24-OHase induction by $1,25(OH)_2D_3$ (13, 14, 19, 20). We have evaluated 24-OHase in skin fibroblasts from four additional, unrelated patients with DD II and have correlated this marker of $1,25(OH)_2D_3$ bioeffect in vitro with two clinical variables: alopecia and the variable calcemic response to treatment with high doses of calciferol analogues.

Table I. Clinical and Laboratory Data for Patients with Resistance to $1,25(OH)_2D$

	Patient			
	1a	2b	3	7
Total alopecia	−	+	+	+
Eucalacemia with high dose calciferol analogue*	+	+	−	−
Normal receptor binding of $[^3H]1,25(OH)_2D_3$	+	+	+	+
Nuclear uptake of $[^3H]1,25(OH)_2D_3$	−	−	+‡	+‡

* The criteria for a sufficient trial of calciferol analogue were appropriately high dosage of calciferol analogue and appropriate duration of calciferol therapy (for $1,25(OH)_2D_3$, at least 5 μg/d for 3 mo; for $1\alpha(OH)D_3$, at least 15 μg/d for 3 mo; for $25(OH)D_3$, at least 1 mg/d for 3 mo).

‡ In these patients the nuclear uptake capacity was 30–50% of the normal mean. This decrease is of uncertain significance.

Methods

Subjects. Fibroblasts from 10 normal subjects were studied. Each patient (Table I), representing a separate unrelated kindred, exhibited typical features of hereditary resistance to $1,25(OH)_2D$ or DD II including rickets, hypocalcemia, secondary hyperparathyroidism, and elevated serum levels of $1,25(OH)_2D$. Clinical details for one or more affected members of these kindreds have previously been reported as follows: kindred 1 (4, 16), kindred 2 (5, 16), and kindred 3 (7). We have reported the characteristics of cytosol binding and nuclear uptake of $[^3H]1,25(OH)_2D_3$ in cultured skin fibroblasts from members of kindreds 1–3 (15–17); these are summarized in Table I. Kindred 7 is newly recognized (Liberman, U. A., unpublished observations) and has only been incompletely described (39).

Materials. Collagenase (type I from *Clostridium histolyticum*), Tricine (*n*-Tris-[hydroxymethyl]methyl glycine), and glutamine were from Sigma Chemical Co., St. Louis, MO. Fetal calf serum (mycoplasma and virus screened), trypsin, penicillin, streptomycin, and trypan blue stain were from Gibco Laboratories, Grand Island, NY. Improved Eagle's minimal essential medium was from Associated Biomedical Systems, Inc., Buffalo, NY. $1,25(OH)_2D_3$, $25(OH)D_3$, and $24,25(OH)_2D_3$ were from Hoffmann-LaRoche, Nutley, NJ (gift of M. Uskokovic). $[26,27(N)^3H]25(OH)D_3$ (specific activity 22 Ci/mmol) was from Amersham Corp., Arlington Heights, IL. Hexane was from Waters Associates, Inc., Milford, MA, isopropanol (2-propanol) from Fisher Scientific Co., Fair Lawn, NJ, and liquid scintillation fluid (Aquasol) from New England Nuclear, Boston, MA. Tissue culture flasks (25 cm^2, 75 cm^2, and 150 cm^2) were purchased from either Falcon Labware, Div. Becton-Dickinson & Co., Oxnard, CA or Corning Glass Works, Corning, NY. Petri dishes (60 mm in diameter) were also from Falcon Labware. Insulin (Iletin U-100) was obtained from Eli Lilly & Co., Indianapolis, IN.

Cell culture. Fibroblasts from the normal controls and from patients resistant to $1,25(OH)_2D$ were established from punch biopsies (4 mm diameter) of arm, thigh, or mons pubis, or from genital skin obtained at surgery and processed as described previously (16, 17, 20). Studies were performed with fibroblasts from passages 4–20 and between 1 and 3 d after reaching confluency.

24-OHase induction and assay. The procedure was adapted from the technique of Chandler (35). $1,25(OH)_2D_3$, dissolved in ethanol, was added to culture medium (containing 10% fetal calf serum) in 150-cm^2 flasks to yield concentrations ranging from 10^{-10} M to 10^{-6} M. An equal volume of vehicle alone was added to the unstimulated "control" flasks, giving a 0.1% concentration of ethanol. After 15 h, the cells were harvested with 0.1% trypsin–0.02% EDTA in phosphate-buffered saline. The cells were then washed by suspension and centrifugation in medium A (improved Eagle's minimal essential medium, 0.584 g/liter glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 mM Tricine-HCl, pH 7.4) and resuspended in medium A with 1% fetal calf serum. In some experiments, cell harvest was preceded by a $1,25(OH)_2D_3$ "washout" procedure. This consisted of replacing the preincubation medium (containing $1,25(OH)_2D_3$) with medium A containing 10% fetal calf serum but no added $1,25(OH)_2D_3$ at 30-min intervals 1–3 times. Following dilution (1:10) of a small aliquot of cell suspension with trypan blue stain (1 g/liter), the total number of viable cells was determined using a hemocytometer. Viable cells were always >95% of total cells.

24-OHase activity in suspensions of dispersed skin fibroblasts was quantitated by measuring the conversion of $[^3H]25(OH)D_3$ to $[^3H]24,25(OH)_2D_3$. The $[^3H]25(OH)D_3$ was always purified by high performance liquid chromatography (HPLC) within a month prior to use (details of the HPLC system are presented under "metabolite identification"). Initial experiments employed $[^3H]25(OH)D_3$ diluted with radioinert $25(OH)D_3$ to give a specific activity of 5.5 Ci/mmol, while for later assays the $[^3H]25(OH)D_3$ was diluted further to a specific activity of 1.1 Ci/mmol. Unless indicated otherwise, assay conditions were as follows: 100 pmol of $[^3H]25(OH)D_3$ in ethanol was added to 12 × 75 borosilicate glass tubes (Kimble Glass Co., Div. of Owens-Illinois, Toledo, OH) and allowed to dry. 10^6 cells, suspended in 0.2 ml of medium A with 1% fetal calf serum, were added and the mixture was incubated in a shaking water bath at 37°C. After 30 min, we added the reference standards [$25(OH)D_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$ (150 pmol each)] in 5 μl ethanol and then 0.62 ml methanol-chloroform (2:1).

Extraction of the calciferols was by the method of Bligh and Dyer (40). The solvent/cell mixtures were transferred to 1.5 ml polypropylene tubes (Eppendorf, Brinkmann Inst., Inc., Westbury, NY) and incubated at 24°C for 1 h. Cell debris was sedimented by centrifugation for 1.5 min (Eppendorf Centrifuge Model 5412, Brinkmann Inst., Inc.), and the supernates were added to 1.5-ml polypropylene tubes containing 0.2 ml chloroform and 0.1 ml H_2O. After vigorous mixing (Vortex Genie, Scientific Industries, Inc., Springfield, MA) and centrifugation

2. Responsiveness is measured by bioeffect of maximal levels of a factor. Sensitivity is measured by the level of a factor required to give half of maximal bioeffect (21).

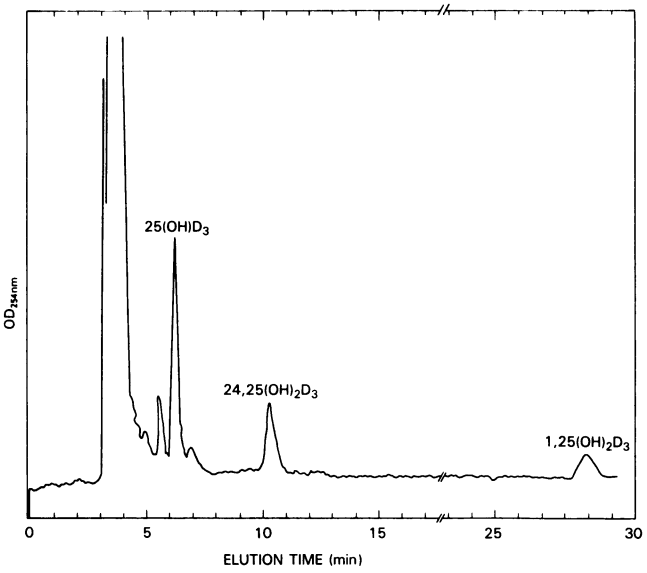

Figure 1. HPLC analysis of vitamin D analogues. Chromatogram shows resolution of radioinert standards $25(OH)D_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$.

for 1 min, the lower organic phase was separated from the aqueous phase. Reextraction of the remaining aqueous phase was accomplished as before by the addition of 0.4 ml chloroform, and the second organic phase was combined with the first. These extracts were then dried under N_2 and stored in 0.2 ml toluene-ethanol (1:1) at −20°C for analysis.

Metabolite identification. Production of $24,25(OH)_2D_3$ was assessed by HPLC with a model 322 gradient liquid chromatograph (Altex Scientific Inc., Berkeley, CA) with a 25 × 4-cm μPorasil column (Waters Associates, Inc., Milford, MA). Hexane/isopropanol (92:8) served as the elution solvent and was passed through a 0.5-μM filter (Millipore Corp., Medford, MA) just prior to use. The assay extracts, which had been stored in toluene/ethanol (1:1), were dried under N_2 and redissolved in 0.025 ml hexane/isopropanol (92:8) prior to injection. The column was eluted isocratically at a flow rate of 1 ml/min and 0.5-ml fractions were collected. Absorbance was measured at a wavelength of 254 nm with a sensitivity of 0.02 absorbance units full scale. Following the addition of 6 ml Aquasol, radioactivity was quantitated by a Mark III, model 6882 liquid scintillation counting system (Tracor Analytic, Elk Grove Village, IL). In addition to co-migration with the $24,25(OH)_2D_3$ reference standard, the putative $[^3H]24,25(OH)_2D_3$ was analyzed in some experiments by periodate oxidation of the fractions co-migrating with authentic $24,25(OH)_2D_3$ (41). These fractions were dried under N_2 and redissolved in 0.1 ml methanol. This volume was then divided equally and treated with either 0.1 ml H_2O or 0.1 ml 10% $NAIO_4$, for 1 h at 24°C. At the end of this period the samples were re-extracted, chromatographed, and radioactivity quantitated as before.

Results

Conditions for 24-OHase induction and assay in normal skin fibroblasts. The separation of vitamin D metabolite standards was readily accomplished by HPLC (Fig. 1). In addition to co-migration with the reference standard (Fig. 2 *A*), the identity

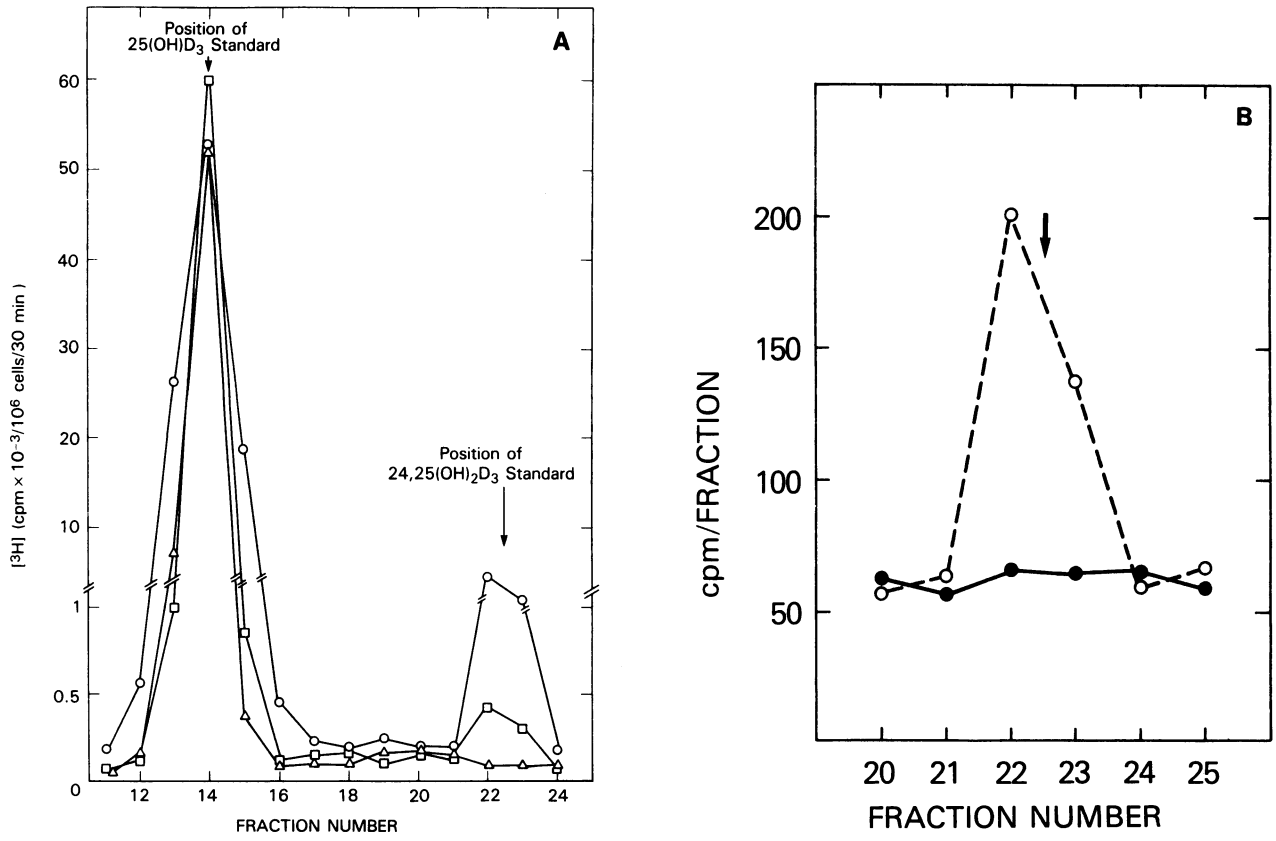

Figure 2. Characterization of calciferol metabolites from cultured skin fibroblasts. (*A*) $[^3H]24,25(OH)_2D_3$ production by fibroblasts from a normal subject (○) following preincubation with 10^{-8} M $1,25(OH)_2D_3$ (the Y axis values for the normal in fractions 22 and 23 are 4.4 and 1.8, respectively), from a patient (patient 2b) with hereditary vitamin D-dependency type II (□) following induction with 10^{-6} M $1,25(OH)_2D_3$ without washout, and from an incubation of $[^3H]25(OH)D_3$ without cells (△). (*B*) The effect of periodate treatment in fractions 21–25 (from patient 2b, see *A*) prior to rechromatography, as described in Methods: (○), control; (●), periodate-treated. The arrow indicates the position of elution for authentic $24,25(OH)_2D_3$.

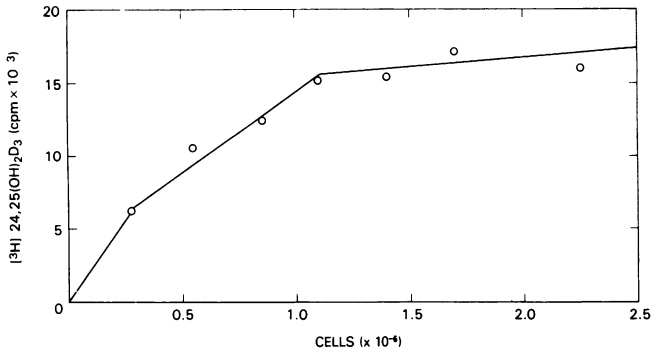

Figure 3. Effect of a cell number on [^{3}H]24,25(OH)$_2$D$_3$ formation in skin fibroblasts from a normal subject following induction with 10^{-8} M 1,25(OH)$_2$D$_3$.

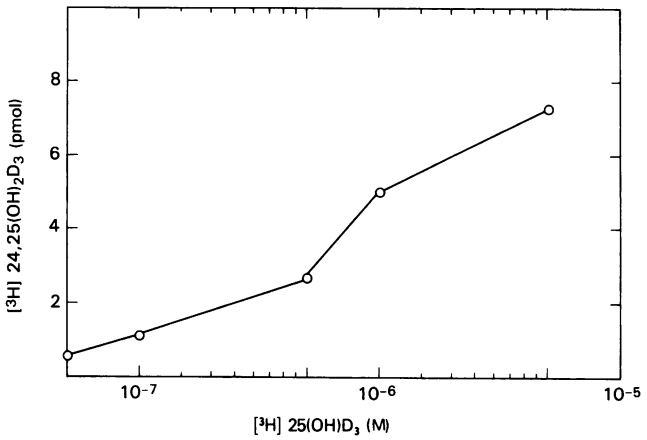

Figure 5. Effect of substrate [^{3}H]25(OH)D$_3$ concentration on [^{3}H]24,25(OH)$_2$D$_3$ formation in skin fibroblasts from a normal subject following induction with 10^{-8} M 1,25(OH)$_2$D$_3$.

of [^{3}H]24,25(OH)$_2$D$_3$ in media from cells of normals and DD II patients was further verified with periodate oxidation, which consistently abolished virtually all (98–99%) of the putative [^{3}H]24,25(OH)$_2$D$_3$ (Fig. 2 *B*).

We examined several variables which affect 24,25(OH)$_2$D$_3$ synthesis in a normal skin fibroblast line: cell number (Fig. 3), induction time (not shown), incubation time (Fig. 4), concentration of substrate ([^{3}H]25(OH)D$_3$) (Fig. 5), and concentration of inducer (1,25(OH)$_2$D$_3$) (Fig. 6). Accumulation of [^{3}H]24,25(OH)$_2$D$_3$ as a function of induction time was initially detectable at 4 h, peaked at 16 h, and decreased thereafter (data not shown). The conditions for subsequent studies (15 h induction time, 30 min assay incubation time, 10^6 cells, and 0.5 μM [^{3}H]25(OH)D$_3$ substrate) were chosen to maximize the likelihood of detecting product in cell lines with presumed deficient enzyme induction. Cell viability, as determined by trypan blue exclusion, remained unaltered during incubation for at least 2 h under these assay conditions.

Initial experiments in normal fibroblasts showed diminished [^{3}H]24,25(OH)$_2$D$_3$ production following induction with high concentrations of 1,25(OH)$_2$D$_3$ (10^{-7} M, 10^{-6} M). At 10^{-7} M, a 50–75% decrease was noted in some, while at 10^{-6} M, [^{3}H]24,25(OH)$_2$D$_3$ formation was reduced to barely measurable levels in all (data not shown). Two consecutive changes in preincubation medium (1,25(OH)$_2$D$_3$ washouts) resulted in [^{3}H]24,25(OH)$_2$D$_3$ production at 10^{-6} M 1,25(OH)$_2$D$_3$, which was similar to that following 10^{-8} M 1,25(OH)$_2$D$_3$ (Fig. 6). A third "washout" failed to increase [^{3}H]24,25(OH)$_2$D$_3$ formation further. Therefore, results of 24-OHase induction in fibroblasts from normals as well as DD II patients following induction with 10^{-7} M and 10^{-6} M 1,25(OH)$_2$D$_3$ were included only if two consecutive 1,25(OH)$_2$D$_3$ monolayer washouts had been introduced prior to cell harvest.

Induction of 24-OHase by 1,25(OH)$_2$D$_3$ in skin fibroblasts from normal subjects. The lower detection limit for 24-OHase was ~0.2 pmol/30 min per 10^6 cells. There was no measurable [^{3}H]24,25(OH)$_2$D$_3$ production following induction for 15 h with vehicle alone in fibroblasts from normal subjects even when the assay incubation was carried to 120 min (Fig. 4). Fibroblasts from two of five normals showed detectable 24-OHase induction after incubation with 10^{-10} M 1,25(OH)$_2$D$_3$ (Fig. 7 *A*). In fibroblasts from all normal subjects 24-OHase was detectable following exposure to 1,25(OH)$_2$D$_3$ at 10^{-9} M and reached apparent maximal levels (8.8±1.6 [SEM] pmol/30 min per 10^6 cells) at 10^{-8} M (Fig. 7 *A*).

Induction of 24-OHase by 1,25(OH)$_2$D$_3$ in skin fibroblasts

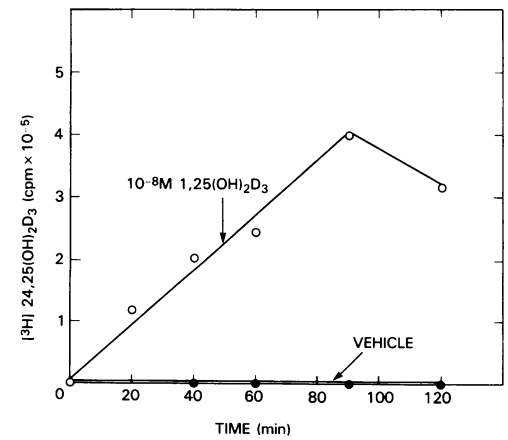

Figure 4. Effect of assay incubation time on [^{3}H]24,25(OH)$_2$D$_3$ formation in skin fibroblasts from a normal subject following induction with vehicle (●) or with 10^{-8} M 1,25(OH)$_2$D$_3$ (○).

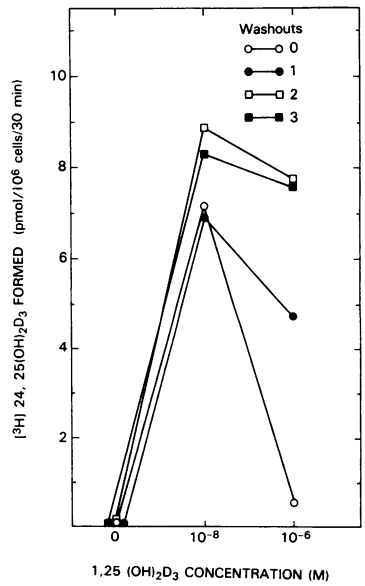

Figure 6. Effect of number of monolayer medium changes (1,25(OH)$_2$D$_3$ washout) after induction with 1,25(OH)$_2$D$_3$ upon subsequent conversion of [^{3}H]25(OH)D$_3$ to [^{3}H]24,25(OH)$_2$D$_3$ with normal fibroblasts.

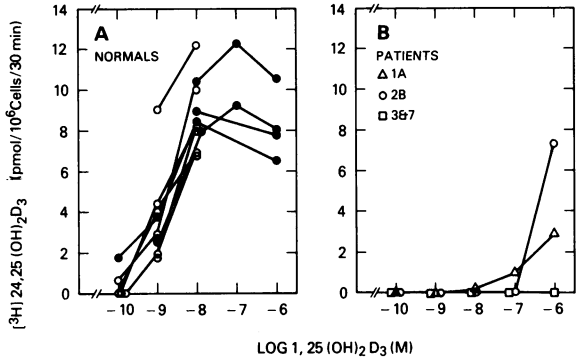

Figure 7. [^{3}H]24,25(OH)$_2$D$_3$ formation in skin fibroblasts following induction with varying concentrations of 1,25(OH)$_2$D$_3$ for 15 h. (*A*) Normal subjects. (*B*) Patients with hereditary vitamin D dependency type II. For all patients with DD II, the data are means of two assays. Values of duplicate assays (in pmol/10^6 cells per 30 min), wherein 24-OHase induction was detectable with patient cells, are as follows: for patient 1a at 10^{-8} M (0.2, 0.2); at 10^{-7} M (0.6, 1.3); at 10^{-6} M (2.4, 3.3); for patient 2b at 10^{-6} M (7.4, 7.1).

from subjects with DD II. Fibroblasts from two patients with DD II, representing kindreds three and seven, had no detectable [^{3}H]24,25(OH)$_2$D$_3$ formation following induction with concentrations of 1,25(OH)$_2$D$_3$ up to 10^{-6} M (Fig. 7 *B*). However, fibroblasts from two patients with DD II representing kindreds one and two, clearly demonstrated [^{3}H]24,25(OH)$_2$D$_3$ production following induction with 1,25(OH)$_2$D$_3$ (Fig. 7 *B*). In cells from patient 1a, [^{3}H]24,25(OH)$_2$D$_3$ production was only barely detectable following 10^{-8} M 1,25(OH)$_2$D$_3$ and rose to 2.9 pmol/30 min per 10^6 cells following induction with 10^{-6} M 1,25(OH)$_2$D$_3$ (30% of normal production at 10^{-6} M 1,25(OH)$_2$D$_3$). Even with the double washout procedure, 24-OHase activity in cells induced with 10^{-5} M 1,25(OH)$_2$D$_3$ was too variable to compare with normal cells. In cells from patient 2b, [^{3}H]24,25(OH)$_2$D$_3$ production was unmeasurable following 10^{-8} M and 10^{-7} M 1,25(OH)$_2$D$_3$; however, it rose to 7.3 pmol/30 min per 10^6 cells following induction with 10^{-6} M 1,25(OH)$_2$D$_3$ (90% normal production at 10^{-6} M 1,25(OH)$_2$D$_3$). For the DD II fibroblasts, all experiments were performed twice and incorporated two consecutive 1,25(OH)$_2$D$_3$ "washouts" prior to cell harvest.

Discussion

Until recently, there has been no available marker of 1,25(OH)$_2$D action distal to its receptor in cultured skin fibroblasts. However, Clemens et al. (18) have reported that 1,25(OH)$_2$D can decrease cell division rate in cultured human skin fibroblasts; additionally, both Feldman et al. (13) and Griffin and Zerwekh (14) have demonstrated induction of 24-OHase by 1,25(OH)$_2$D$_3$ in this tissue from normal subjects. 24-OHase activity appears to be a useful index of the 1,25(OH)$_2$D effector pathway, since it changes in parallel with modulation of receptors during changes in cell division rate (42). And, it is deficient in a simian renal cell line that fails to show receptors for 1,25(OH)$_2$D by radioligand studies (35). Moreover, this bioeffect is deficient in fibroblasts from seven patients with hereditary resistance to 1,25(OH)$_2$D (13, 14, 19, 20). The absent or blunted [^{3}H]24,25(OH)$_2$D$_3$ formation following 1,25(OH)$_2$D$_3$ induction with fibroblasts from all four of our patients with hereditary DD II provides further support for the validity of this enzyme marker of 1,25(OH)$_2$D action.[3] The values we observed in normal fibroblasts for apparent maximal 24-OHase and for 1,25(OH)$_2$D$_3$ concentration to give half maximal induction agree with those from another laboratory using similar methods (13, 19). And the differential 24-OHase responsivity to 1,25(OH)$_2$D$_3$ at 10^{-7} M and 10^{-6} M extends the heterogeneity of cellular defects associated with hereditary DD II (17). In light of the results of the monolayer "washout" studies (Fig. 6), the marked suppression of [^{3}H]24,25(OH)$_2$D$_3$ accumulation in cells from normal subjects following preincubation with high concentrations of 1,25(OH)$_2$D$_3$ (10^{-6} M) appears to have resulted from competition of residual 1,25(OH)$_2$D$_3$ with [^{3}H]25(OH)D$_3$ for the substrate site on the 24-OHase enzyme. These findings are consistent with data suggesting greater apparent affinity of 1,25(OH)$_2$D$_3$ than 25(OH)D$_3$ at the substrate site of the 24-OHase (43). The washout procedure is of particular importance in 24-OHase assays of mutant fibroblasts where hormone responsiveness must be analyzed with high concentrations of 1,25(OH)$_2$D$_3$ inducer.

Just as the characteristics of receptor binding and nuclear uptake of [^{3}H]1,25(OH)$_2$D$_3$ have not been fully predictive of responsiveness to 1,25(OH)$_2$D$_3$ in vivo (16, 17), these radioligand binding studies have also failed to correlate with responsiveness to 1,25(OH)$_2$D$_3$ in vitro as measured by 24-OHase induction. Cells from the two patients (patients three and seven) with normal receptor binding and measurable nuclear uptake had no detectable 24-OHase activity following preincubation with 1,25(OH)$_2$D$_3$. A similar finding of "receptor positive" resistance has been noted previously (14). Cells of the two patients (patients 1a and 2b), which had inducible 24-OHase activity, lacked measurable nuclear uptake of [^{3}H]1,25(OH)$_2$D$_3$. Since most of the available evidence is strongly in favor of 1,25(OH)$_2$D acting through a classical steroid hormone mechanism (44), these data would appear to be in conflict.

However, all the findings in vivo and in vitro can be reconciled when one considers technical aspects of the assays for the in vitro studies. The nuclear uptake assay does not assess the 1,25(OH)$_2$D effector pathway distal to the process of receptor localization in the nucleus. Thus, normal nuclear localization with deficient 24-OHase induction would reflect an abnormal receptor unable to function after uptake in the nucleus or a normal receptor with an abnormality of post-receptor events (14). The lack of detectable [^{3}H]1,25(OH)$_2$D$_3$ nuclear uptake in cells from patients 1a and 2b probably reflects the relatively low maximal concentration (5×10^{-9} M) of [^{3}H]1,25(OH)$_2$D$_3$ required by that assay. Patients with this variety of DD II appear to have receptors for 1,25(OH)$_2$D which have decreased ability to localize hormone in the nucleus properly, and therefore an impaired ability to activate 1,25(OH)$_2$D sensitive genes. It is presently unclear whether this defect(s) resides in the receptor or elsewhere in the cell. Apparently, in our two patients, the defect(s) can be partially (patient 1a) or completely (patient 2b) overcome by high

3. We also noted unmeasurable 24-OHase induction in cells from an additional patient whose fibroblasts had no binding with [^{3}H]1,25-(OH)$_2$D$_3$ and who showed no calcemic response to calciferol in vivo (12, 17). Since these cells were not analyzed with the 1,25(OH)$_2$D$_3$ washout procedure, the data are only meaningful up to a 1,25(OH)$_2$D$_3$ concentration of 10^{-8} M.

concentrations of $1,25(OH)_2D$. Affinity of the 24-OHase induction process for $1,25(OH)_2D_3$ cannot be accurately derived because of inability to attain substrate saturation (Fig. 5). However, the affinity must be strikingly decreased in both patients 1a and 2b because responses to $1,25(OH)_2D_3$, while measurable, are shifted to far higher concentrations of $1,25(OH)_2D_3$ (Fig. 7 *B*). The reproducibly differing slopes of 24-OHase response in cells from patients 1a and 2b suggest subtle differences in the types of defects in these two cell lines.

The heterogeneity of 24-OHase inducibility by $1,25(OH)_2D_3$ has not been evaluated by prior studies. However, slight induction was suggested at 10^{-9} M and 5×10^{-8} M in two prior cases (14, 20) that were analyzed with somewhat different methods. It is notable that these patients showed calcemic responses with serum levels of $1,25(OH)_2D$ that were only modestly elevated and that neither patient had alopecia. Induction of 24-OHase was undetectable at 10^{-6} M $1,25(OH)_2D_3$ in one case (13) and at 10^{-7} M in four cases from three kindreds in another study (19). All affected members of these four kindreds had alopecia, and none showed a calcemic response to calciferols. However, available data from only one of these cases (45) meet our criteria for unresponsiveness to calciferols in vivo (see Table I), and 24-OHase was not analyzed at high $1,25(OH)_2D_3$ with a washout protocol.

Testing for bioeffects of calciferol analogues in vivo can be time consuming, costly, and dangerous (if rickets is at an advanced stage and is allowed to progress in the face of futile therapy). Thus, an assay for bioeffect of $1,25(OH)_2D$ in vitro has potential for important clinical application. Our data suggest that 24-OHase activity provides a meaningful index of responsiveness and sensitivity to $1,25(OH)_2D$. Cells from two patients with no calcemic response to prolonged administration of calciferol analogues at high dosage in vivo exhibited no 24-OHase response to $1,25(OH)_2D_3$ in vitro, while cells from two affected patients with a calcemic response to high dose therapy in vivo had a clearly detectable response of 24-OHase in vitro.

While the cause of alopecia in DD II remains an enigma, our data and those from others suggest that this finding may be related to the severity of resistance to $1,25(OH)_2D$. Among our four patients, only the cells from the one lacking alopecia showed a 24-OHase response at either 10^{-8} M or 10^{-7} M $1,25(OH)_2D_3$. Of the six kindreds with DD II without alopecia reported to date (3, 4, 6, 8, 11, 20), all have shown calcemic responses to high endogenous levels or high dose of calciferol analogues exogenously. Conversely, of the 11 kindreds reported with alopecia (5, 7, 9, 10–12, 13, 15, 19, 45), only three (5, 10, 15) have shown calcemic responses with such treatment. Thus, our findings further strengthen the relationship between alopecia and profound resistance to $1,25(OH)_2D$. In this regard, it is of interest that Stumpf et al. (46) have reported high affinity nuclear uptake of $[^3H]1,25(OH)_2D_3$ in the outer root sheath cells of the rat hair follicle.

In summary, we have examined 24-OHase induction by $1,25(OH)_2D_3$ in skin fibroblasts from members of four kindreds with DD II and from 10 normal subjects. Deficient 24-OHase induction was documented in all four patients with DD II. Moreover, the presence or absence of a response to $1,25(OH)_2D_3$ in vitro correlated with the presence or absence of a calcemic response to calciferols in vivo. Studies of $1,25(OH)_2D_3$ bioeffect in cultured skin fibroblasts provide a useful tool for the characterization of hereditary resistance to $1,25(OH)_2D$ including prediction of response to therapy with calciferol analogues.

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