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)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 μ M [³H]25(OH)D₃ at 37°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/10⁶ 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 $\sim 3 \times 10^{-10} \,\mathrm{M}$ 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/10⁶ 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/10⁶ 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, [3 H]24,25(OH)₂D₃ production achieved normal levels, 7.3 pmol/106 cells per 30 min after 10⁻⁶ M 1,25(OH)₂D₃.

The two patients whose cells had a detectable 24-OHase induction by 1,25(OH)₂D₃ 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

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.

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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)₂D₃ 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 $[^{3}H]1,25(OH)_{2}D_{3}$ (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 [3H]1,25(OH)2D3 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

^{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 responsiveness² 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)D₃-24-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)₂D₃ (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)₂D₃ bioeffect in vitro with two clinical variables: alopecia and the variable calcemic response to treatment with high doses of calciferol analogues.

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)₂D₃ 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)₂D₃, 25(OH)D₃, and 24,25(OH)₂D₃ were from Hoffmann-LaRoche, Nutley, NJ (gift of M. Uskokovic). [26,27(N)³H]25(OH)D₃ (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 cm², 75 cm², and 150 cm²) 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)₂D₃, dissolved in ethanol,

Table I. Clinical and Laboratory Data for Patients with Resistance to 1,25(OH)₂D

	Patient			
	la	2ь	3	7
Total alopecia	_	+	+	+
Eucalacemia with high				
dose calciferol				
analogue*	+	+	_	_
Normal receptor binding				
of $[^{3}H]1,25(OH)_{2}D_{3}$	+	+	+	+
Nuclear uptake of				
[³ H]1,25(OH) ₂ D 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 $5 \mu g/d$ for 3 mo; for $1\alpha(OH)D_3$, at least $15 \mu g/d$ 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.

was added to culture medium (containing 10% fetal calf serum) in 150-cm² 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 phosphatebuffered 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)₂D₃ "washout" procedure. This consisted of replacing the preincubation medium (containing 1,25(OH)₂D₃) with medium A containing 10% fetal calf serum but no added 1,25(OH)₂D₃ 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)D₃ to [3H]24,25(OH)₂D₃. The [3H]25(OH)D₃ 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)D₃ diluted with radioinert 25(OH)D₃ to give a specific activity of 5.5 Ci/mmol, while for later assays the [3H]25(OH)D3 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)D₃ in ethanol was added to 12×75 borosilicate glass tubes (Kimble Glass Co., Div. of Owens-Illinois, Toledo, OH) and allowed to dry. 106 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)D₃, 24,25(OH)₂D₃, and $1,25(OH)_2D_3$ (150 pmol each)] in 5 μ l 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 H₂O. 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).

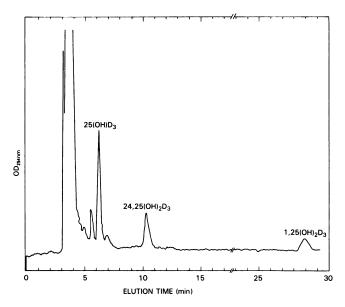


Figure 1. HPLC analysis of vitamin D analogues. Chromatogram shows resolution of radioinert standards 25(OH)D₃, 24,25(OH)₂D₃, and 1,25(OH)₂D₃.

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

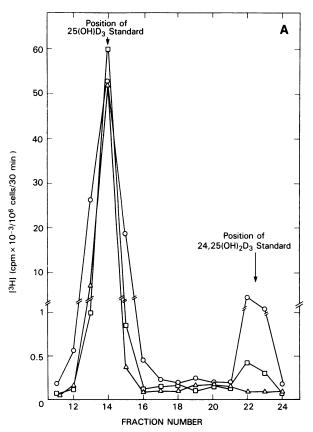


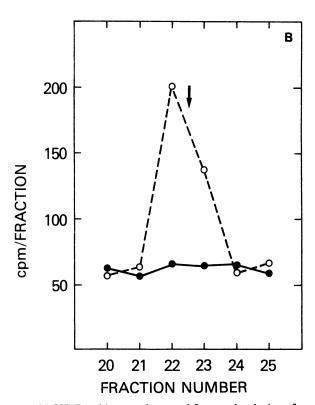
Figure 2. Characterization of calciferol metabolites from cultured skin fibroblasts. (A) [3 H]24,25(OH) $_{2}$ D $_{3}$ production by fibroblasts from a normal subject (O) following preincubation with 10^{-8} M 1,25(OH) $_{2}$ D $_{3}$ (the Y axis values for the normal in fractions 22 and 23 are 4.4 and 1.8, respectively), from a patient (patient 2b) with hereditary vitamin D-dependency type II (\square) following induction with 10^{-6}

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)₂D₃ was assessed by HPLC with a model 322 gradient liquid chromatograph (Altex Scientific Inc., Berkeley, CA) with a 25 \times 4-cm μ Porasil 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 N₂ 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)₂D₃ reference standard, the putative [³H]24,25(OH)₂D₃ was analyzed in some experiments by periodate oxidation of the fractions co-migrating with authentic 24,25(OH)₂D₃ (41). These fractions were dried under N2 and redissolved in 0.1 ml methanol. This volume was then divided equally and treated with either 0.1 ml H₂O or 0.1 ml 10% NAIO₄, 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 comigration with the reference standard (Fig. 2 A), the identity



M 1,25(OH)₂D₃ without washout, and from an incubation of $[^3H]25(OH)D_3$ without cells (\triangle). (B) The effect of periodate treatment in fractions 21–25 (from patient 2b, see A) prior to rechromatography, as described in Methods: (\bigcirc), control; (\bullet), periodate-treated. The arrow indicates the position of elution for authentic 24,25(OH)₂D₃.

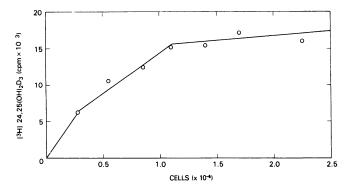


Figure 3. Effect of a cell number on $[^3H]24,25(OH)_2D_3$ formation in skin fibroblasts from a normal subject following induction with 10^{-8} M $1,25(OH)_2D_3$.

of $[^3H]24,25(OH)_2D_3$ 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 $[^3H]24,25(OH)_2D_3$ (Fig. 2 B).

We examined several variables which affect $24,25(OH)_2D_3$ synthesis in a normal skin fibroblast line: cell number (Fig. 3), induction time (not shown), incubation time (Fig. 4), concentration of substrate ([³H]25(OH)D_3) (Fig. 5), and concentration of inducer $(1,25(OH)_2D_3)$ (Fig. 6). Accumulation of [³H]24,25(OH)₂D₃ 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 $0.5 \,\mu$ M [³H]25(OH)D₃ 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 $[^3H]24,25(OH)_2D_3$ production following induction with high concentrations of 1,25(OH)₂D₃ (10⁻⁷ M, 10⁻⁶ M). At 10⁻⁷ M, a 50-75% decrease was noted in some, while at 10⁻⁶ M, $[^3H]24,25(OH)_2D_3$ formation was reduced to barely measurable levels in all (data not shown). Two consecutive changes in preincubation medium (1,25(OH)₂D₃ washouts) resulted in

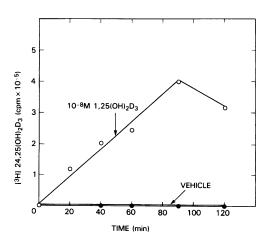


Figure 4. Effect of assay incubation time on $[^3H]^24,25(OH)_2D_3$ formation in skin fibroblasts from a normal subject following induction with vehicle (\bullet) or with 10^{-8} M 1,25(OH)₂D₃ (O).

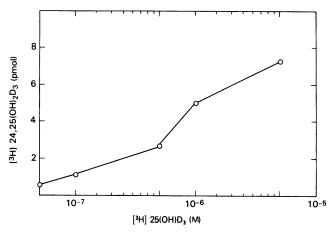


Figure 5. Effect of substrate [${}^{3}H$]25(OH)D₃ concentration on [${}^{3}H$]24,25(OH)₂D₃ formation in skin fibroblasts from a normal subject following induction with 10^{-8} M 1,25(OH)₂D₃.

[³H]24,25(OH)₂D₃ production at 10⁻⁶ M 1,25(OH)₂D₃, which was similar to that following 10⁻⁸ M 1,25(OH)₂D₃ (Fig. 6). A third "washout" failed to increase [³H]24,25(OH)₂D₃ 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)₂D₃ were included only if two consecutive 1,25(OH)₂D₃ monolayer washouts had been introduced prior to cell harvest.

Induction of 24-OHase by $1,25(OH)_2D_3$ 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 $[^3H]24,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ (Fig. 7 A). In fibroblasts from all normal subjects 24-OHase was detectable following exposure to $1,25(OH)_2D_3$ at 10^{-9} M and reached apparent maximal levels $(8.8\pm1.6 \text{ [SEM] pmol/} 30 \text{ min per } 10^6 \text{ cells})$ at 10^{-8} M (Fig. 7 A).

Induction of 24-OHase by 1,25(OH)₂D₃ in skin fibroblasts

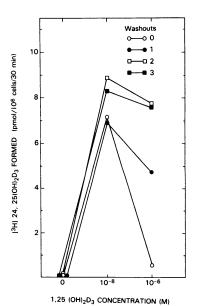


Figure 6. Effect of number of monolayer medium changes (1,25(OH)₂D₃ washout) after induction with 1,25(OH)₂D₃ upon subsequent conversion of [³H]25(OH)D₃ to [³H]24,25(OH)₂D₃ with normal fibroblasts.

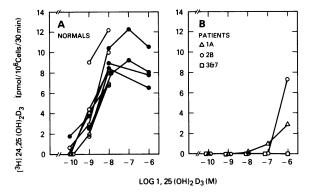


Figure 7. [³H]24,25(OH)₂D₃ formation in skin fibroblasts following induction with varying concentrations of 1,25(OH)₂D₃ for 15 h. (A) Normal subjects. (B) Patients with hereditary vitamin D dependency type II. For all patients with DD II, the data are means of two assays. Values of duplicate assays (in pmol/10⁶ 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 [3H]24,25(OH)₂D₃ formation following induction with concentrations of 1,25(OH)₂D₃ up to 10⁻⁶ M (Fig. 7 B). However, fibroblasts from two patients with DD II representing kindreds one and two, clearly demonstrated [3H]24,25(OH)₂D₃ production following induction with 1,25(OH)₂D₃ (Fig. 7 B). In cells from patient 1a, [3H]24,25(OH)₂D₃ production was only barely detectable following 10⁻⁸ M 1,25(OH)₂D₃ and rose to 2.9 pmol/30 min per 10⁶ cells following induction with 10⁻⁶ M $1,25(OH)_2D_3$ (30% of normal production at 10^{-6} M 1,25(OH)₂D₃). Even with the double washout procedure, 24-OHase activity in cells induced with 10⁻⁵ M 1,25(OH)₂D₃ was too variable to compare with normal cells. In cells from patient 2b, [3H]24,25(OH)₂D₃ production was unmeasurable following 10^{-8} M and 10^{-7} M 1,25(OH)₂D₃; however, it rose to 7.3 pmol/30 min per 10⁶ cells following induction with 10⁻⁶ M $1,25(OH)_2D_3$ (90% normal production at 10^{-6} M $1,25(OH)_2D_3$). For the DD II fibroblasts, all experiments were performed twice and incorporated two consecutive 1,25(OH)₂D₃ "washouts" prior to cell harvest.

Discussion

Until recently, there has been no available marker of 1,25(OH)₂D action distal to its receptor in cultured skin fibroblasts. However, Clemens et al. (18) have reported that 1.25(OH)₂D 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)₂D₃ in this tissue from normal subjects. 24-OHase activity appears to be a useful index of the 1,25(OH)₂D 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)₂D by radioligand studies (35). Moreover, this bioeffect is deficient in fibroblasts from seven patients with hereditary resistance to 1,25(OH)₂D (13, 14, 19, 20). The absent or blunted [3H]24,25(OH)₂D₃ formation following 1,25(OH)₂D₃ 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)₂D action.³ The values we observed in normal fibroblasts for apparent maximal 24-OHase and for 1,25(OH)₂D₃ concentration to give half maximal induction agree with those from another laboratory using similar methods (13, 19). And the differential 24-OHase responsivity to $1,25(OH)_2D_3$ 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 [3H]24,25(OH)₂D₃ accumulation in cells from normal subjects following preincubation with high concentrations of 1,25(OH)₂D₃ (10⁻⁶ M) appears to have resulted from competition of residual 1,25(OH)₂D₃ with [³H]25(OH)D₃ for the substrate site on the 24-OHase enzyme. These findings are consistent with data suggesting greater apparent affinity of 1,25(OH)₂D₃ than 25(OH)D₃ 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 $1,25(OH)_2D_3$ inducer.

Just as the characteristics of receptor binding and nuclear uptake of [3H]1,25(OH)₂D₃ have not been fully predictive of responsiveness to 1,25(OH)₂D₃ in vivo (16, 17), these radioligand binding studies have also failed to correlate with responsiveness to 1,25(OH)₂D₃ 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)₂D₃. 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 [3H]1,25(OH)₂D₃. Since most of the available evidence is strongly in favor of 1,25(OH)₂D 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)₂D 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 postreceptor events (14). The lack of detectable [3H]1,25(OH)₂D₃ nuclear uptake in cells from patients 1a and 2b probably reflects the relatively low maximal concentration (5 \times 10⁻⁹ M) of [3H]1,25(OH)₂D₃ required by that assay. Patients with this variety of DD II appear to have receptors for 1,25(OH)₂D which have decreased ability to localize hormone in the nucleus properly, and therefore an impaired ability to activate 1,25(OH)₂D 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 [³H]1,25-(OH)₂D₃ and who showed no calcemic response to calciferol in vivo (12, 17). Since these cells were not analyzed with the 1,25(OH)₂D₃ washout procedure, the data are only meaningful up to a 1,25(OH)₂D₃ concentration of 10⁻⁸ M.

concentrations of 1,25(OH)₂D. Affinity of the 24-OHase induction process for 1,25(OH)₂D₃ 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)₂D₃, while measurable, are shifted to far higher concentrations of 1,25(OH)₂D₃ (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)₂D₃ 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)₂D₃. 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)2D3 in the outer root sheath cells of the rat hair follicle.

In summary, we have examined 24-OHase induction by 1,25(OH)₂D₃ 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)₂D₃ in vitro correlated with the presence or absence of a calcemic response to calciferols in vivo. Studies of 1,25(OH)₂D₃ 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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References

- 1. Prader, V. A., R. Illig, and E. Heidi. 1961. Eine besondere form der primaren vitamin-D-resintenten rachitis mit hypocalcemie unde autosomaldominantem erbgang: die hereditare pseudo-mangelrachitis. *Helv. Paediatr. Acta.* 5/6:452-468.
- 2. Fraser, D., S. W. Kooh, H. P. Kind, M. F. Hollick, Y. Tanaka, and H. F. DeLuca. 1973. Pathogenesis of hereditary vitamin-D-dependent rickets: an inborn error of vitamin D metabolism involving defective conversion of 25-hydroxyvitamin D to 1α ,25-dihydroxyvitamin D. N. Engl. J. Med. 289:817–822.
- 3. Brooks, M. H., N. H. Bell, L. Love, P. H. Stern, E. Orfei, S. F. Queener, A. J. Hamstra, and H. F. DeLuca. 1978. Vitamin-D-dependent rickets type II: resistance of target organs to 1,25-dihydroxyvitamin D. N. Engl. J. Med. 298:996-999.
- 4. Marx, S. J., A. M. Spiegel, E. M. Brown, D. G. Gardner, R. W. Downs, Jr., M. Attie, A. J. Hamstra, and H. F. DeLuca. 1978. A familial syndrome of decrease in sensitivity to 1,25-dihydroxyvitamin D. J. Clin. Endocrinol. Metab. 47:1303-1310.
- 5. Rosen, J. F., A. R. Fleischman, L. Finberg, A. Hamstra, and H. F. DeLuca. 1979. Rickets with alopecia: an inborn error of vitamin D metabolism. *J. Pediatr.* 94:729-735.
- 6. Zerwekh, J. E., K. Glass, J. Jowsey, and C. Y. C. Pak. 1979. An unique form of osteomalacia associated with end organ refractoriness to 1,25-dihydroxy vitamin D and apparent defective synthesis of 25-hydroxyvitamin D. J. Clin. Endocrinol. Metab. 49:171-175.
- 7. Liberman, U. A., R. Samuel, A. Halabe, R. Kauli, S. Edelstein, Y. Weisman, S. E. Papapoulos, T. L. Clemens, L. J. Fraher, and J. L. H. O'Riordan. 1980. End-organ resistance to 1,25-dihydroxycholecalciferol. *Lancet.* 1:504-507.
- 8. Fujita, T., M. Nomura, S. Okajima, and H. Furuya. 1980. Adult-onset vitamin D-resistant osteomalacia with the unresponsiveness to parathyroid hormone. *J. Clin. Endocrinol. Metab.* 50:927–931.
- 9. Sockalosky, J. J., R. A. Ulstrom, H. F. DeLuca, and D. M. Brown. 1980. Vitamin D-resistant rickets: end-organ unresponsiveness to 1,25(OH)₂D₃. *J. Pediatr.* 96:701-703.
- 10. Tsuchiya, Y., N. Matsuo, H. Cho, M. Kumagai, A. Yasaka, T. Suda, H. Orimo, and M. Shiraki. 1980. An unusual form of vitamin D-dependent rickets in a child: alopecia and marked end-organ hyposensitivity to biologically active vitamin D. J. Clin. Endocrinol. Metab. 51-685-690.
- 11. Kudoh, T., T. Kumagai, N. Uetsuji, S. Tsugawa, K. Oyanagi, Y. Chiba, R. Minami, and T. Nakao. 1981. Vitamin D dependent rickets: decreased sensitivity to 1,25-dihydroxyvitamin D. Eur. J. Pediatr. 137:307-311.
- 12. Beer, S., M. Tieder, D. Kohelet, U. A. Liberman, E. Vure, G. Bar-Joseph, D. Gabizon, Z. U. Borochowitz, M. Varon, and D. Modai. 1981. Vitamin-D-resistant rickets with alopecia: a form of end organ resistance to 1,25-dihydroxyvitamin D. Clin. Endocrinol. 14:395-402.
- 13. Feldman, D., T. Chen, C. Cone, M. Hirst, S. Shani, A. Benderli, and Z. Hochberg. 1982. Vitamin D resistant rickets with alopecia: cultured skin fibroblasts exhibit defective cytoplasmic receptors and unresponsiveness to 1,25(OH)₂D₃. *J. Clin. Endocrinol. Metab.* 55: 1020–1022.
- 14. Griffin, J. E., and J. E. Zerwekh. 1983. Impaired stimulation of 25-hydroxyvitamin D-24-hydroxylase in fibroblasts from a patient with vitamin D-dependent rickets, type II. J. Clin. Invest. 72:1190–1199.

- 15. Balsan, S., M. Garabedian, U. A. Liberman, C. Eil, A. Bourdeau, H. Guillozo, R. Grimberg, M. J. LeDeunff, M. Lieberherr, P. Guimbaud, M. Broyer, and S. J. Marx, 1983. Rickets and alopecia in resistance to 1,25-dihydroxyvitamin D: two different clinical courses with two different cellular defects. J. Clin. Endocrinol. Metab. 57:803–811.
- 16. Eil, C., U. A. Liberman, J. F. Rosen, and S. J. Marx. 1981. A cellular defect in hereditary vitamin-D-dependent rickets Type II; defective nuclear uptake of 1,25-dihydroxyvitamin D in cultured skin fibroblasts. N. Engl. J. Med. 304:1588-1591.
- 17. Liberman, U. A., C. Eil, and S. J. Marx. 1983. Resistance to 1,25-dihydroxyvitamin D: association with heterogeneous defects in cultured skin fibroblasts. *J. Clin. Invest.* 71:192-200.
- 18. Clemens, T. L., J. S. Adams, N. Horiuchi, B. A. Gilchrist, H. Cho, Y. Tsuchiya, N. Matsuo, T. Suda, and M. F. Hollick. 1983. Interaction of 1,25-dihydroxy-vitamin-D₃ with keratinocytes and fibroblasts from skin of a subject with vitamin-D-dependent rickets type II: a model for the study of the mode of action of 1,25-dihydroxyvitamin D₃. J. Clin. Endocrinol. Metab. 56:824-830.
- 19. Chen, T. L., M. L. Hirst, C. M. Cone, Z. Hochberg, H.-U Tietze, and D. Feldman. 1984. 1,25-Dihydroxyvitamin D resistance, rickets, and alopecia: analysis of receptors and bioresponse in cultured fibroblasts from patients and parents. *J. Clin. Endocrinol. Metab.* 59: 383–388.
- 20. Adams, J. S., M. A. Gacad, and F. R. Singer. 1984. Specific internalization and action of 1,25-dihydroxyvitamin D_3 in cultured dermal fibroblasts from patients with X-linked hypophosphatemia. *J. Clin. Endocrinol. Metab.* 59:556-560.
- 21. Marx, S. J., U. A. Liberman, C. Eil, G. T. Gamblin, D. A. DeGrange, and S. Balsan. 1984. Hereditary resistance to 1,25-dihydroxyvitamin D. *Recent Prog. Horm. Res.* 40:589-615.
- 22. Feldman, D., T. Chen, M. Hirst, K. Colston, K. Karasek, and C. Cone. 1980. Demonstration of 1,25-dihydroxyvitamin D₃ receptors in human skin biopsies. *J. Clin. Endocrinol. Metab.* 51:1463-1465.
- 23. Eil, C., and S. J. Marx. 1981. Nuclear uptake of 1,25-dihydroxy[³H]cholecalciferol in dispersed fibroblasts cultured from normal human skin. *Proc. Natl. Acad. Sci. USA*. 78:2562-2566.
- 24. Brumbaugh, P. F., and M. R. Haussler. 1974. 1α ,25-dihydroxycholecalciferol receptors in intestine. II. Temperature dependent transfer of the hormone to chromatin via a specific cytosol receptor. *J. Biol. Chem.* 249:1258–1262.
- 25. Chen, T. L., and D. Feldman. 1981. Regulation of 1,25 dihydroxyvitamin D_3 receptors in cultured mouse bone cells: correlation of receptor concentration with the rate of cell division. *J. Biol. Chem.* 256:5561-5566.
- 26. Simpson, R. U., R. T. Franchesi, and H. F. DeLuca. 1980. Characterization of a specific, high affinity binding macromolecule for 1,25 hydroxyvitamin D₃ in cultured chick kidney cells. *J. Biol. Chem.* 255:10160-10166.
- 27. Liberman, U. A., C. Eil, P. Holst, J. F. Rosen, and S. J. Marx. 1983. Hereditary resistance to 1,25-dihydroxyvitamin D: defective function of receptors for 1,25-dihydroxyvitamin D in cells cultured from bone. J. Clin. Endocrinol. Metab. 57:958-962.
- 28. Tanaka, Y., and H. F. DeLuca. 1974. Stimulation of 24,25-dihydroxyvitamin D_3 production by 1,25-dihydroxyvitamin D_3 . Science (Wash. DC). 183:1198–1200.
- 29. Colston, K. W., I. M. Evans, T. C. Spelsberg, and I. MacIntyre. 1977. Feedback regulation of vitamin D metabolism by 1,25-dihydroxycholecalciferol. *Biochem. J.* 164:83-89.
- 30. Spanos, E., D. I. Barrett, K. T. Chang, and I. MacIntyre. 1978. Effect of oestrogen and 1,25-dihydroxycholecalciferol on 25-hydroxy-

- cholecalciferol metabolism in primary chick kidney-cell cultures. *Biochem. J.* 174:231-236.
- 31. Henry, H. L. 1979. Regulation of the hydroxylation of 25-hydroxyvitamin D₃ in vivo and in primary cultures of chick kidney cells. *J. Biol. Chem.* 254:2722-2729.
- 32. Trechsel, U., J. P. Bonjour, and H. Fleisch. 1979. Regulation of the metabolism of 25-hydroxyvitamin D₃ in primary cultures of chick kidney cells. *J. Clin. Invest.* 64:206-217.
- 33. Omdahl, J. L., L. A. Hunsaker, A. P. Evan, and P. Torrez. 1980. In vitro regulation of kidney 25-hydroxyvitamin D₃-hydroxylase enzyme activities by vitamin D₃ metabolites: molecular specificity and mechanism of action. *J. Biol. Chem.* 255:7460-7466.
- 34. Colston, K., and D. Feldman. 1981. 1,25-dihydroxyvitamin D_3 receptors and functions in cultured pig kidney cells (LLC PK): regulation of 24,25-dihydroxyvitamin D_3 production. J. Biol. Chem. 257:2504–2508
- 35. Chandler, J. J., S. K. Chandler, J. W. Pike, and M. R. Haussler. 1984. 1,25-dihydroxyvitamin D₃ induces 25-hydroxyvitamin D₃-24-hydroxylase in a cultured monkey kidney cell line (LLC-MK₂) apparently deficient in the high affinity receptor for the hormone. *J. Biol. Chem.* 259:2214–2222.
- 36. Kumar, R., H. K. Schnoes, and H. F. DeLuca. 1978. Rat intestinal 25-hydroxyvitamin D_3 and 1α ,25-dihydroxyvitamin D_3 -24-hydroxylase. *J. Biol. Chem.* 253:3804–3809.
- 37. Turner, R. T., J. E. Puzas, M. D. Forte, G. E. Lester, T. K. Gray, G. A. Howard, and D. J. Baylink. 1980. In vitro synthesis of $1\alpha,25$ -dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol by isolated calvarial cells. *Proc. Natl. Acad. Sci. USA*. 77:5720–5724.
- 38. Howard, G. A., R. T. Turner, D. J. Sherrard, and D. J. Baylink. 1981. Human bone cells in culture metabolize 25-hydroxyvitamin D₃ to 1,25-dihydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃. J. Biol. Chem. 256:7738-7740.
- 39. Pike, J. W., S. Dokoh, M. R. Haussler, U. A. Liberman, S. J. Marx, and C. Eil. 1984. Vitamin D₃-resistant fibroblasts have immunoassayable 1,25-dihydroxyvitamin D₃ receptors. *Science (Wash. DC)*. 224:879–881.
- 40. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.
- 41. Knutson, J. C., and H. F. DeLuca. 1974. 25-hydroxyvitamin D₃-24-hydroxylase: subcellular location and properties. *Biochemistry*. 13:1543-1548.
- 42. Hirst, M., and D. Feldman. 1983. Regulation of 1,25(OH)₂ vitamin D₃ receptor content in cultured LLC-PK₁ kidney cells limits hormone responsiveness. *Biochem. Biophys. Res. Commun.* 116:121–127
- 43. Danan, J.-L, A.-C. Delorme, and H. Mathieu. 1982. Presence of 25-hydroxyvitamin D₃ and 1,25-dihydroxyvitamin D₃ 24-Hydroxylase in vitamin D target cells of rat yolk sac. *J. Biol. Chem.* 257:10715–10721.
- 44. Grody, W. W., W. T. Schrader, and B. W. O'Malley. 1982. Activation, transformation, and subunit structure of steroid hormone receptors. *Endocr. Rev.* 3:141-163.
- 45. Tietze, H.-U., A. Burgert, A. Schaff, and U. Hennes. 1981. Familial rickets with alopecia: inborn end organ unresponsiveness to 1,25(OH)₂D₃. Acta Endocrinol. (Supp. 240):35-36. (Abstr.)
- 46. Stumpf, W. E., M. Sar, F. A. Reid, Y. Tanaka, and H. F. DeLuca. 1979. Target cells for 1,25-dihydroxyvitamin D₃ in intestinal tract, stomach, kidney, skin, pituitary, and parathyroid. *Science (Wash. DC)*. 206:1188-1190.