Impaired Stimulation of 25-Hydroxyvitamin D-24-hydroxylase in Fibroblasts from a Patient with Vitamin D-dependent Rickets, Type II

A FORM OF RECEPTOR-POSITIVE RESISTANCE TO 1,25-DIHYDROXYVITAMIN D_3

JAMES E. GRIFFIN and JOSEPH E. ZERWEKH, *Department of Internal Medicine, University of Texas Southwestern Medical School, Dallas, Texas 75235*

ABSTRACT We describe studies of the molecular defect in 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] action in cultured skin fibroblasts from a patient previously reported to have vitamin D-dependent rickets, type II. Binding of [^{3}H]1,25-$(OH)_2D_3$ in fibroblast cytosol was normal with a B_{max} (amount of high affinity binding) of 26 fmol/mg protein and a half-maximal saturation of 0.2 nM. Nuclear binding of [^{3}H]1,25-$(OH)_2D_3$ following whole cell uptake was 1.5 fmol/μg DNA in patient fibroblasts compared with a range of 0.5–2.9 fmol/μg DNA in five control strains. The size of the [^{3}H]1,25-$(OH)_2D_3$-receptor complex on sucrose density gradients, 3.8 S, was the same as in normal cells. This patient, therefore, appeared to have a receptor-positive form of resistance to 1,25-$(OH)_2D_3$. To document resistance to 1,25-$(OH)_2D_3$ in the fibroblasts we developed a method for detection of 1,25-$(OH)_2D_3$ action in normal skin fibroblasts. Following treatment of normal cell monolayers with 1,25-$(OH)_2D_3$ there was more than a 20-fold increase of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) activity. Treatment of 10 control cell strains with 1,25-$(OH)_2D_3$ for 8 h increased the formation of 24,25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3 in cell sonicates from <0.02 to 0.11–0.27 pmol/min per mg protein. When cells from the patient with vitamin D-dependent rickets, type II were treated with 1,25-$(OH)_2D_3$ in a similar manner, maximal 24-hydroxylase activity was only 0.02 pmol/min per mg protein, less than a fifth the lower limit of normal. 24-Hydroxylase activity in fibroblasts from the parents of the patient increased normally following treatment with 1,25-$(OH)_2D_3$. We conclude that impaired induction of 24-hydroxylase in the presence of normal receptor binding is evidence for postreceptor resistance to the action of 1,25-$(OH)_2D_3$.

INTRODUCTION

Rickets due to resistance to the action of 1,25-dihydroxyvitamin D [1,25-$(OH)_2D$][1] has been described in several families (1–9). This condition has come to be known as vitamin D-dependent rickets, type II, a term that fails to convey the concept that this disorder is a form of steroid hormone resistance. The characteristic clinical features are defective bone mineralization, usually beginning as rickets in childhood, impaired intestinal calcium absorption, and increased plasma levels of 1,25-$(OH)_2D$. In contrast to patients with vitamin D-dependent rickets, type I, whose plasma levels of 1,25-$(OH)_2D$ are decreased and whose bone disease can be cured by physiological replacement doses of 1,25-$(OH)_2D_3$, these patients either respond only to pharmacological doses of the hormone (1–3, 6) or do not respond at all with the doses tested (7–9).

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; 24-hydroxylase, 25-hydroxyvitamin D-24-hydroxylase; 1,25-$(OH)_2D$, 1,25-dihydroxyvitamin D; TEDMK buffer; 10 mM Tris-Cl, 1.5 mM EDTA, 1 mM dithiothreitol, 10 mM sodium molybdate, 300 mM KCl, pH 7.4.

Studies of the molecular nature of the defects in this disorder were initially limited due to the difficulty of studying human intestinal mucosa, the primary site of hormone action. More recently, methods have been developed to measure 1,25-$(OH)_2D_3$ receptors in cytosol from cultured skin fibroblasts (10) and to assess retention of [^{3}H]1,25-$(OH)_2D_3$ in fibroblast nuclei following binding in dispersed whole cells (11). Eil et al. (12) initially used the latter method to evaluate two families with vitamin D-dependent rickets, type II, and reported an apparent lack of nuclear retention of the steroid. More recently, Liberman, Eil, and Marx (13) have presented evidence for genetic heterogeneity in fibroblasts from affected patients with some families having deficiency of cytosol receptor binding, others having impaired nuclear retention of the steroid, and at least one family having no identifiable abnormality.

In the present report a method of assessing 1,25-$(OH)_2D_3$ action in cultured skin fibroblasts is described. Normal skin fibroblasts respond to 1,25-$(OH)_2D_3$ treatment by enhancement of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase). Using this method the nature of the molecular defect in 1,25-$(OH)_2D_3$ action is evaluated in cultured skin fibroblasts from a previously reported patient with vitamin D-dependent rickets, type II (4), in whom 1,25-$(OH)_2D_3$ receptor binding is normal.

TABLE I
Identification of Cell Strains

	Cell strain	Diagnosis	Site of biopsy	Age
				yr
Controls with no bone disease	72	Normal	Arm	26
	160	Incomplete testicular feminization	Abdomen	11
	232	5α-Reductase deficiency	Abdomen	18
	233	Mixed gonadal dysgenesis	Abdomen	1
	374	Normal	Abdomen	1
	402	Normal	Thigh	
	446	5α-Reductase deficiency	Abdomen	
	466	Normal	Abdomen	5
	495	Normal	Breast	22
Control with osteomalacia	82	Vitamin D-dependent rickets, type I	Arm	24
Patient	460	Vitamin D-dependent rickets, type II	Arm	30
Parents	87	Father of patient	Arm	57
	98	Mother of patient	Arm	53

METHODS

Materials. Materials used for cell culture have been described previously (14), except that fetal and newborn calf sera were obtained from Gibco Laboratories (Grand Island, NY). Tissue culture dishes (15 cm in diameter; No. 3025) from Falcon Labware, Div. of Becton, Dickinson & Co. (Oxnard, CA) were used to grow cells for experiments. 1α,25-Dihydroxy [23,24(*n*)-^{3}H]vitamin D_3 91 Ci/mmol and 25-hydroxy [26(27)-*methyl*-^{3}H]vitamin D_3 20 Ci/mmol were from Amersham Corp. (Arlington Heights, IL). 1,25-Dihydroxy[26(27)-*methyl*-^{3}H]vitamin D_3 and 24,25-dihydroxy [26(27)-*methyl*-^{3}H]vitamin D_3 were prepared from 25-hydroxy[26(27)-methyl-^{3}H]vitamin D_3 and kidney homogenates from either vitamin D-deficient chicks [for the preparation of 1,25-$(OH)_2D_3$] or from vitamin D-treated chicks [for the preparation of 24,25-$(OH)_2D_3$] using the method of Haussler (15). [^{14}C]Albumin was prepared by acetylation of bovine serum albumin (BSA) (Armour Pharmaceuticals, Phoenix, AZ) with [^{14}C]acetic anhydride (New England Nuclear, Boston, MA) (14). 25-Hydroxyvitamin D_3 [25-$(OH)D_3$] and 1,25-$(OH)_2D_3$ were gifts of Upjohn Co. (Kalamazoo, MI). 24,25-$(OH)_2D_3$ was provided by M. Uskokovic of Hoffmann-La Roche, Inc. (Nutley, NJ). Hydroxylapatite was obtained from Bio-Rad Laboratories (Richmond, CA). Liquid scintillation counting cocktail (Budget-Solve) was from Research Products International Corp. (Mount Prospect, IL).

Cell culture. The fibroblast strains used in these experiments were established from explants of skin and are listed in Table I. Cell strain 460 is from a patient previously reported to have vitamin D-dependent rickets, type II (4). Cell strains 87 and 98 are from her father and mother, respectively. Cell strain 82 is from a patient with the tentative diagnosis of vitamin D-dependent rickets, type I, due to 1α-hydroxylase deficiency and was made available to us by Dr. N. A. Breslau, University of Texas Health Science Center, Dallas, TX. Fibroblast storage and maintenance of stock cultures have been described previously (14) except that newborn calf serum was substituted for fetal calf serum for routine maintenance of stock cultures. To grow fibroblasts for the various assays cells from stock flasks were dissociated with 0.05% trypsin-0.02% EDTA at 37°C for 3 min and seeded (day 0) at a density of ~500,000 cells/dish in 15-cm Falcon dishes containing 25 ml of medium with 10% newborn calf serum. On day 3, 15 ml of fresh medium with serum were added to each dish. On day 6 the medium was removed, each monolayer was rinsed once with 10 ml of phosphate-buffered saline (PBS), and 25 ml of fresh medium without serum were added to each dish. The fibroblasts were assayed on day 7.

Cytosol-binding assay. Cells were scraped from plastic dishes into cold Tris-saline (50 mM Tris-Cl, 150 mM NaCl, pH 7.4). The cells were pelleted by centrifugation at 800 *g*, rinsed twice in Tris-saline, and resuspended in 2–3 vol of TEDMK buffer (10 mM Tris-Cl, 1.5 mM EDTA, 1 mM dithiothreitol, 10 mM sodium molybdate, 300 mM KCl, pH 7.4). The cell suspension was subjected to sonic disruption in the cup horn attachment of a model W-185 sonifier cell disrupter (Heat Systems-Ultrasonics, Inc., Plainview, NY) using six 10-s bursts, and the sonicate was centrifuged at 100,000 *g* for 1 h at 0°C. The resultant high salt extract supernatant was saved and, though it probably contains receptors from both the cytosolic and nuclear compartment, will be referred to as cytosol. 200 μl of the cytosol were added to polypropylene microfuge tubes (capacity 1.5 ml) containing various concentrations of [^{3}H]1,25-$(OH)_2D_3$ with or without a 250-fold excess of nonradioactive 1,25-$(OH)_2D_3$.

The tubes were incubated for 4 h at 0°C, and bound and free steroid were separated by hydroxylapatite as described by Wecksler and Norman (16). In this procedure 0.4 ml of a 50% slurry of hydroxylapatite in 10 mM Tris-Cl 0.1 M KCl (pH 7.5) were added, the tubes were mixed, and the hydroxylapatite pelleted by centrifugation for 2–3 s in a microfuge. The hydroxylapatite pellets were washed three times with 10 mM Tris-Cl 0.5% Triton X-100 (pH 7.5), extracted twice with 0.9 ml ethanol, and counted in 10 ml of Budget-Solve. Protein was measured by the method of Lowry et al. (17) using BSA as standard. The amount of high affinity binding or B_{max} was estimated by linear regression of the plot of total binding using the higher steroid concentrations. Half-maximal saturation was estimated from this same plot as the concentration of steroid at which the binding was half-maximal.

Nuclear localization of [^{3}H]*1,25-(OH)$_2$D$_3$.* The nuclear binding of [^{3}H]1,25-$(OH)_2D_3$ was assessed by a modification of a method developed in this laboratory for the measurement of the intranuclear localization of the androgen receptor (18). Cells cultured as described above were harvested from the 15-cm Falcon dishes by addition of 4 ml of stock trypsin-EDTA solution diluted 1:1 with PBS followed by incubation at 37°C for 5 min. The dishes were removed from the incubator and tapped gently, and the cells were detached by gentle trituration with a Pasteur pipette. The cells were pelleted by centrifugation at 800 *g* and resuspended with mixing in cold PBS. Following repeat pelleting at 800 *g*, the cells were resuspended in bicarbonate-free Eagle's minimal essential medium buffered with Hepes instead of tricine at a concentration of $1–2 \times 10^6$ cells/ml. 5 ml of the cell suspension were incubated in 15-ml polypropylene tubes containing 1 nM [^{3}H]1,25-$(OH)_2D_3$ with or without 250 nM 1,25-$(OH)_2D_3$ at 37°C for 45 min in a shaking water bath. The tubes were mixed on a vortex apparatus every 10 min during the incubation. Following the incubation the cells were centrifuged at 800 *g* and rinsed by resuspension and centrifugation five times in 5 ml of 50 mM Tris-Cl (pH 7.4) containing 150 mM NaCl and 0.2% BSA, and twice with 5 ml of 150 mM NaCl. The final supernatant was aspirated, and the cell pellet was resuspended by mixing in 5 ml Tris-sucrose buffer (20 mM Tris-Cl, 0.32 M sucrose, and 1 mM $MgCl_2$, pH 7.5). The cell suspension was transferred to glass tubes, and the remainder of the nuclear binding procedure was as described (18). In brief, following incubation in hypotonic buffer (20 mM Tris-Cl, 0.5 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.5) for 15 min to allow cell swelling, the cells were disrupted by passage 10 times through a 25-gauge needle. The solution was made isotonic by the addition of hypertonic Tris-sucrose buffer (20 mM Tris-Cl, 2.1 M sucrose, 0.5 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.5), the nuclei were collected by centrifugation at 2,000 *g* for 20 min, and the nuclear pellet was resuspended in 5 ml Tris-sucrose buffer. Following repeat centrifugation, the nuclei were resuspended in 2.6 ml Tris-sucrose and layered over 2.6 ml of hypertonic Tris-sucrose buffer. The mixture was centrifuged at 100,000 *g* for 1 h, the resultant purified nuclear pellet was resuspended by passage through a 25-gauge needle, and aliquots for DNA quantitation and radioactivity measurement were taken. Specific nuclear binding was estimated by subtraction of the amount bound in the incubation containing added nonradioactive 1,25-$(OH)_2D_3$ from that bound in the incubation with radioactive hormone alone.

Density gradients. Cells cultured as described above were harvested from 15-cm Falcon dishes with trypsin-EDTA solution and incubated at a density of $1–2 \times 10^6$ cells/ml medium with 1 nM [^{3}H]1,25-$(OH)_2D_3$ with or without 250 nM 1,25-$(OH)_2D_3$ at 37°C for 45 min as described for the nuclear localization studies above. After rinsing, the cell pellets were resuspended in an equal volume of TEDMK buffer and subjected to sonic disruption as described for the cytosol-binding assay. The sonicate was centrifuged at 100,000 *g* for 1 h. 200 μl of the supernatant were layered on the top of 5.3 ml 5–20% sucrose gradients prepared in TEDMK buffer containing 10% glycerol. [^{14}C]Albumin was added to the top of the gradient as an internal marker. Density gradients were centrifuged in polyallomer tubes for 18 h in a SW 50.1 rotor at 50,000 rpm (250,000 *g*) at 0°C in an ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). 4-drop fractions were collected from the top of the tube using an ISCO gradient fractionater (model 640, ISCO, Inc., Lincoln, NE) and assayed for radioactivity in 10 ml of Budget-Solve. Sedimentation coefficients were estimated by the relative sedimentation of BSA taken as 4.6 S.

24-Hydroxylase assay. Cells were grown under standard conditions. At various times before harvesting on day 7 of culture, 25 μl of either ethanol alone or of ethanol containing various concentrations of 1,25-$(OH)_2D_3$ were added to 25 ml of medium without serum in the 15-cm Falcon dishes. At the indicated times thereafter the medium was removed from the dishes and discarded. The cell monolayers were rinsed with 10 ml of Tris-saline, harvested by scraping into Tris-saline, and collected by centrifugation at 800 *g* at 4°C. After two rinses with Tris-saline, the cells were resuspended in 50 mM Tris-citrate, pH 7.5, and the cell suspension was subjected to sonic disruption as described for the cytosol-binding assay above. 100 μl of the sonicate (1–2 mg protein/ml) was added to polypropylene tubes containing 100 μl of Tris-citrate buffer, 10 mM sodium succinate, 10 mM NADPH, and various concentrations of [^{3}H]25-$(OH)D_3$. The assay tubes were then incubated for various times at 25°C in a shaking water bath. The routine incubation time was 30 min. The assay was stopped by addition of 0.64 ml of methanol/chloroform (2:1, vol/vol), and 70 ng of nonradioactive 24,25-$(OH)_2D_3$ was added to each assay tube as an internal recovery standard for the subsequent extraction procedure.

The steroids were extracted in 1.5-ml polypropylene microfuge tubes by a modification of the procedure of Bligh and Dyer (19). Following centrifugation of the chloroform/methanol/buffer mixture for 5 min at maximum speed in the microfuge, the supernatant was transferred to another microfuge tube containing 0.2 ml chloroform and 0.1 ml water, and the pellet was discarded. The tube was shaken and then centrifuged for 15 s. The lower phase was removed and transferred to a clean 1.5-ml microfuge tube. The remaining upper phase was washed with 0.4 ml chloroform, and the lower phase from this mixture was collected and added to the first lower phase. The combined chloroform extracts were dried under a stream of nitrogen and the residue was dissolved in 0.1 ml toluene/ethanol (1:1, vol/vol) and stored at −20°C until analysis by high-performance liquid chromatography (HPLC).

Reversed-phase HPLC was performed on the extracts using a Beckman model 334 system equipped with an Altex Ultrasphere-ODS column (4.6 mm × 25 cm) (Altex Scientific, Inc., Berkeley, CA). The extracts were dried under a stream of nitrogen, dissolved in 25 μl of 85% methanol in water, and applied to the column. The flow rate was 1 ml/min using a program that increased the percent methanol from 85 to 100% over a 2-min period beginning at 7 min after injection of sample, maintained 100% methanol for 1 min, and returned to 85% methanol over an additional 2 min. 20-s fractions were collected into scintillation vials, and radioactivity was measured following addition of 10 ml

Budget-Solve. Recovery of standard [24,25-$(OH)_2D_3$] averaged ~70% and was used to correct the amount of radioactivity in the peak eluting in the region of 24,25-$(OH)_2D_3$ in each assay.

Identification of [^3H]24,25-$(OH)_2D_3$ by straight-phase HPLC and periodate oxidation. The fractions from the reversed-phase HPLC eluting in the region of authentic 24,25-$(OH)_2D_3$ were pooled and dried under a stream of nitrogen. The dried residue was reconstituted in 150 μl of 7% 2-propanol in hexane and applied to a HPLC system (Glenco Scientific, Inc., Houston, TX) equipped with a Supelcosil column (4.6 mm × 25 cm, Supelco Inc., Belleforte, PA). The flow rate was 1 ml/min using 7% 2-propanol in hexane as the mobile phase. 10-s fractions were collected beginning 2 min before the elution of standard 24,25-$(OH)_2D_3$ and continuing for 2 min following its elution. Utilizing this system 25-$(OH)D_3$ eluted between 3'15" and 3'45", and 24,25-$(OH)_2D_3$ eluted between 5'15" and 6'15". One aliquot of the eluate was assessed for radioactivity, and the remainder of the pooled samples from the peak was analyzed further. The putative [^3H]24,25-$(OH)_2D_3$ obtained after straight-phase HPLC was subjected to sodium periodate oxidation according to the procedure of Tanaka et al. (20). The extract was treated with a 5% aqueous solution of sodium metaperiodate and extracted with methanol/chloroform (2:1, vol/vol). The recovery of radioactivity in the organic extract was assessed and compared with authentic [^3H]24,25-$(OH)_2D_3$ and [^3H]1,25-$(OH)_2D_3$ treated in the same manner.

RESULTS

Incubation of skin fibroblast cytosols prepared from a normal control cell strain and from a strain derived from the patient with vitamin D-dependent rickets, type II, with increasing concentrations of [^3H]1,25-$(OH)_2D_3$ demonstrated high affinity saturable binding in both (Fig. 1). The amount and affinity of binding were similar to those previously reported for normal fibroblasts by this assay (10). The amount of high affinity cytosol [^3H]1,25-$(OH)_2D_3$ binding in the patient's cells was 26 fmol/mg protein, and the half-maximal saturation was ~0.2 nM. The nuclear binding of [^3H]1,25-$(OH)_2D_3$ was assessed following uptake of the hormone by suspended whole cells at 37°C at a saturating concentration of [^3H]1,25-$(OH)_2D_3$. Specific [^3H]1,25-$(OH)_2D_3$ binding to purified nuclei in five control cell strains ranged from 0.5 to 2.9 fmol/μg DNA. Specific purified nuclear [^3H]1,25-$(OH)_2D_3$ binding in the patient with vitamin D-dependent rickets, type II, averaged 1.5 fmol/μg DNA in three determinations. The sedimentation characteristics of the [^3H]1,25-$(OH)_2D_3$-receptor complex were assessed on sucrose density gradients (Fig. 2). Both control (Fig. 2 *A*) and patient (Fig. 2 *B*) fibroblasts had a peak of [^3H]1,25-$(OH)_2D_3$ binding that sedimented at 3.8 S.

The effect of pretreatment of intact monolayers with 1,25-$(OH)_2D_3$ on the metabolism of [^3H]25-$(OH)D_3$ was then evaluated. When a cell sonicate from a control strain not previously treated with 1,25-$(OH)D_3$ was

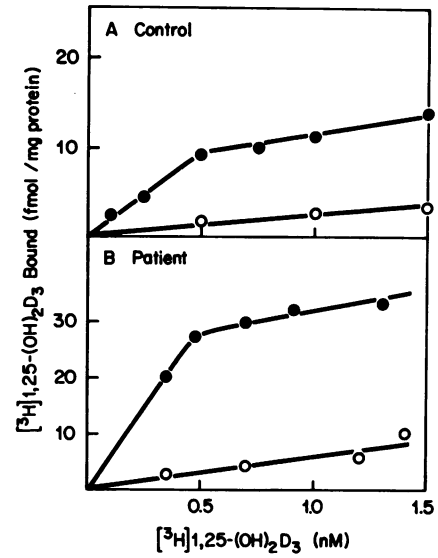

FIGURE 1 [^3H]1,25-$(OH)_2D_3$ binding in fibroblast cytosol. Fibroblasts from a normal subject (strain 402) (*A*) and from the patient with vitamin D-dependent rickets, type II (strain 460) (*B*) were grown under standard conditions. The cells were harvested by scraping and cytosol prepared as described. 200-μl aliquots of cytosol were incubated with various concentrations of [^3H]1,25-$(OH)_2D_3$ in the presence or absence of a 250-fold excess of nonradioactive 1,25-$(OH)_2D_3$ for 4 h at 0°C. Bound and free steroid was separated by hydroxylapatite. Protein concentration of each cytosol was 2 mg/ml. (●): total binding; (○): nonspecific binding.

incubated for 30 min with 0.05 μM [^3H]25-$(OH)D_3$ and the metabolism of the substrate was assessed by reversed-phase HPLC of the extract, little or no evidence of metabolism of [^3H]25-$(OH)D_3$ was found (Fig. 3 *A*). However, when parallel dishes of the same control strain were treated with 1 nM 1,25-$(OH)_2D_3$ for 8 h before harvesting the cells, several [^3H]25-$(OH)D_3$ metabolites were detected after incubation of the cell sonicate with [^3H]25-$(OH)D_3$ (Fig. 3 *B*). The major metabolite peak coeluted with authentic 24,25-$(OH)_2D_3$.

When the fractions in the region of the peak coeluting with 24,25-$(OH)_2D_3$ were pooled and rechromatographed on straight-phase HPLC as described in the Methods, 87% of the radioactivity from the reversed-phase HPLC peak coeluted with authentic 24,25-$(OH)_2D_3$. Periodate oxidation of the putative [^3H]24,25-$(OH)_2D_3$ peak that eluted from the straight-phase column was then compared with oxidation of authentic [^3H]1,25-$(OH)_2D_3$ and authentic [^3H]24,25-$(OH)_2D_3$ (Table II). Whereas there was no loss of radioactivity following periodate oxidation of authentic [^3H]1,25-$(OH)_2D_3$, both the authentic and putative [^3H]24,25-$(OH)_2D_3$ had >95% loss of radioactivity. We concluded that the major radioactive metabolite was

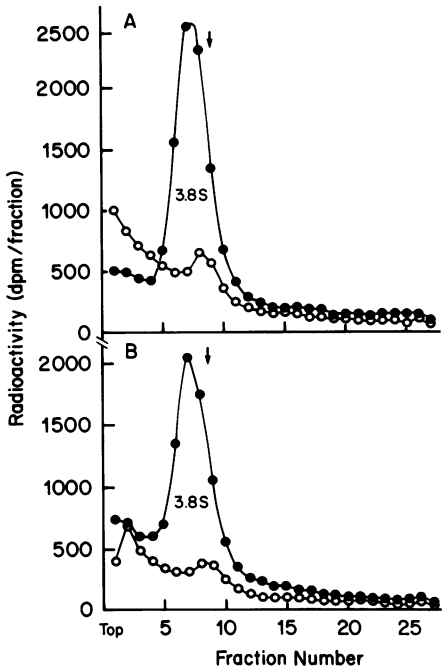

FIGURE 2 Analysis of [^{3}H]1,25-$(OH)_2D_3$ binding on sucrose density gradients. Fibroblasts from a control subject (strain 466) (*A*) and from the patient with vitamin D-dependent rickets, type II (strain 460) (*B*) were grown under standard conditions. The cells were harvested with trypsin-EDTA and incubated with 1 nM [^{3}H]1,25-$(OH)_2D_3$ with or without 250 nM 1,25-$(OH)_2D_3$ at 37°C for 45 min (Methods). Following rinsing, the cells were sonicated in TEDMK buffer, cytosol was prepared, and 200 μl were layered on 5–20% sucrose gradients in TEDMK buffer. The gradients were centrifuged and fractionated as described in the Methods. The vertical arrow indicates the position of the [^{14}C]albumin marker. The protein concentrations in the control cytosols (*A*) were 4.3 and 5.3 mg/ml for the incubations in the absence and presence of added nonradioactive 1,25-$(Oh)_2D_3$, respectively. The protein concentrations in the patient cytosols (*B*) were 3.6 and 5.3 mg/ml for the incubations in the absence and presence of added nonradioactive 1,25-$(OH)_2D_3$, respectively. (●); 1 nM [^{3}H]1,25-$(OH)_2D_3$; (○); 1 nM [^{3}H]1,25-$(OH)_2D_3$ and 250 nM 1,25-$(OH)_2D_3$.

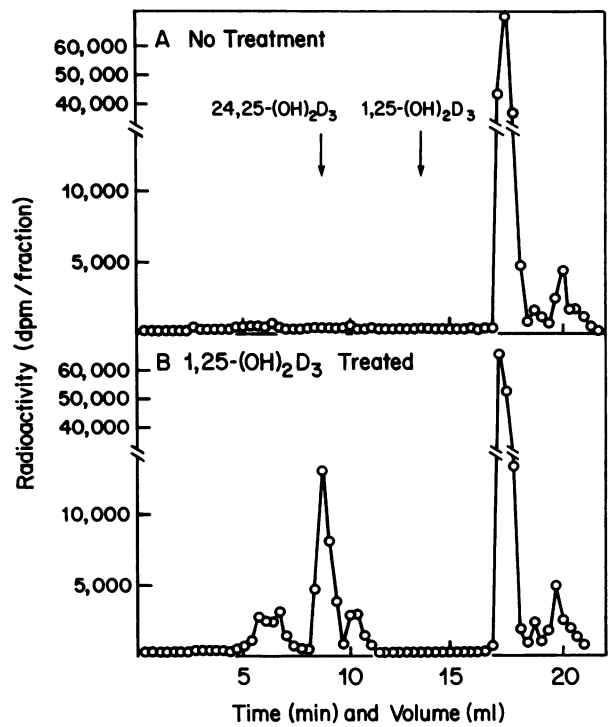

FIGURE 3 Metabolism of [^{3}H]25-$(OH)D_3$ by control fibroblast sonicates as assessed by reversed-phase HPLC. Fibroblasts from a control subject (strain 446) were grown under standard conditions. 8 h before harvesting, one 15-cm dish was treated with 25 μl ethanol (*A*) or 25 μl ethanol containing 1 nM 1,25-$(OH)_2D_3$ (*B*). Cells were harvested by scraping, rinsed with Tris-saline, and sonicated in 50 mM Tris-citrate, pH 7.5. 100 μl of each sonicate containing 0.22 mg protein (*A*) and 0.18 mg protein (*B*) were incubated with 100 μl of buffer and cofactors (Methods) and 0.05 μM [^{3}H]25-$(OH)D_3$ for 30 min at 25°C. The reaction was stopped by the addition of methanol/chloroform and 70 ng 24,25-$(OH)_2D_3$ was added as recovery standard. The lipid extract was redissolved in 85% methanol in water and subjected to reversed-phase HPLC as described. The arrows indicate the elution positions of authentic 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$. The recovery percentages of the 24,25-$(OH)_2D_3$ internal standards were 43% (*A*) and 59% (*B*).

indeed 24,25-$(OH)_2D_3$ and that 1,25-$(OH)_2D_3$ treatment of control fibroblasts causes a marked increase in 24-hydroxylase activity. The small peak eluting immediately after 24,25-$(OH)_2D_3$ in Fig. 3 *B* is in the position of elution of 25,26-$(OH)_2D_3$. The minor peak(s) eluting before 24,25-$(OH)_2D_3$ have not been identified.

The effect of 1,25-$(OH)_2D_3$ treatment on the metabolism of [^{3}H]25-$(OH)D_3$ in fibroblasts from the patient with vitamin D-dependent rickets, type II, is shown in Fig. 4. Sonicates of untreated fibroblasts did not metabolize [^{3}H]25-$(OH)D_3$ significantly as analyzed on reversed-phase HPLC (Fig. 4 *A*). When parallel dishes were treated with 1 nM 1,25-$(OH)_2D_3$ 8 h before harvest, there was a small peak of radioactivity coeluting with authentic 24,25-$(OH)_2D_3$ (Fig. 4 *B*) that was <10% of the peak seen in control cells (Fig. 3 *B*). This peak was usually no more than twice the substrate blank. When analyzed further to confirm that this putative 24,25-$(OH)_2D_3$ peak was actually 24,25-$(OH)_2D_3$, the amount of radioactivity eluting with authentic 24,25-$(OH)_2D_3$ from the straight-phase column was <3% of the radioactivity of a simultaneously run control. This small amount of radioactivity was sensitive to periodate oxidation. Thus a portion of the radioactivity was in fact 24,25-$(OH)_2D_3$.

TABLE II
Periodate Oxidation of Vitamin D_3 Metabolites

Metabolite	Loss of ^3H
	%
Authentic [^{3}H]1,25-$(OH)_2D_3$	0
Authentic [^{3}H]24,25-$(OH)_2D_3$	98±2
Putative [^{3}H]24,25-$(OH)_2D_3$	96±3

Authentic vitamin D metabolites labeled with ^3H in positions 26 and 27 and putative [^{3}H]24,25-$(OH)_2D_3$ formed from [^{3}H]25-$(OH)D_3$ labeled in positions 26 and 27 were treated with sodium metaperiodate as described in the Methods. The loss of radioactivity as estimated by the amount recovered in an organic extract is given as a percentage of the starting radioactivity. Results are expressed as mean±SE of three determinations.

The conditions for assessing the 24-hydroxylase activity in fibroblasts were evaluated. The formation of 24,25-$(OH)_2D_3$ as a function of increasing concentration of [^{3}H]25-$(OH)D_3$ was assessed. The average calculated K_m for the substrate in two experiments was 0.04 μM. A standard concentration of 0.05 μM substrate was chosen because it was near saturation and similar to the normal plasma level of 25-$(OH)D_3$. The 24-hydroxylase assay was shown to be linear with time to 50 min (Fig. 5 *A*) and with amount of protein to 0.1 mg/assay (Fig. 5 *B*). A standard assay time of 30 min was chosen.

When control fibroblasts were incubated with increasing concentrations of 1,25-$(OH)_2D_3$ for 8 h before harvesting, the formation of 24,25-$(OH)_2D_3$ was increased by concentrations, of 1,25-$(OH)_2D_3$ as low as 0.02 nM (Fig. 6). Maximal induction occurred at ~0.1 nM 1,25-$(OH)_2D_3$. The effects of similar treatments on 24-hydroxylase activity in fibroblasts from the patient with vitamin D-dependent rickets, type II, are also shown in Fig. 6. At the lowest concentration of 1,25-$(OH)_2D_3$ studied a small increase in 24-hydroxylase could be detected. With increasing concentrations of 1,25-$(OH)_2D_3$ slightly more 24,25-$(OH)_2D_3$ formation was detected. However, even at 2.5 nM 1,25-$(OH)_2D_3$ (or 25 times the maximally effective dose in control cells) the rate of formation of 24,25-$(OH)_2D_3$ was only a small fraction of that seen in control cells (Fig. 6). In other studies not shown, doses of 1,25-$(OH)_2D_3$ as

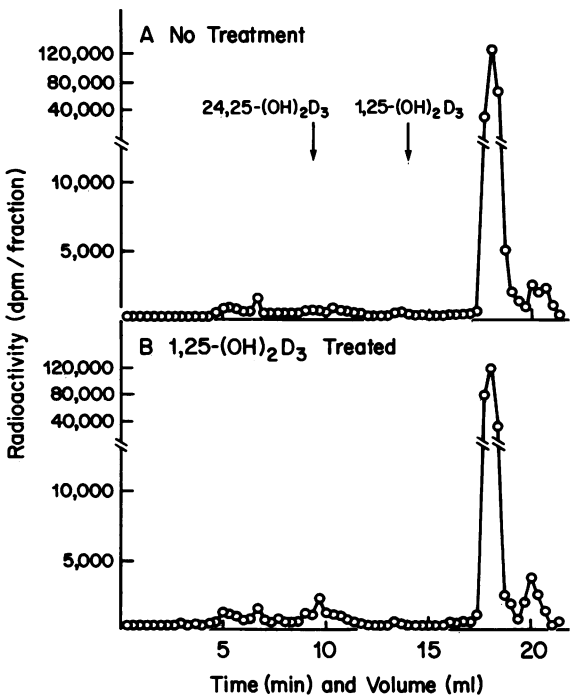

FIGURE 4 Metabolism of [^{3}H]25-$(OH)D_3$ by patient fibroblast sonicates as assessed by reversed-phase HPLC. Fibroblasts from the patient with vitamin D-dependent rickets, type II (strain 460) were grown under standard conditions. 8 h before harvesting one 15-cm dish was treated with 25 μl ethanol (*A*) or 25 μl ethanol containing 1 nM 1,25-$(OH)_2D_3$ (*B*). Cells were harvested, sonicated, and incubated as described in the legend to Fig. 3 and the extracts subjected to analysis by reversed-phase HPLC. The incubations contained 0.18 mg protein (*A*) and 0.15 mg protein (*B*). The recoveries of the 24,25-$(OH)_2D_3$ internal standard were 79% (*A*) and 87% (*B*).

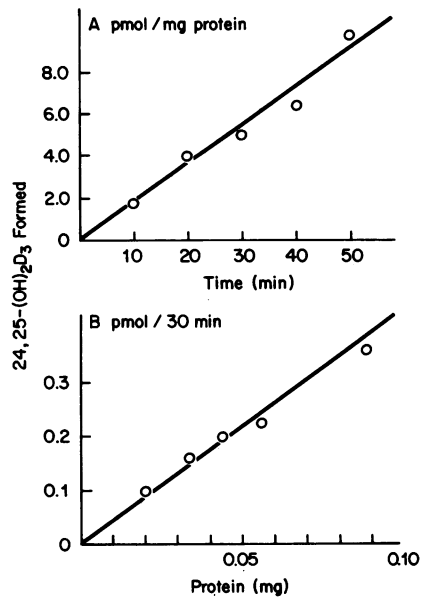

FIGURE 5 24-Hydroxylase activity in control skin fibroblast sonicates. Fibroblasts from a normal subject (strain 402) were grown under standard conditions and treated with 1 nM 1,25-$(OH)_2D_3$ 8 h before harvesting. Fibroblast sonicate was prepared and incubated for varying periods of time at various dilutions with [^{3}H]25-$(OH)D_3$ as described in the Methods. The formation of 24,25-$(OH)_2D_3$ was assessed by reversed-phase HPLC.

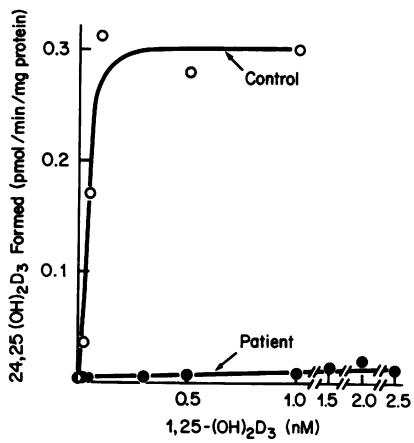

FIGURE 6 Induction of 24-hydroxylase activity in skin fibroblasts as a function 1,25-$(OH)_2D_3$ concentration. Fibroblasts from a normal subject (strain 402) and from the patient with vitamin D-dependent rickets, type II (strain 460) were grown under the standard conditions. 8 h before harvesting parallel dishes of cells were treated with either vehicle alone or various concentrations of 1,25-$(OH)_2D_3$. Cells were harvested, sonicated, and incubated with [^{3}H]25-$(OH)D_3$ as described. The formation of 24,25-$(OH)_2D_3$ was assessed by reversed-phase HPLC. (○): control fibroblasts; (●): patient fibroblasts.

high as 100 nM were ineffective in stimulating 24-hydroxylase activity.

The time courses of increase in 24-hydroxylase activity by 1,25-$(OH)_2D_3$ in control and patient fibroblasts are shown in Fig. 7. Following addition of 1 nM 1,25-$(OH)_2D_3$ to control cells, enhancement of 24-hydroxylase activity could be detected as early as 2 h and appeared to plateau between 6 and 12 h. The level of 24-hydroxylase activity in control cells declined somewhat by 16 h in this experiment and was even lower at 16 h in other experiments (results not shown). The increase of 24,25-$(OH)_2D_3$ in the patient's cells was maximal at 8 h and nearly undetectable at 16 h (Fig. 7). A standard time of 8 h treatment with 1,25-$(OH)_2D_3$ was chosen for comparing cell strains.

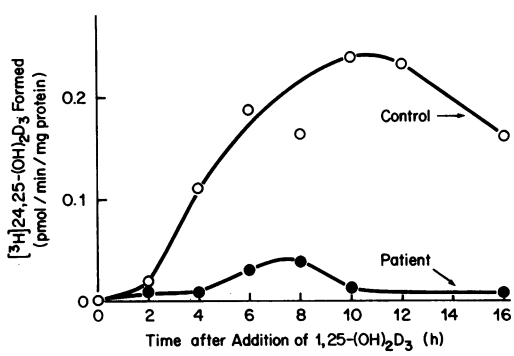

FIGURE 7 Time course of induction of 24-hydroxylase activity in skin fibroblasts by 1,25-$(OH)_2D_3$. Fibroblasts from a control subject (strain 446) and from the patient with vitamin D-dependent rickets type II (strain 460) were grown under the standard conditions. At various times before harvesting the monolayers were treated with 1 nM 1,25-$(OH)_2D_3$. The cells were harvested, sonicated, incubated with [^{3}H]25-$(OH)D_3$, and 24,25-$(OH)_2D_3$ formation was assessed as described. (○): control fibroblasts; (●): patient fibroblasts.

The effects of 1,25-$(OH)_2D_3$ on 24-hydroxylase activity in control fibroblast strains, and in fibroblasts from the patient with vitamin D-dependent rickets, type II, and from her parents are shown in Fig. 8. In all 13 strains 24-hydroxylase activity in the absence of pretreatment with 1,25-$(OH)_2D_3$ was <0.02 pmol/min per mg protein and similar to the substrate blank. Following treatment with increasing concentrations of 1,25-$(OH)_2D_3$, 24-hydroxylase activity was increased to maximal levels of from 0.11 to 0.27 pmol/min per mg protein in the 10 control fibroblast strains. The control strains included nine patients without bone disease and the patient with osteomalacia due to vitamin D-dependent rickets, type I, whose stimulated value was 0.27 pmol/min per mg protein. The concentration of 1,25-$(OH)_2D_3$ that resulted in half-maximal level of 24-hydroxylase averaged 0.06 nM in these 10 strains. The average maximal 24-hydroxylase activity in the patient with vitamin D-dependent rickets, type II, was 0.02 pmol/min per mg protein, a value about one-fifth the lower limit of the control group. The maximal level of 24-hydroxylase activity in the

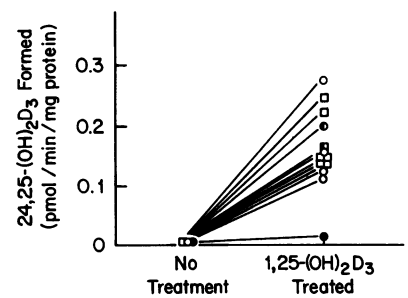

FIGURE 8 24-Hydroxylase activity in skin fibroblasts. Fibroblasts from 10 control subjects, the patient with vitamin D-dependent rickets, type II, and her parents were grown under the standard conditions. 8 h before harvesting parallel dishes of cells were treated with vehicle alone or increasing concentrations of 1,25-$(OH)_2D_3$ ranging from 0.02 to 1.0 nM. The fibroblasts were harvested, sonicated, incubated with [^{3}H]25-$(OH)D_3$, and the formation of 24,25-$(OH)_2D_3$ was assessed as described in the Methods. The average maximal 24,25-$(OH)_2D_3$ formation from two or more such experiments with each cell strain is plotted as the value for 1,25-$(OH)_2D_3$-treated. ○: control women; □: control men; ●: patient with vitamin D-dependent rickets, type II; ◐: the patient's mother; ◧: the patient's father.

cells from the parents of the patient was in the mid-normal range (Fig. 8).

DISCUSSION

Three general types of resistance to steroid hormones are now recognized: impaired formation of an active metabolite, abnormalities of the steroid receptor protein, and so-called receptor-positive resistance in which hormone metabolism and receptor binding are normal and the defect appears to reside at some subsequent phase of hormone action (21). Vitamin D-dependent rickets, type I, is an example of a disorder of impaired formation of an active metabolite, i.e., 1,25-$(OH)_2D_3$. Most patients with vitamin D-dependent rickets type II have been found to have an abnormality of the 1,25-$(OH)_2D_3$ receptor (9, 12, 13). However, at least one previously reported patient with the disorder was found to have normal receptor binding in cultured skin fibroblasts and, by definition, receptor-positive resistance.

Receptor-positive resistance has been the most difficult of these types of abnormalities to investigate at the molecular level for two reasons. On the one hand, hormone resistance in the intact subject could be due to transient or hormonal factors and thus not be expressed in vitro in cultured cells. On the other hand, what appears to be a normal receptor initially may in fact be a qualitatively abnormal receptor when examined more carefully and thus not actually an example of receptor-positive resistance. This has proven to be the case in many prior instances of glucocorticoid and androgen resistance.

The concept of a postreceptor defect in steroid hormone action was first proposed by studies of glucocorticoid-resistant lymphoma cells (22–25). Absent binding, decreased amount of receptor, and a qualitatively abnormal receptor are defects commonly seen in these glucocorticoid-resistant cells, while the inability to identify a receptor abnormality is rare (22–25). Amrhein et al. (26) were the first to report the presence of normal androgen receptor binding in a family with testicular feminization and to raise the possibility of a postreceptor defect in androgen action. However, when more sensitive techniques were developed to assess qualitative abnormalities of the androgen receptor, only a small fraction of the families with androgen resistance had no identifiable receptor abnormality (27). And, in fact, the first family reported to have receptor-positive androgen resistance (26) has recently been shown to have a qualitatively abnormal androgen receptor (28). Thus, defects in steroid hormone action at a postreceptor site are uncommon.

One of the limitations of prior evaluations of receptor-positive steroid hormone resistance in man has been the lack of a marker of hormone action in cultured cells. In the studies of fibroblasts from families with androgen resistance (27) and from the single family with glucocorticoid resistance (29), receptor binding is the only parameter of normal hormone action thus far available for assessment.

The present report describes studies in fibroblasts from a patient with vitamin D-dependent rickets, type II. Cytosol 1,25-$(OH)_2D_3$ receptor binding and nuclear uptake of 1,25-$(OH)_2D_3$ in fibroblasts cultured from skin were normal. Thus, despite in vivo evidence for resistance to the action of the hormone, the in vitro assessment of the 1,25-$(OH)_2D_3$ receptor function seemed to be normal in this patient. To document that the defect in 1,25-$(OH)_2D_3$ action in this patient was due to an abnormality at some postreceptor level we utilized 25-hydroxyvitamin D_3-24-hydroxylase activity as a marker for 1,25-$(OH)_2D_3$ action in cultured skin fibroblasts. The induction of this enzyme has recently been reported to be deficient in fibroblasts from a patient with receptor-negative vitamin D-dependent rickets, type II (9). Compared with control fibroblasts the increase in 24-hydroxylase activity following treatment with 1,25-$(OH)_2D_3$ in our patient's cells was markedly diminished. Thus, this is the first instance in which a marker for a steroid hormone action in normal cultured fibroblasts has been identified and used to evaluate the overall pathway of hormone action in cells from a patient with hormone resistance in which hormone binding is normal. We cannot exclude the possibility that the abnormality in this patient involves the receptor protein (for example, the binding of the hormone-receptor complex to receptor sites in the chromatin), but, regardless, the availability of such a mutation should make it possible to design experiments to investigate the events in 1,25-$(OH)_2D_3$ action between receptor binding and ultimate nuclear action. Indeed, the use of 24-hydroxylase as a marker of 1,25-$(OH)_2D_3$ action might be the most reliable means of documenting resistance to the hormone.

The utilization of 24-hydroxylase as a marker for 1,25-$(OH)_2D_3$ action has a solid rationale. In addition to the actions of 1,25-$(OH)_2D_3$ to stimulate intestinal absorption of calcium and mobilization of calcium from bone, 1,25-$(OH)_2D_3$ regulates the metabolism of 25-$(OH)D_3$. Vitamin D-deficient animals metabolize 25-$(OH)D_3$ primarily by 1α-hydroxylation to form 1,25-$(OH)_2D_3$. Treatment with 1,25-$(OH)_2D_3$ inhibits 1α-hydroxylation and induces the 25-hydroxyvitamin D_3-24-hydroxylase; this results in increased plasma levels of 24,25-$(OH)_2D_3$ (30). Although the primary site of induction of 24-hydroxylase activity is thought to be the kidney (31), recent studies in the rat suggest that extrarenal 24-hydroxylase activity accounts for a third of the 24,25-$(OH)_2D_3$ production, and that the

extrarenal 24-hydroxylase is also under regulatory control by 1,25-$(OH)_2D_3$ (32). The sites of extrarenal 24-hydroxylase activity are incompletely defined. Initial studies of 24-hydroxylase activity in cultured cells were limited to primary cultures of chick kidney cells (33). Primary cultures of human bone cells also have 24-hydroxylase activity that is responsive to 1,25-$(OH)_2D_3$ (34), and a number of established mammalian cell lines possess both receptors for 1,25-$(OH)_2D_3$ and the capacity to respond to the hormone by induction of 24-hydroxylase activity (35, 36). Because of these findings we assessed the effects of 1,25-$(OH)_2D_3$ on the 24-hydroxylase activity in normal and patient skin fibroblasts. Since the presence of a normal 1,25-$(OH)_2D_3$ receptor and presumably normal receptor function are required for induction of 24-hydroxylase (9, 36), we conclude that impaired stimulation of the enzyme in the presence of normal receptor binding is evidence for postreceptor resistance to the action of the hormone.

The relation of the impaired induction of 24-hydroxylase activity in fibroblasts to the in vivo formation of 24,25-$(OH)_2D_3$ in the patient is unclear. Although receiving treatment with 25-$(OH)D_3$ to raise the plasma level of 25-$(OH)D_3$ to five times normal, the patient's plasma level of 24,25-$(OH)_2D_3$ was in the normal range (unpublished observations). (No pretreatment level was obtained.) This "normal" value may be inappropriately low for an elevated plasma level of 1,25-$(OH)_2D_3$ and the increased level of the substrate (37). Alternatively the low 24-hydroxylase in the fibroblasts may not reflect the activity of the enzyme in the renal tubular epithelium, the primary site of formation of plasma 24,25-$(OH)_2D_3$, but rather may be more indicative of the enzyme in bone or other extrarenal tissues. There is precedent for this impaired induction of 24-hydroxylase in fibroblasts in spite of "normal" 24,25-$(OH)_2D$ levels in vivo in the previously mentioned patient with receptor-negative vitamin D-dependent rickets, type II (9). In this patient the 24,25-$(OH)_2D$ level was higher than the mean level in normal subjects (9).

The finding of impaired formation of 24,25-$(OH)_2D_3$ in cells from a patient with osteomalacia raises the question of the importance of this vitamin D metabolite in normal vitamin D action. Although some investigators have concluded that 24,25-$(OH)_2D_3$ does not have effects distinct from those of 25-$(OH)D_3$ (38), others have concluded that 24,25-$(OH)_2D_3$ plays an important role in mineralization of bone (39), in suppression of parathyroid hormone secretion (40), in skeletal maturation during embryogenesis (41, 42), and in ameliorating the effect of nephrectomy on bone resorption (43).

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REFERENCES

1. 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.
2. 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.
3. 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.
4. 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-dihydroxyvitamin D and apparent defective synthesis of 25-hydroxyvitamin D. *J. Clin. Endocrinol. Metab.* 49:171–175.
5. Adams, J. S., T. O. Wahl, W. V. Moore, W. A. Horton, and B. P. Lukert. 1979. Familial vitamin D-dependent rickets: further evidence for end-organ resistance to active vitamin D metabolites. Abstracts of the 61st Annual Meeting of the Endocrine Society. p. 264.
6. 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.
7. Liberman, U. A., R. Samuel, A. Halabe, and R. Kauli. 1980. End-organ resistance to 1,25-dihydroxycholecalciferol. *Lancet.* I:504–507.
8. Beer, S., M. Tieder, D. Kohelet, O. 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.
9. 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$(OH)_2D_3$. *J. Clin. Endocrinol. Metab.* 55:1020–1022.
10. Feldman, D., T. Chen, M. Hirst, K. Colston, M. Karasek, and C. Cone. 1980. Demonstration of 1,25-dihydroxyvitamin D_3 receptors in human skin biopsies. *J. Clin. Endocrinol. Metab.* 51:1463–1465.
11. Eil, C., and S. J. Marx. 1981. Nuclear uptake of 1,25-dihydroxy[^{3}H]cholecalciferol in dispersed fibroblasts

cultured from normal human skin. *Proc. Natl. Acad. Sci. USA.* 78:2562–2566.
12. 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.
13. 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.
14. Griffin, J. E., K. Punyashthiti, and J. D. Wilson. 1976. Dihydrotestosterone binding by cultured human fibroblasts: comparison of cells from control subjects and from patients with hereditary male pseudohermaphroditism due to androgen resistance. *J. Clin. Invest.* 57:1342–1351.
15. Haussler, M. R. 1972. Characterization of the metabolites of vitamin D_3 in the chick. *Steroids.* 20:639–650.
16. Wecksler, W. R., and A. W. Norman. 1979. A hydroxylapatite batch assay for the quantitation of 1α,25-dihydroxyvitamin D_3-receptor complexes. *Anal Biochem.* 92:314–323.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
18. Collier, M. E., J. E. Griffin, and J. D. Wilson. 1978. Intranuclear binding of [^{3}H]dihydrotestosterone by cultured human fibroblasts. *Endocrinology.* 103:1499–1505.
19. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.
20. Tanaka, Y., H. K. Schroes, C. M. Smith, and H. F. DeLuca. 1981. 1,25,26-Trihydroxyvitamin D_3: isolation, identification, and biological activity. *Arch. Biochem. Biophys.* 210:104–109.
21. Griffin, J. E., and J. D. Wilson. 1980. The syndromes of androgen resistance. *N. Engl. J. Med.* 302:198–209.
22. Yamamoto, K. R., U. Gehring, M. R. Stampfer, and C. H. Sibley. 1976. Genetic approaches to steroid hormone action. *Recent Prog. Horm. Res.* 32:3–32.
23. Bourgeois, S., and R. F. Newby. 1977. Diploid and haploid states of the glucocorticoid receptor gene of mouse lymphoid cell lines. *Cell.* 11:423–430.
24. Bourgeois, S., R. F. Newby, and M. Huet. 1978. Glucocorticoid resistance in murine lymphoma and thymoma lines. *Cancer Res.* 38:4279–4284.
25. Gehring, U. 1980. Cell genetics of glucocorticoid responsiveness. *In* Biochemical Actions of Hormones. G. Litwack, editor. Academic Press, Inc., New York. 205–232.
26. Amrhein, J. A., W. J. Meyer III, H. W. Jones, Jr., and C. J. Migeon. 1976. Androgen insensitivity in man: evidence for genetic heterogeneity. *Proc. Natl. Acad. Sci. USA.* 73:891–894.
27. Griffin, J. E., and J. L. Durrant. 1982. Qualitative receptor defects in families with androgen resistance: failure of stabilization of the fibroblast cytosol androgen receptor. *J. Clin. Endocrinol. Metab.* 55:465–474.
28. Brown, T. R., M. Maes, S. W. Rothwell, and C. J. Migeon. 1982. Human complete androgen insensitivity with normal dihydrotestosterone receptor binding capacity in cultured genital skin fibroblasts: evidence for a qualitative abnormality of the receptor. *J. Clin. Endocrinol. Metab.* 55:61–69.
29. Chrousos, F. P., A. Vingerhoeds, D. Brandon, C. Eil, M. Pugeat, M. DeVroede, D. L. Loriaux, and M. B. Lipsett. 1982. Primary cortisol resistance in man. A glucocorticoid receptor-mediated disease. *J. Clin. Invest.* 69:1261–1269.
30. Tanaka, Y., and H. F. DeLuca. 1974. Stimulation of 24,25-dihydroxy-vitamin D_3 production by 1,25-dihydroxyvitamin D_3. *Science (Wash. DC).* 183:1198–1200.
31. Tanaka, Y., R. S. Lorenc, and H. F. DeLuca. 1975. The role of 1,25-dihydroxyvitamin D_3 and parathyroid hormone in the regulation of chick renal 25-hydroxyvitamin D_3-24-hydroxylase. *Arch. Biochem. Biophys.* 171:521–526.
32. Turner, R. T., R. C. Avioli, and N. H. Bell. 1982. Extrarenal metabolism of 25-hydroxyvitamin D_3 in the rat is regulated by 1,25-dihydroxyvitamin D_3. The American Society for Bone and Mineral Reserach: Abstracts of the Fourth Annual Scientific Meeting. p. S47.
33. Henry, H. L. 1977. Metabolism of 25-hydroxyvitamin D_3 by primary cultures of chick kidney cells. *Biochem. Biophys. Res. Commun.* 74:768–775.
34. Howard, G. A., R. T. Turner, D. J. Sherrard, and D. J. Baylink. 1981. Human bone cells in culture metabolize 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3. *J. Biol. Chem.* 256:7738–7740.
35. Chandler, J. S., S. Dokoh, J. W. Pike, and M. R. Haussler. 1981. 1,25-Dihydroxyvitamin D_3-receptors and responsiveness in established cell lines from mammalian kidney, breast, and pituitary. *Calcif. Tissue Int.* 33:331*a*. (Abstr.)
36. Colston, K., and D. Feldman. 1981. 1,25-Dihydroxyvitamin D_3 receptors and functions in cultured pig kidney cells (LLC PK_1). *J. Biol. Chem.* 257:2504–2508.
37. Taylor, C. M., E. B. Mawer, J. E. Wallace, J. St. John, M. Cochran, R. G. G. Russell, and J. A. Kanis. 1978. The absence of 24,25-dihydroxy-cholecalciferol in anephric patients. *Clin. Sci. Mol. Med.* 55:541–547.
38. Tanaka, Y., H. F. DeLuca, Y. Kobayashi, T. Taguchi, N. Ikekawa, and M. Morisaki. 1979. Biological activity of 24,25-difluoro-25-hydroxyvitamin D_3. *J. Biol. Chem.* 254:7163–7167.
39. Ornoy, A., D. Goodwin, D. Noff, and S. Edelsein. 1978. 24,25-Dihydroxyvitamin D is a metabolite of vitamin D essential for bone formation. *Nature (Lond.).* 276:517–519.
40. Henry, H. L., A. N. Taylor, and A. W. Norman. 1977. Response of chick parathyroid glands to the vitamin D metabolites, 1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol. *J. Nutr.* 107:1918–1926.
41. Henry, H. L., and A. W. Norman. 1978. Vitamin D: two dihydroxylated metabolites are required for normal chicken egg hatchability. *Science (Wash. DC).* 201:835–837.
42. Endo, H., M. Kiyoki, K. Kawashima, T. Naruchi, and Y. Hashimoto. 1980. Vitamin D_3 metabolites and PTH synergistically stimulate bone formation of chick embryonic femur *in vitro*. *Nature (Lond.).* 286:262–264.
43. Pavlovitch, J. H., G. Cournot-Witmer, A. Bourdeau, S. Balsan, J. A. Fischer, and G. Heynen. 1981. Suppressive effects of 24,25-dihydroxycholecalciferol on bone resorption induced by acute bilateral nephrectomy in rats. *J. Clin. Invest.* 68:803–810.