Calvarial Cells Synthesize 1α,25-Dihydroxyvitamin D_3 from 25-Hydroxyvitamin D_3†

Russell T. Turner,* Guy A. Howard, J. Edward Puzas, David J. Baylink, and Daniel R. Knapp

ABSTRACT: A metabolite of vitamin D has been isolated in pure form from incubation of 25-hydroxyvitamin D_3 with embryonic chick calvarial cells that had been grown on Cytodex 1 microcarrier beads. The isolation involved dichloromethane extraction of the cells and incubation medium, followed by Sephadex LH-20 column chromatography and high-performance liquid chromatography of the extract. The metabolite was identified as 1α,25-dihydroxyvitamin D_3 by means of ultraviolet absorption spectroscopy, mass spectrometry, and sensitivity to oxidation by periodate. This metabolite was not produced by cell-free medium or by cells from embryonic chick liver, skin, or heart. In conclusion, (1) kidney cells are not unique in having 25-hydroxyvitamin D_3:1α-hydroxylase activity as previously believed and (2) vitamin D target tissues such as the skeleton may play a direct role in mediating the metabolism of 25-hydroxyvitamin D_3 to 1α,25-dihydroxyvitamin D_3, a vitamin D metabolite active at those sites.

The mineral homeostatic hormone 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$][1] affects bone cell proliferation and differentiation, bone matrix synthesis and mineralization, and bone resorption. Clearly, 1,25$(OH)_2D_3$ influences all aspects of bone formation and turnover and as a consequence each of the major functions of the skeleton: regulation of mineral homeostasis, acid–base balance, and mechanical support (Baylink et al., 1980). It is widely accepted that 1,25$(OH)_2D_3$ is produced only by the kidneys (Fraser & Kodicek, 1970; Gray et al., 1971). However, recent evidence suggests 25-hydroxyvitamin D_3:1-hydroxylase (1-hydroxylase) activity in cultured human bone biopsy cells (Howard et al., 1981), cultured human osteosarcoma cells (Howard et al., 1981), and cultured embryonic chick calvarial cells (Turner et al., 1980), as well as in intact human spongiosa bone samples obtained during orthopaedic surgery from 15 individual patients (Keck et al., 1981). If true, 1,25$(OH)_2D_3$ may be produced at the site of its action in the skeleton. Until now, the basis of identification of the 1,25$(OH)_2D_3$ produced by bone cells was limited to chromatographic properties and thus equivocal. In this report we present unambiguous evidence that embryonic chick calvarial cells produce 1,25$(OH)_2D_3$.

Experimental Procedures

Isolation of Calvarial Cells. Calvariae were dissected from 16-day-old chicken embryos. Cells from the calvariae were isolated as previously described (Turner et al., 1980). Briefly, the frontal and parietal bones and intervening suture line were aseptically dissected, rinsed, and incubated for up to 120 min in Tris-HCl–NaCl, pH 7.4 (five calvaria/mL), containing collagenase (2 mg/mL; Worthington, Type I). The cells were recovered as described (Puzas et al., 1979), washed twice in enzyme-free Hank's balanced salt solution (pH 7.4), and diluted in BGJ_b medium (Fitton-Jackson modification, Gibco) (Biggers et al., 1961) containing 100 units of penicillin and 100 μg of streptomycin per mL. Cell number was determined by counting with a hemacytometer. A total of approximately 10^8 cells were harvested from 2-dozen embryos, not including any red blood cells in the count.

Culture Conditions. Calvarial cells were suspended in serum-free BGJ_b medium and allowed to attach to Cytodex 1 microcarrier beads (Pharmacia), in 500-mL spinner–culture flasks (2.0 g of beads in 400 mL of medium) (Levine et al., 1977). The cells were incubated at 37 °C in 5% CO_2–95% air and stirred at 70 rpm. Cell number was determined by counting cell nuclei in a hemacytometer after incubation (1 h, 37 °C) of the cells in an aqueous solutions of 0.1 M citrate and 0.1% crystal violet. The cells increased in number to 2.0 × 10^6 cells/mL in 5–7 days.

Metabolism of 25(OH)D_3. 25-Hydroxyvitamin D_3 [25(OH)D_3] (100 nM) was incubated with the cultured cells for 4 h (with ethanol at a final concentration of 0.1% as the carrier). To aid in separation of the 1,25$(OH)_2D_3$ produced by calvarial cells 0.1 nM [^{3}H]25(OH)D_3 (25-hydroxy[26(27)-*methyl*-^{3}H]cholecalciferol; 23 Ci/mmol; Amersham) was added to the incubation as a tracer. Dichloromethane extracts of the cultured cells and medum were evaporated to dryness under a gentle stream of N_2, resuspended in a small volume of hexane–chloroform–methanol (9:1:1), and chromatographed on a Sephadex LH-20 column (1 × 56 cm, 15 g of Sephadex LH-20; Pharmacia) previously equilibrated with the same solvent system. Authentic [^{3}H]25(OH)D_3 and [^{3}H]1,25-$(OH)_2D_3$ were used to calibrate the column. The 1,25$(OH)_2D_3$ region (180–212 mL of eluant) was collected, evaporated to dryness under N_2, and resuspended in a small volume of hexane–2-propanol (90:10).

High-Performance Liquid Chromatography of Putative 1,25(OH)$_2D_3$. The material in the 1,25$(OH)_2D_3$ region from the Sephadex LH-20 column chromatography was then chromatographed on a high-performance liquid chromatography (HPLC) system (Waters Associates) by using a hexane–isopropyl alcohol (90:10) solvent system at a flow rate of 1 mL/min. The column (μPorasil, Waters Associates) was precalibrated with synthetic 1,25$(OH)_2D_3$. The material

† From the Veterans Administration Medical Center, Charleston, South Carolina 29402 (R.T.), the Department of Pharmacology, Medical University of South Carolina, Charleston, South Carolina 29425 (R.T.T. and D.R.K.), the Veterans Administration Medical Center, Tacoma, Washington 98493 (G.A.H.), the Department of Orthopaedics, University of Rochester School of Medicine, Rochester, New York 14642 (J.E.P.), and the Veterans Administration Hospital, Loma Linda, California 92357 (D.J.B.). *Received March 22, 1982; revised manuscript received August 10, 1982.* This work was supported by the Veterans Administration and by National Institutes of Health Grant DE 02600.

[1] Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D_3; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1-hydroxylase, 25-hydroxyvitamin D_3:1α-hydroxylase.

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eluting in the $1,25(OH)_2D_3$ region (25–35 mL) was collected, evaporated to dryness with N_2, resuspended in hexane–isopropyl alcohol (87:13), and rechromatographed by HPLC on recycling mode at a flow rate of 1.5 mL/min. After three cycles, the $1,25(OH)_2D_3$ region (80–87 mL) was collected, dried under N_2, resuspended in hexane–isopropyl alcohol (87:13), and rechromatographed on HPLC at a flow rate of 1.0 mL/min. The purified putative $1,25(OH)_2D_3$ eluting at 25–26.5 mL was collected.

Ultraviolet Absorption Spectrometry. Ultraviolet (UV) spectroscopy of the purified putative $1,25(OH)_2D_3$ and synthetic $1,25(OH)_2D_3$ was recorded from ethanol solutions by using a Beckman recording photometer.

Mass Spectrometry. A 1-μg sample of purified putative $1,25(OH)_2D_3$ was subjected to mass spectrometry on a Finnigan MAT 212 mass spectrometer by direct probe inlet. Electron impact spectra were collected repetitively at 70-eV ionizing energy while programming the probe temperature from ambient to ca. 280 °C. The total ion current reached a peak at ca. 200 °C probe temperature.

Periodate Oxidation. A total of 0.1 μg of the putative $1,25(OH)_2D_3$ was dissolved in 30 μL of methanol and treated with 20 μL of a 5% aqueous solution of sodium metaperiodate. After 16 h in the dark at ambient temperature the putative $1,25(OH)_2D_3$ was extracted from the periodate solution 3 times with chloroform. Synthetic $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ standards were treated in the same manner. The chloroform extracts were evaporated to dryness with N_2 and chromatographed on HPLC as previously described by using a hexane–isopropyl alcohol (87:13) solvent system. Under the above conditions periodate did not alter elution volume or peak size of the synthetic $1,25(OH)_2D_3$ but completely eradicated the synthetic $24,25(OH)_2D_3$ peak.

Results

We have demonstrated previously (Turner et al., 1980; Drivdahl et al., 1981) that the calvaria from 16–17-day-old chicks contain a high percentage of osteoblasts and their precursors, as indicated by the diffuse metachromatic staining, negative Golgi, and high alkaline phosphatase content [regarded as a marker in bone for osteoblasts and their precursors (McComb et al., 1979)] which are known to be characteristic of osteoblasts in vivo (Thompson et al., 1975; Farley et al., 1980). Moreover, the cultured cells which were alkaline phosphatase positive, as assessed by enzyme cytochemistry (Gruber et al., 1982), were enriched in cells with osteoblast characteristics by discarding the large number of cells removed during the first 20 min of collagenase digestion, since they were found to be red blood cells and superficial connective tissue cells (Drivdahl et al., 1981).

These embryonic chick calvarial cells readily attached to the microcarrier spheres and proliferated to a density that involved a confluent layer of cells on the surface of the spheres as determined by scanning electron microscopy (unpublished experiments). Cultured embryonic chick calvarial cells produced a metabolite of $25(OH)D_3$ that chromatographed on Sephadex LH-20 column chromatography (Figure 1) and straight-phase HPLC (Figures 2 and 3) at the same position as $1,25(OH)_2D_3$. We recovered 2 μg of this material; a value determined by comparing the UV absorption of the putative $1,25(OH)_2D_3$ at 254 nm with authentic standards. The UV absorption spectra of this compound is shown in Figure 4. The purified putative $1,25(OH)_2D_3$ had a UV absorption maximum at 265 nm and a minimum at 225 nm, and the spectrum was superimposable on the spectrum taken on synthetic $1,25(OH)_2D_3$. This spectrum is typical of the vitamin D_3 chro-

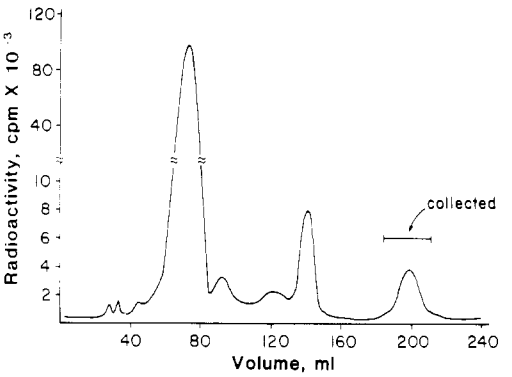

FIGURE 1: Separation of vitamin D metabolites by Sephadex LH-20 column chromatography. Primary cultures of chicken embryo calvarial cells (8×10^8) grown on microcarrier beads were incubated for 2 h with $[^3H]25(OH)D_3$ (100 nM) and extracted with dichloromethane. The extracted vitamin D metabolites were separated on Sephadex LH-20 (1 × 56 cm; 15 g) columns by elution with hexane–chloroform–methanol (9:1:1). Radioactivity is plotted as a function of elution volume. 2.5-mL fractions were collected. Radioactivity was measured in aliquots of each fraction by liquid scintillation counting. The material in the $1,25(OH)_2D_3$ region (185–212.5 mL) was saved for further purification.

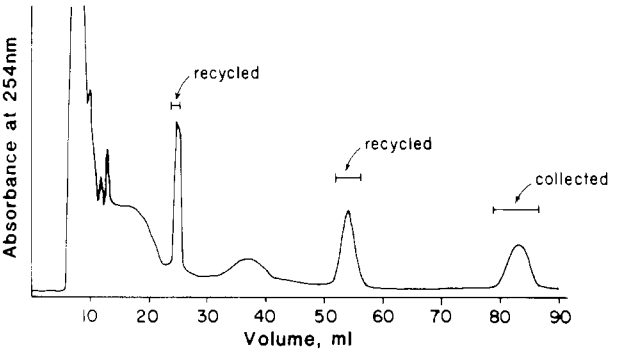

FIGURE 2: Separation of vitamin D metabolites by high-performance liquid chromatography (HPLC). The $1,25(OH)_2D_3$ region from the Sephadex LH-20 column shown in Figure 1 was dried under N_2, resuspended in hexane–isopropyl alcohol (90:10), and eluted by HPLC using the same solvent system (not shown). The $1,25(OH)_2D_3$ region was dried under N_2, resuspended in hexane–isopropyl alcohol (87:13), and eluted with the same solvent system on the recycling mode (shown) by using a μPorasil column. The $1,25(OH)_2D_3$ region was recycled twice and then collected (80–87 mL). UV absorption is recorded as a function of elution volume. The flow rate was 1.5 mL/min.

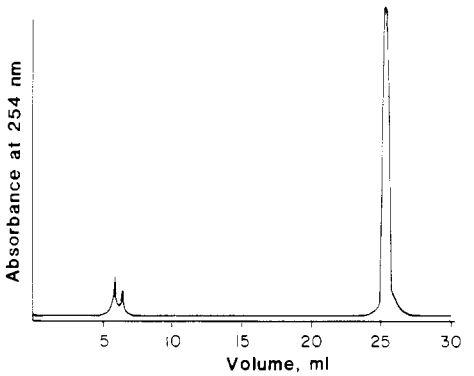

FIGURE 3: Final purification of putative $1,25(OH)_2D_3$ by HPLC. The $1,25(OH)_2D_3$ region following recycling on straight-phase HPLC (Figure 2) was evaporated under N_2, resuspended in hexane–isopropyl alcohol (87:13), and rechromatographed on HPLC with the same solvent. The purified putative $1,25(OH)_2D_3$ eluting at 25–26.5 mL was collected for structural determination. UV absorption at 254 nm is recorded as a function of elution volume. The flow rate was 1.0 mL/min.

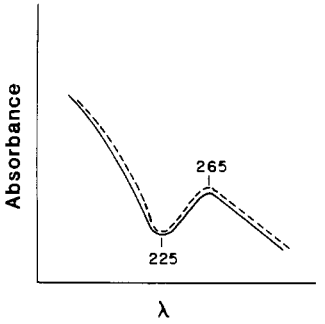

FIGURE 4: Ultraviolet (UV) absorption spectrum of purified putative $1,25(OH)_2D_3$. UV spectra for purified putative $1,25(OH)_2D_3$ is represented by (—) and synthetic $1,25(OH)_2D_3$ represented by (---). The spectra were taken in 100% alcohol.

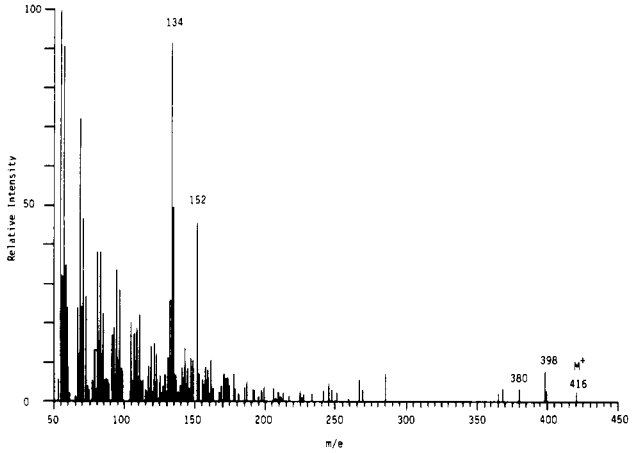

FIGURE 5: Mass spectrum of purified putative $1,25(OH)_2D_3$. The spectrum from 1 μg of purified putative $1,25(OH)_2D_3$ was obtained with a Finnigan MAT 212 mass spectrometer by direct probe inlet.

matophore and reflects an intact 5,6-cis triene system.

The mass spectrum for the putative $1,25(OH)_2D_3$ is shown in Figure 5. The mass spectrum was virtually identical with that of a reference standard. A molecular ion (M^+) of 416 was noted, indicating that an additional oxygen was added to $25(OH)D_3$. Fragments were noted at mass-to-charge ratios (m/z) of 398 ($M^+ - H_2O$), 380 ($M^+ - 2H_2O$), 152 (ring A + C6 and C7), and 134 (152 – H_2O; base peak). The fragments at m/z 134 and 152 correspond to the same fragments arising from $25(OH)D_3$ with an increase in 16, indicating that the added oxygen is on the A ring. The intensity of the 134 fragment compared to the 152 fragment was nearly identical with the $1,25(OH)_2D_3$ reference standard. This ratio is considered to be diagnostic of a $1\alpha,3\beta$-diol function (Paaren et al., 1977).

The position of the added oxygen function on the A ring was confirmed by HPLC of the product recovered following treatment of the putative $1,25(OH)_2D_3$ with periodate (Figure 6). Had the added oxygen function been inserted at positions 2 or 4, the A ring would have become sensitive to oxidation by periodate which would have been readily detected chromatographically. Since no change in retention volume occurred after treatment with periodate, the oxygen function must have been added at the 1 position.

The mass spectra, together with the UV absorption spectra and cochromatography with authentic $1,25(OH)_2D_3$ on HPLC before and after treatment with periodate, clearly demonstrate that embryonic chick calvarial cells produce $1,25(OH)_2D_3$ in culture. The production of $1,25(OH)_2D_3$ by cells in culture is tissue specific. No $1,25(OH)_2D_3$ was produced by incubation of $25(OH)D_3$ with medium alone or with cells isolated in the same manner from liver, heart, or skin.

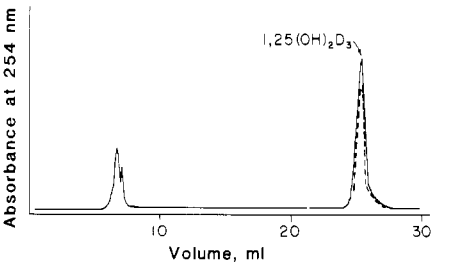

FIGURE 6: Sensitivity of purified putative $1,25(OH)_2D_3$ to oxidation by periodate. UV absorbed at 254 nm is recorded as a function of elution volume. The flow rate was 1.0 mL/min. Untreated purified putative $1,25(OH)_2D_3$ is represented by (—) and periodate-treated purified putative $1,25(OH)_2D_3$ by (---).

Discussion

In 1970 Fraser & Kodicek (1970) showed that anephric rats failed to produce the metabolite of vitamin D subsequently identified as $1,25(OH)_2D_3$. This result has been substantiated in several laboratories (Gray et al., 1971, Gray T. K., et al., 1979) and forms the basis of the long accepted belief that the kidney is the exclusive site of production of $1,25(OH)_2D_3$. This concept is further supported in humans by reports that anephric patients have nondetectable levels of the hormone. However, the human studies are subject to the criticism that the receptor assays (Shephard et al., 1979; Gray, R. W., et al., 1979; Taylor et al., 1979; Haussler et al., 1979; Bouillon et al., 1980) and radioimmunoassays (Clemens et al., 1979; Bouillon et al., 1980) used to measure $1,25(OH)_2D_3$ are relatively insensitive and do not detect decreased but potentially physiologically important hormone levels. In this regard, a recent study using a more sensitive receptor assay procedure (Jongen et al., 1981) and a study using both a receptor assay and a bioassay (Lambert et al., 1982) have detected $1,25(OH)_2D_3$ in serum from anephric patients. Also, high levels of $1,25(OH)_2D_3$ have been reported in an anephric patient with sarcoid (Barbour et al., 1981). Thus, the evidence in humans that the $1,25(OH)_2D_3$ in the peripheral circulation is exclusively of renal origin is not convincing. Furthermore, there is in vitro evidence that tissues in addition to the kidney produce $1,25(OH)_2D_3$. Weisman et al. (1979) reported evidence of 1-hydroxylase activity in homogenates of human decidua, Whitsett et al. (1981) reported that physiologically relevant quantities of $1,25(OH)_2D_3$ were synthesized by homogenates of villous tissue from term human placentas, and we (Howard et al., 1981) reported substantial 1-hydroxylase activity in cultured cells from human bone biopsies. Moreover, Keck et al. (1981) recently reported the formation of a metabolite with the chromatographic features of $1,25(OH)_2D_3$ by intact human bone samples from 15 individuals. Birds also appear to produce extrarenal $1,25(OH)_2D_3$. 1-Hydroxylase activity has been detected in chorioallantoic membrane cells (Puzas et al., 1980), the avian equivalent to the placenta, intestine (Puzas et al., 1983), and embryonic chick calvarial cells (Turner et al., 1980). Even in the rat, the kidney does not appear to be the exclusive site of production of $1,25(OH)_2D_3$ as it is clear that rat placenta homogenates produce $1,25(OH)_2D_3$ in vitro (Tanaka et al., 1979).

The evidence for 1-hydroxylase activity at sites other than rat kidney and rat placenta have been based mainly [$1,25(OH)_2D_3$ produced by human placenta was also identified by the chick cytosol binding assay (Whitsett et al., 1981)] on comigration of radioactive metabolites with authentic standards during chromatography. Thus (with the exception of the human placenta studies), there is some ambiguity as to the

identification of extrarenal metabolites, particularly in light of the previously unexpected large number of metabolites produced from vitamin D (Ribovich & DeLuca, 1978). It is therefore significant that in the present study the $1,25(OH)_2D_3$ produced by chick calvarial cells in conclusively identified.

The physiological significance of extrarenal production of $1,25(OH)_2D_3$ is not yet known, nor is there any information as to whether extrarenal 1-hydroxylases are regulated in vivo in a manner analogous to that of the kidney enzyme. It is of considerable interest that 1-hydroxylase activity is associated with cells and tissues, from embryonic animals as well as from adult humans, which are involved in bulk transport of calcium: kidney, intestine, chorioallantoic membrane, and skeleton. These tissues are also vitamin D targets. Although not shown specifically for bone in vivo, the in vitro evidence both from studies with cultured cells and from intact human bone biopsies would seem to support the postulate that at least some vitamin D target organs are capable of converting the major circulating metabolite of vitamin D, $25(OH)D_3$, to $1,25(OH)_2D_3$ at the site of the hormone's action.

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Registry No. 25-Hydroxyvitamin D_3, 19356-17-3; 1α,25-dihydroxyvitamin D_3, 32222-06-3; 25-hydroxyvitamin D_3:1α-hydroxylase, 9081-36-1.

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