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Neonatal Human Foreskin Keratinocytes Produce 1,25-Dihydroxyvitamin D_3†

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ABSTRACT: Primary cultures of neonatal human foreskin keratinocytes converted 25-hydroxyvitamin D in high yield to a metabolite with the chromatographic behavior of 1,25-dihydroxyvitamin D_3. The identity of this metabolite as 1,25-dihydroxyvitamin D_3 was confirmed both by its potency in displacing 1,25-dihydroxyvitamin D_3 in the chick cytosol receptor assay and by mass spectral analysis. These results suggest that 1,25-dihydroxyvitamin D_3 may be formed in the epidermis to regulate vitamin D production by the epidermis and to provide an alternative to 1,25-dihydroxyvitamin D_3 production by the kidneys.

Vitamin D_3 is produced in the epidermis from 7-dehydrocholesterol under the influence of ultraviolet irradiation (Holick et al., 1980). This process has been studied in vitro by using primary cultures of human foreskin keratinocytes (Nemanic et al., 1983b). Although the production of vitamin D_3 from 7-dehydrocholesterol is viewed as a sequence of photochemical and thermal reactions without hormonal regulation, some data suggest that 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$],[1] the most biologically active metabolite of vitamin D_3, regulates the amount of 7-dehydrocholesterol available for conversion to vitamin D_3 (Nemanic et al., 1983a; Esvelt et al., 1980). This possibility is supported by the presence of high-affinity receptors for 1,25$(OH)_2D_3$ in the skin (Stumpf et al., 1979; Feldman et al., 1980). Furthermore, 1,25$(OH)_2D_3$ has been shown to influence differentiation of the keratinocytes (Hosomi et al., 1983).

1,25$(OH)_2D_3$ is produced primarily if not exclusively in the kidney under normal physiologic circumstances (Fraser et al., 1970; Reeve et al., 1983; Shultz et al., 1983; Brumbaugh et al., 1974). However, some studies suggest that 1,25$(OH)_2D_3$ may also be produced by human bone cells in culture (Howard et al., 1981), in melanomas (Frankel et al., 1983), in sarcoid tissue (Barbour et al., 1981; Mason et al., 1984), and in placenta (Gray et al., 1979; Whitsett et al., 1981; Weisman et al. 1979). Furthermore, anephric humans (Lambert et al., 1982) and anephric pigs (Littledike et al., 1982) have been noted to have circulating levels of what appears to be 1,25$(OH)_2D_3$ which are most readily detected after vitamin D_3 or 25-hydroxyvitamin D_3 (25OHD_3) administration, although other recent studies (Manolagas et al., 1983; Reinhardt et al., 1984) have not detected 1,25$(OH)_2D_3$ in anephric humans. Therefore, the possibility that other tissues produce 1,25$(OH)_2D_3$ when production by the kidney is reduced needs further consideration. We tested the possibility that cells from the epidermis produce 1,25$(OH)_2D_3$—a mechanism that could provide a means to regulate vitamin D_3 production in the epidermis as well as an alternative source of 1,25$(OH)_2D_3$ production in patients with renal failure.

Experimental Procedures

Fresh human neonatal foreskin was obtained and keratinocytes (readily distinguished by morphologic criteria) were prepared by the method of Rheinwald and Green (1975) using a mitomycin-treated 3T3 cell feeder layer and Dulbecco's modified Eagle's medium (DME-H21) containing 20% fetal calf serum. The cells were studied after one or two passages. Just prior to use, contaminating cells were removed (>95%) with 0.1% ethylenediaminetetraacetate.

To assay for 25OHD_3 metabolism, the cells were grown to confluence in plates 3 cm in diameter. The growth medium was then replaced with 1 mL of serum-free medium for 36–40 h before [^{3}H]25OHD_3 was added to a final concentration of 7×10^{-10} M (0.1 μCi per well; specific activity, 148 Ci/mmol). After a defined period of incubation at 37 °C under 1 atm of 5% CO_2–air, the cells and medium were extracted by the

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[1] Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25OHD_3, 25-hydroxyvitamin D_3; DME-H21, Dulbecco's modified Eagle's medium.

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method of Bligh and Dyer (1959), and the chloroform extract was prepared for chromatography using a Waters high-performance liquid chromatography system containing a Dupont Zorbax Sil column (4.6 mm × 25 cm). A nonlinear gradient from 97:3 to 90:10 hexane–2-propanol at 2 mL/min was used to elute the metabolites. Output was monitored by UV absorbance and by radioactivity with appropriate detectors. Under these conditions, up to 44% of the substrate was converted to a metabolite eluting in the $1,25(OH)_2D_3$ position.

To produce adequate amounts of the presumptive $1,25(OH)_2D_3$ for structural identification, the keratinocytes were grown to confluence on twenty 10 cm diameter plates and then serum deprived for 36 h, as above. The medium was then supplemented with 1 mM isobutylmethylxanthine and 1 μg/mL bovine parathyroid hormone-1–34 [to maximize $1,25(OH)_2D_3$ production] for 4 h before [^{3}H]$25OHD_3$ was added to a final concentration of 500 × 10^{-9} M (specific activity, 2 Ci/mol). The incubation was continued for an additional 2 h; cells and medium were then extracted as above, and the chloroform extract was chromatographed on a 7.8 mm × 30 cm μPorasil column, eluted with 90:10 hexane–2-propanol. The presumptive $1,25(OH)_2D_3$ peak (the major metabolite produced under these conditions) was further purified by using three chromatographic systems. The reverse-phase system used a 3.0 mm × 30 cm μBondapak C_{18} column eluted with 75:25 MeOH–H_2O. The two straight-phase systems each used a 4.6 mm × 25 cm Zorbax Sil column eluted either with a 97:3 to 90:10 hexane–2-propanol nonlinear gradient or with a 96:4 dichloromethane–2-propanol isocratic system. Under these conditions, 5.6% of the substrate was converted to a metabolite eluting in the $1,25(OH)_2D_3$ position, and 70–80% of this metabolite was recovered from each of the chromatographic steps in the same position as authentic $1,25(OH)_2D_3$. With this method, nearly 2 nmol of purified material was available for structural identification.

The intestinal cytosol receptor assay for $1,25(OH)_2D_3$ described by Eisman et al. (1976) was used to determine the biologic potency of the presumptive $1,25(OH)_2D_3$ peak relative to authentic $1,25(OH)_2D_3$, which was obtained from M. R. Uskokovic (Hoffmann-La Roche, Nutley, NJ). Mass spectrometric determinations of the presumptive $1,25(OH)_2D_3$ peak were performed with a Kratos MS-12 mass spectrometer equipped with an Incos Data system using a direct insertion probe at temperatures 100–200 °C above ambient and a scan rate of 30 s/decade.

Results

Figure 1 illustrates a chromatogram of the metabolites produced during a 1-h incubation of keratinocytes with 7 × 10^{-10} M [^{3}H]$25OHD_3$ (bottom panel) compared with the chromatogram of known standards (top panel). The metabolite produced in the greatest amounts (24% of original substrate) is the one eluting in the same position as $1,25(OH)_2D_3$ (i.e., at 23 min in this system; this metabolite will be referred to as peak 23). The chromatographic elution pattern of the presumptive $1,25(OH)_2D_3$ in two additional chromatographic systems is shown in Figures 2 and 3. Figures 2A and 3A show the elution pattern of authentic $1,25(OH)_2D_3$; Figures 2B and 3B show the elution pattern of keratinocyte peak 23. The chromatographic system used to obtain the data in Figure 2 was a reverse-phase system (a μBondapak C_{18} column eluted with 75:25 MeOH–H_2O). The UV and radioactivity monitor recordings are shown. Peak 23 eluted in a position identical with that of the authentic $1,25(OH)_2D_3$ standard. In Figure 3, data are shown for a straight-phase system (a Zorbax Sil column eluted with 96:4 dichloromethane–2-propanol). Again,

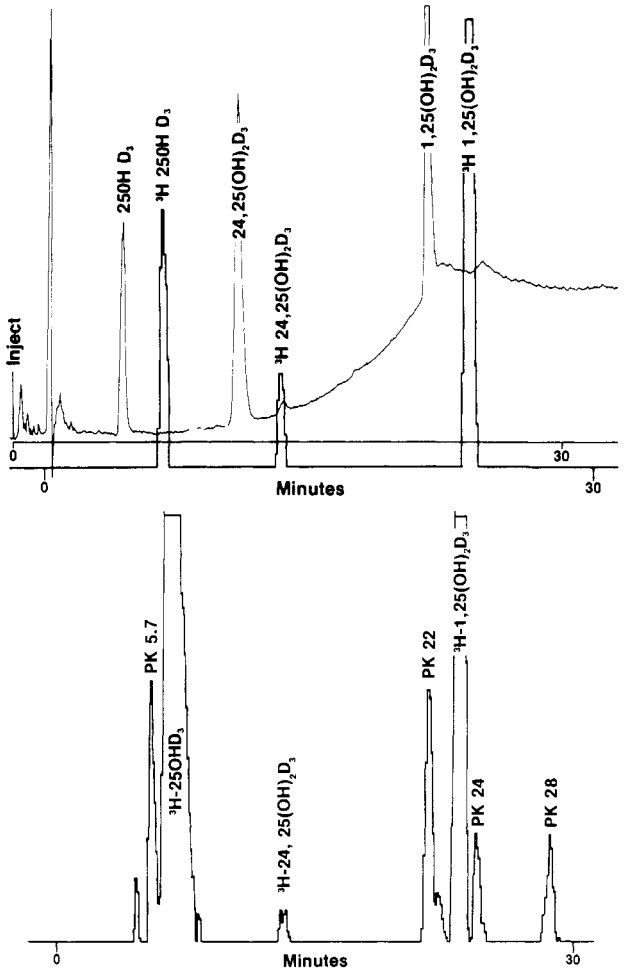

FIGURE 1: Representative chromatogram of the metabolites of $25OHD_3$ produced by keratinocytes. The chromatographic system used was a 4.6 mm × 25 cm Zorbax Sil column eluted with a 97:3 to 90:10 hexane–2-propanol nonlinear gradient at 2 mL/min with effluent monitored by UV absorbance at A_{254} (light line) and by a Radiomatics Flo One radioactivity monitor (dark line). The offset between UV absorbance and radioactivity peaks reflects the distance between pens on the recorder (note different "0" starting points), plus a small displacement due to the volume of tubing (1 mL) connecting the monitors. In the top panel, the elution patterns of radioactive and nonradioactive standards $25OHD_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$ are shown. In the bottom panel, the elution pattern of the chloroform extract from keratinocytes incubated for 1 h with 0.1 μCi of [^{3}H]-$25OHD_3$ (specific activity, 148 Ci/mmol) is shown. Only the tracing from the radioactivity monitor is depicted. The presumptive $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ peaks are indicated. The presumptive $1,25(OH)_2D_3$ peak is referred to as peak 23 throughout the text.

peak 23 elutes in the same position as authentic $1,25(OH)_2D_3$.

Peak 23 was then assayed for its ability to displace [^{3}H]-$1,25(OH)_2D_3$ from the chick intestinal cytosol receptor. The results are shown in Figure 4. On the basis of the specific activity of the $25OHD_3$ used as substrate to produce peak 23, this metabolite is as effective as authentic $1,25(OH)_2D_3$ in displacing tracer $1,25(OH)_2D_3$ from the receptor.

Mass spectrometric determinations of peak 23 (Figure 5B) and authentic $1,25(OH)_2D_3$ (Figure 5A) were then performed. The molecular ion is seen at m/z 416, and fragment ions are seen at m/z 398, 380, and 362 (indicating losses of one, two, and three molecules of water, respectively) and m/z 383, 365, and 347 (indicating the additional loss of CH_3). Other characteristic ions in the spectrum include m/z 287, 269, and 251 (indicating a loss of the eight-membered side chain and losses of one and two water molecules, respectively) and m/z 152 and 134 (indicating fission between C_7 and C_8 and the loss of one water molecule, respectively). The latter frag-

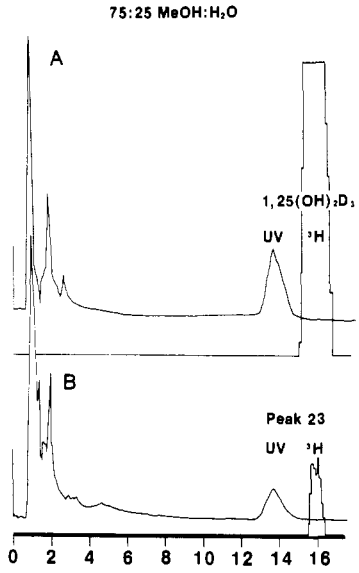

FIGURE 2: Elution pattern of radioactive and nonradioactive 1,25-$(OH)_2D_3$ standards (panel A) compared with that of purified peak 23 (panel B); a 3.9 mm × 30 cm μBondapak C_{18} column eluted with 75:25 MeOH–H_2O at 2 mL/min was used. The presumptive 1,25-$(OH)_2D_3$ was generated by keratinocytes incubated with 500×10^{-9} M [^{3}H]25OHD$_3$ (specific activity, 2 Ci/mol) and initially purified by using a 7.8 cm × 30 cm μPorasil column prior to this chromatographic analysis.

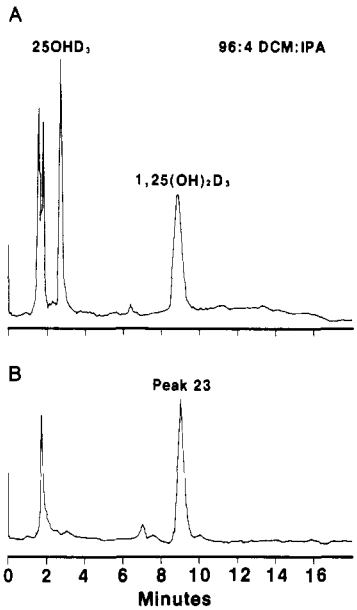

FIGURE 3: Elution pattern of 25OHD$_3$ and 1,25$(OH)_2D_3$ standards (panel A) compared with that of purified peak 23 [presumptive 1,25$(OH)_2D_3$] (panel B); a 4.6 mm × 25 cm Zorbax Sil column eluted with 96:4 dichloromethane–2-propanol at 2 mL/min was used. Only the UV monitor trace is shown since dichloromethane quenched the radioactivity below the limits of detection under these conditions. The presumptive 1,25$(OH)_2D_3$ was prepared as described in Figure 2 prior to this chromatographic analysis.

mentations are diagnostic of the intact nuclear triene system of vitamin D metabolites. The essential features of the mass spectrum of peak 23 are identical with those of the authentic 1,25$(OH)_2D_3$ standard that was analyzed at the same time and with those of the mass spectrum that Holick et al. (1971) used to originally identify the structure of 1,25$(OH)_2D_3$.

DISCUSSION

These results indicate for the first time that neonatal human foreskin keratinocytes produce a metabolite indistinguishable from authentic 1,25$(OH)_2D_3$. Fibroblasts isolated from the same tissue and assayed with [^{3}H]25OHD$_3$ at 148 Ci/mmol (to maximize conversion rates) do not produce this metabolite (data not shown) under conditions in which keratinocytes convert at least 20% of the substrate to 1,25$(OH)_2D_3$. Because these keratinocyte preparations are primary cultures of normal epidermal cells, one might assume that the epidermis produces 1,25$(OH)_2D_3$ in vivo; however, this has not yet been shown. Furthermore, we cannot dismiss the possibility that keratinocytes isolated from other sites or older donors would produce less or no 1,25$(OH)_2D_3$. Conceivably, 1,25$(OH)_2D_3$ pro-

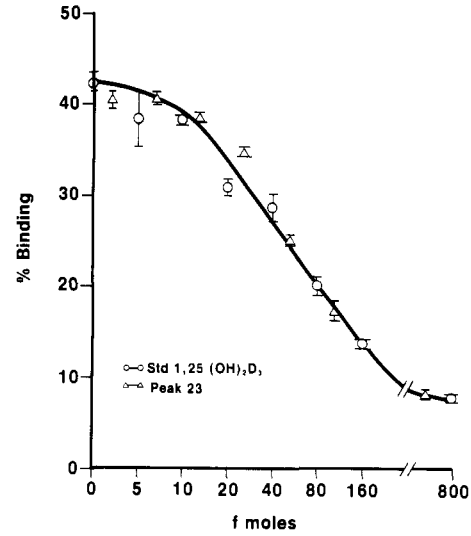

FIGURE 4: Comparison of the ability of peak 23 (▲) and authentic 1,25$(OH)_2D_3$ (○) to displace [^{3}H]1,25$(OH)_2D_3$ from the chick intestinal cytosol receptor. The amount of peak 23 that was added was determined by the specific activity of the initial substrate (25OHD$_3$) and the radioactivity in the final purified product (peak 23). Peak 23 and authentic 1,25$(OH)_2D_3$ have comparable affinity for the intestinal 1,25$(OH)_2D_3$ receptor. Error bars enclose mean and range of duplicate determinations.

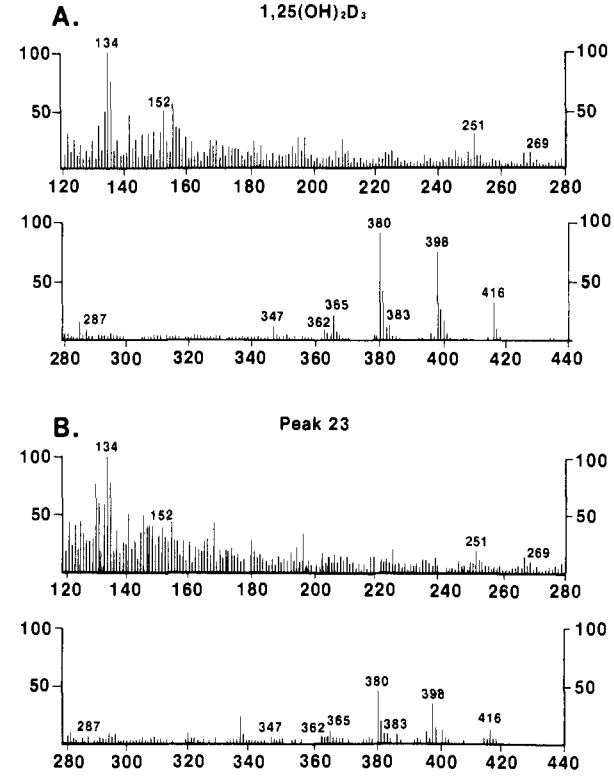

FIGURE 5: Mass spectrum determinations of authentic 1,25$(OH)_2D_3$ (panel A) and purified peak 23 (panel B). Fragmentations characteristic of 1,25$(OH)_2D_3$ are indicated and described in the text.

duction by the epidermis is suppressed in vivo, or if production does occur, the epidermis may produce only enough 1,25-$(OH)_2D_3$ for use in situ (for example, to regulate vitamin D_3 production or cellular differentiation). In either case, production of 1,25$(OH)_2D_3$ by the epidermis does not seem to play a major role in maintaining circulating levels of 1,25-$(OH)_2D_3$ under normal conditions, as evidenced by the observations that acutely nephrectomized rats fail to produce 1,25$(OH)_2D_3$ from 25OHD_3 (Reeve et al., 1983; Shultz et al., 1983) and anephric humans generally have undetectable levels of serum 1,25$(OH)_2D_3$ (Brumbaugh et al., 1974; Manolagas et al., 1983; Reinhardt et al., 1984). However, one can speculate that the epidermis may produce some or all of the 1,25$(OH)_2D_3$ found in anephric patients (Lambert et al., 1982) or anephric pigs (Littledike & Horst, 1982) given large amounts of vitamin D or 25OHD_3. This possibility has important therapeutic implications for patients whose renal production of 1,25$(OH)_2D_3$ is impaired.

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