UVB-Induced Conversion of 7-Dehydrocholesterol to 1α,25-Dihydroxyvitamin D_3 in an *In Vitro* Human Skin Equivalent Model

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We have previously shown that keratinocytes *in vitro* can convert biologically inactive vitamin D_3 to the hormone calcitriol (1α,25-dihydroxyvitamin D_3). This study was initiated to test whether the ultraviolet-B-induced photolysis of provitamin D_3 (7-dehydrocholesterol), which results in the formation of vitamin D_3, can generate calcitriol in an *in vivo*-like human skin equivalent model made of fibroblasts in a collagen matrix as the dermal component and keratinocytes as the epidermal component. Cultures were preincubated with increasing concentrations of 7-dehydrocholesterol (0.53–5.94 nmol per cm^2 human skin equivalent) at 37°C and irradiated with monochromatic ultraviolet B at wavelengths ranging from 285 to 315 nm (effective ultraviolet doses 7.5–45 mJ per cm^2). In our *in vitro* model irradiation with ultraviolet B resulted in a sequential metabolic process with generation of previtamin D_3 followed by the time-dependent formation of vitamin D_3, 25-hydroxyvitamin D_3, and ultimately calcitriol in the femtomolar range. Unirradiated cultures and irradiated cultures without keratinocytes generated no calcitriol. Irradiation of skin equivalents at wavelengths > 315 nm generated no or only trace amounts of calcitriol. The ultraviolet-B-triggered conversion of 7-dehydrocholesterol to calcitriol was strongly inhibited by ketoconazole indicating the involvement of P450 mixed function oxidases. The amount of calcitriol generated was dependent on the 7-dehydrocholesterol concentration, on wavelength, and on ultraviolet B dose. Hence, keratinocytes in the presence of physiologic concentrations of 7-dehydrocholesterol and irradiated with therapeutic doses of ultraviolet B may be a potential source of biologically active calcitriol within the epidermis. *Key words: keratinocyte/metabolism/vitamin D3. J Invest Dermatol 117:1179–1185, 2001*

Calcitriol (1α,25-dihydroxyvitamin D_3, 1α,25$(OH)_2$-D_3), the most potent biologically active form of vitamin D_3 (VD_3), is produced by a cascade of reactions including photochemical synthesis of VD_3 in the skin and after release into the circulation subsequent hydroxylation at the C25 atom in the liver and at the C-1α position in the kidney (Haussler, 1986). Calcitriol and other vitamin D analogs have antiproliferative and prodifferentiative effects on epidermal keratinocytes (Hosomi *et al*, 1983; Smith *et al*, 1986; McLane *et al*, 1990) and have become potent therapeutic agents for the treatment of proliferative skin disorders such as psoriasis. It has been shown that cultured keratinocytes can convert exogenous calcidiol (25-hydroxyvitamin D_3, 25OHD_3) to calcitriol (Bikle *et al*, 1986; Matsumoto *et al*, 1991; Lehmann, 1997) and we have previously demonstrated that keratinocytes can convert exogenous alphacalcidol (1α-hydroxyvitamin D_3, 1α-OHD_3) and VD_3 to calcitriol (Lehmann *et al*, 1998; 2000a) implicating functionally active 1α- and 25-hydroxylases present in keratinocytes. Provitamin D_3 (7-dehydrocholesterol, 7-DHC) exposed to ultraviolet B (UVB) radiation (spectral range 290–315 nm) converts *in vivo* (Holick *et al*, 1980) and *in vitro* (Nemanic *et al*, 1985) to previtamin D_3 (pre-VD_3), which in turn isomerizes to VD_3. Until now, the complete pathway from 7-DHC to 1α,25$(OH)_2D_3$ has only been shown in the transformed cell line HaCaT (Lehmann *et al*, 2000b) and not under *in vivo*-like conditions. Furthermore, the question of possible intermediate products on the pathway from VD_3 to 1α,25$(OH)_2D_3$ remains unanswered. It was the aim of this study to demonstrate that photolysis of 7-DHC results in the formation of 1α,25$(OH)_2D_3$ not only in HaCaT cells but also in an *in vivo*-like human skin equivalent (HSE) model. Our skin equivalent culture system consists of fibroblasts in a collagen matrix as the dermal component and keratinocytes as the epidermal component (Bell *et al*, 1981; Prunieras *et al*, 1983).

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Abbreviations: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α-OHD_3, 1α-hydroxyvitamin D_3; D_{eff}, effective ultraviolet B dose; 7-DHC, 7-dehydrocholesterol; E_e, irradiance; GC-MS, gas chromatography–mass spectrometry; HSE, human skin equivalent; 19-nor,10-keto-25OHD_3, 19-nor,10-keto-25-hydroxyvitamin D_3; pre-VD_3, previtamin D_3; 25OHD_3, 25-hydroxyvitamin D_3; VD_3, vitamin D_3; VDR, vitamin D receptor.

MATERIALS AND METHODS

Chemicals and reagents Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were provided from Gibco

(Eggenstein, Germany). Keratinocyte growth medium (KGM) and keratinocyte basal medium (KBM) were purchased from Clonetics (San Diego, CA). Culture dishes (→ 30 mm) were from Falcon (Heidelberg, Germany). 1α,25$(OH)_2D_3$ was kindly provided by Hoffmann-La Roche (Basel, Switzerland). 25-hydroxy[26,27-methyl-^{3}H]vitamin D_3 (^{3}H-25OHD_3, 177 Ci per mmol), 1α,25-dihydroxy[26,27-methyl-^{3}H]vitamin D_3 (^{3}H-1α,25$(OH)_2D_3$, 173.5 Ci per mmol), and 24R,25-dihydroxy-[26,27-methyl-^{3}H]vitamin D_3 (^{3}H-24R,25$(OH)_2D_3$, 170 Ci per mmol) were purchased from Amersham, Braunschweig, Germany. The 1α,25$(OH)_2D_3$-radioreceptor assay kit from Nichols Institute (Bad Nauheim, Germany) was used. VD_3 solvents for high performance liquid chromatography (HPLC) (n-hexane, 2-propanol, and methanol) were provided by Merck (Darmstadt, Germany). Lumisterol and tachysterol were donated by Dr. A. Kissmeyer (Leo Pharmaceutical Products, Ballerup, Denmark). Ketoconazole was bought from Paesel & Lorei (Frankfurt/M, Germany). Bovine serum albumin (BSA), purity ⩾ 99% (product number A 0281), and 7-DHC were from Sigma (Deisenhofen, Germany). Scintillation cocktail, Ready Protein+, was purchased from Beckman Instruments (Fullerton). N,O-bis(trimethylsilyl) acetamide (BSA), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), N-trimethylsilylimidazole (TMSI), and trimethylchlorosilane (TMCS) were purchased from Macherey-Nagel (Dueren, Germany). Pre-VD_3 was prepared by thermal treatment of an ethanolic solution of VD_3 (1.0 μg per ml) at 60°C for 16 h under nitrogen according to the literature (Isler and Brubacher, 1982). VD_3 (retention time 10.57 min) and generated pre-VD_3 (retention time 8.22 min) were separated by normal phase (NP) HPLC (eluent 1, see *HPLC analysis*). Pre-VD_3 was fractionated from 8.00 to 9.00 min. The UV spectrum of pre-D_3 (λ_{max} 260 nm, λ_{min} 230 nm) was identical to that described in the literature (Holick *et al*, 1977).

Preparation of the HSE model Keratinocytes from the epidermis of normal skin from young patients undergoing plastic surgery were used as second-passage cells. Dermal fibroblasts from the same patients were cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum. The fibroblasts were embedded in rat tail collagen type 1 lattices 2 d before the keratinocytes were inoculated onto the gel matrix in the culture dishes at a density of 4×10^4 per cm^2. Cultivation was carried out in 3 ml KGM at 37°C in a humidified atmosphere of 5% CO_2 in air. After achieving preconfluence (equivalent to $0.45–0.65 \times 10^6$ cells per dish) fresh KBM (1.2 ml) supplemented with 1.0% (wt/vol) of BSA was added. Cell numbers and cellular viability were assessed using a CASY® one-cell counter (Schärfe System, Reutlingen, Germany). The viability of keratinocytes in HSE was always ⩾ 92%.

Incubation conditions Different concentrations of 7-DHC (3.13–35 μM) dissolved in 6 μl ethanol (final concentration 0.5%) were added to the medium of HSE. Control experiments were carried out (i) in the presence of 7-DHC without irradiation, (ii) in the absence of 7-DHC with and without irradiation, and (iii) in the presence of collagen matrix without keratinocytes with 7-DHC and irradiation. After UVB irradiation and incubation in the dark the medium and detached keratinocytes were separately extracted with methanol:chloroform (1:1). The chloroform phases were used for the determination of pre-VD_3, VD_3, and 1α,25$(OH)_2D_3$.

UV irradiation Samples were exposed to UVB emitted by a tunable high intensity monochromator (FWHM, 5 nm) (part of the Dermolum Um, Fa. Müller Optik-Elektronik, Moosinning, Germany). The irradiance E_e = 0.28 mW per cm^{-2} (inhomogeneity within the irradiation spot of → 15 mm, ± 10%) was measured at the bottom of the culture dish and controlled by a thermopile TS 50-1 (Physikalisch-Technische Werkstätten, Jena, Germany), calibrated with PTB (Braunschweig, Germany). The culture dish (→ 30 mm) was continously rotated round the axis. The UV spot (→ 15 mm) was positioned at the radius of the rotating dish. The UVB doses used were adapted to these experimental conditions (effective dose D_{eff} = applied UVB dose × 0.25).

HPLC analysis NP-HPLC [Merck/Hitachi; column LiChroCART 250-4, Superspher Si 60, 5 μm; solvent system 1 (n-hexane:2-propanol 95:5 vol/vol; flow rate 0.5 ml per min)] was used for the determination of pre-VD_3, VD_3, and 7-DHC. The peaks of pre-VD_3 and VD_3 (retention times 8.22 min and 10.57 min) were quantified by UV detection at 265 nm. The peak homogeneity was checked using a diode array detector (L-4500, Merck/Hitachi). Solvent system 2 (n-hexane:2-propanol:methanol 87:10:3 vol/vol; flow rate 1.0 ml per min) was used for separation of 25OHD_3 and 1α,25$(OH)_2D_3$ (retention times 7.01 min and 21.10 min). Fractions containing putative 25OHD_3 (fraction number 6–8) and 1α,25$(OH)_2D_3$ (fraction number 20–22) were collected and, after drying, analyzed for calcidiol [gas chromatography–mass spectrometry (GC-MS)] and calcitriol (GC-MS and radioreceptor assay). In reversed phase (RP) HPLC the calcitriol generated was identified by cochromatography of the ^{3}H-labeled standard using a Hibar© column, 250-4, LiChrospher 100RP-18, 5 μm (Merck, Darmstadt, Germany), with solvent system 3 (methanol:water 85:15 vol/vol, flow rate 1 ml per min). Calcitriol-containing fractions were dried and analyzed for their content of calcitriol. Concentrations of VD_3 and calcitriol were corrected for their recoveries [VD_3 (500 pmol), 63.69% ± 4.61%, N = 5; calcitriol (720 fmol), 50.11% ± 4.87%, N = 5) and normalized to 1 cm^2 HSE.

GC-MS The dried HPLC fractions containing 25OHD_3 or 1α,25$(OH)_2D_3$ were derivatized to the trimethylsilyl ether derivatives and analyzed by GC-MS. The samples were treated with 40 μl of MSTFA:BSA:TMCS:TMSI (10:5:5:2, vol/vol) at 95°C for 45 min. One microliter of the derivatized sample was directly and manually injected into a model 5890/II gas chromatograph equipped with a 25 m × 0.2 mm HP-1 capillary column (cross-linked methylsiloxane, 0.33 μm) and interfaced with a model 5989 A MS-Engine (Hewlett-Packard, Palo Alto, CA). GC conditions were the following: carrier gas, helium; column head pressure, 10 psi; injector temperature, 260°C; oven temperature gradient, maintained at 200°C for 1 min, increased to 260°C at 30°C per min, then increased to 300°C at 20°C per min, held at 300°C for 10 min; interface temperature, 300°C. Electron impact MS conditions were the following: source temperature, 250°C; analyzer temperature, 120°C; energy, 70 eV. All samples were run in triplicate under the control of the HP-ChemStation data system. For selective ion monitoring, the most abundant ions at m/z 452 and m/z 501 were monitored for 1α,25$(OH)_2D_3$. The most abundant ion at m/z 439 was monitored for 25OHD_3.

Statistical analysis Results are presented as mean or mean ± SD. Data were analyzed by one-way analysis of variance (Bonferroni method).

RESULTS

UVB induces conversion of 7-DHC to VD_3 in HSE In order to monitor UVB-induced photolysis under experimental conditions, 7-DHC (25 μM) was added to the culture medium of the HSE model with subsequent exposure to monochromatic UVB at 300 ± 2.5 nm and D_{eff} = 30 mJ per cm^2. **Figure 1** shows three chromatograms obtained from extracts of unirradiated HSE (control) and at 00.17 h and 16 h after UVB irradiation. The photolyzed product pre-VD_3 was identified by cochromatography of pre-VD_3 prepared by thermal isomerization of VD_3 as described in *Materials and Methods* and by its UV spectrum (λ_{max} 260 nm, λ_{min} 230 nm). VD_3 formed was identified by cochromatography of the VD_3 reference substance and by its UV spectrum (λ_{max} 265 nm and λ_{min} 229 nm). The photoisomers of pre-VD_3, lumisterol and tachysterol (retention times 9.00 min and 10.96 min, respectively), were not detectable after irradiation. No 7-DHC was detected in detached keratinocytes whereas the concentration of 7-DHC in the gel matrix supplemented with fibroblasts was determined as 0.23 ± 0.14 nmol per cm^2 (N = 5). The time course of the UVB-induced conversion of 7-DHC via pre-VD_3 to VD_3 indicates that the isomerization of pre-VD_3 to VD_3 is complete after 15–20 h (data not shown in detail). The VD_3 synthesis in HSE is dependent on the wavelength of UV radiation. The maximum synthesis rates of VD_3 were found at 302 nm. After irradiation at wavelengths ⩾ 315 nm no *de novo* VD_3 production was detectable in the HSE model.

Photosynthesized VD_3 is converted to 1α,25$(OH)_2D_3$ in HSE Extracts of irradiated HSE (300 ± 2.5 nm, D_{eff} = 30 mJ per cm^2) and of nonirradiated controls were fractionated by NP-HPLC (solvent system 2) and fractions (Nos 1–25) were analyzed for calcitriol (**Fig 2**). The peak in fractions 20–22 is identical with calcitriol. Two additional minor peaks at 7 min and 17 min were found. The radioactivity peaks of ^{3}H-25OHD_3 and ^{3}H-24R,25$(OH)_2D_3$ appear after 7 min and 12 min, respectively. We could not quantify 25OHD_3 in the same NP-HPLC step by UV detection at 265 nm, however (analytical sensitivity ≈2 ng per ml; data not shown). No calcitriol was detectable in analogous

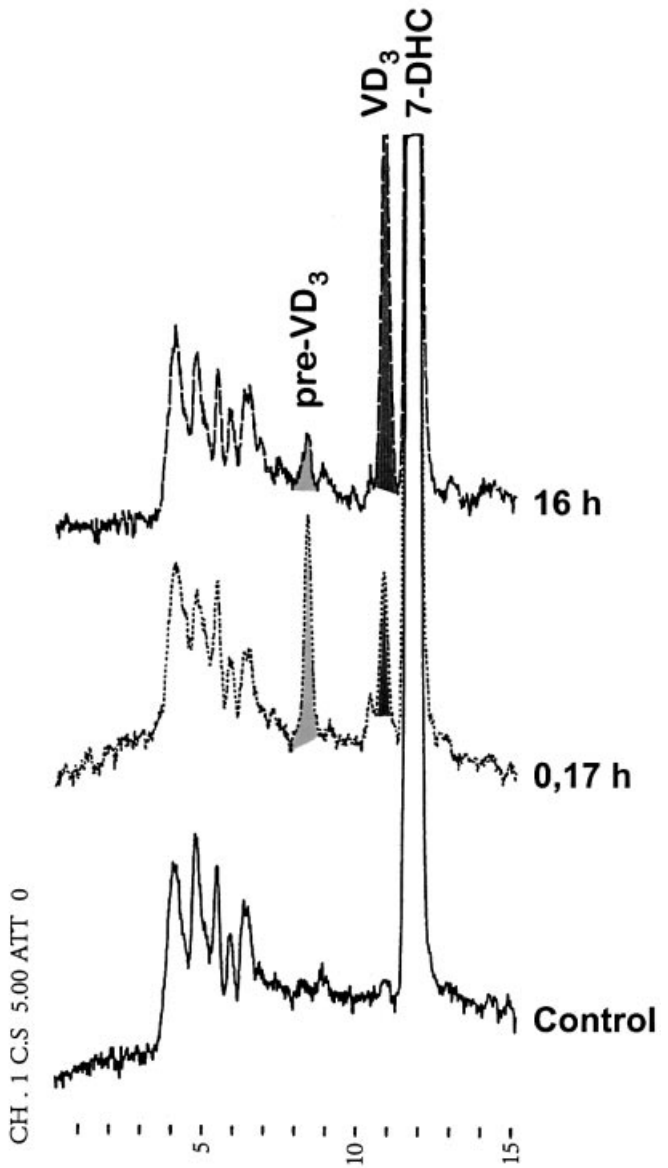

Figure 1. HPLC chromatograms after photochemical transformation of 7-DHC via pre-VD_3 into VD_3 in an HSE culture model. Culture medium (KBM) supplemented with 1% BSA (wt/vol) was incubated with 25 μM 7-DHC and irradiated at 300 nm (D_{eff} = 30 mJ per cm^2) or not irradiated (control). Peaks represent pre-VD_3, VD_3, and 7-DHC at 00.17 h and 16 h incubation time after UVB exposure.

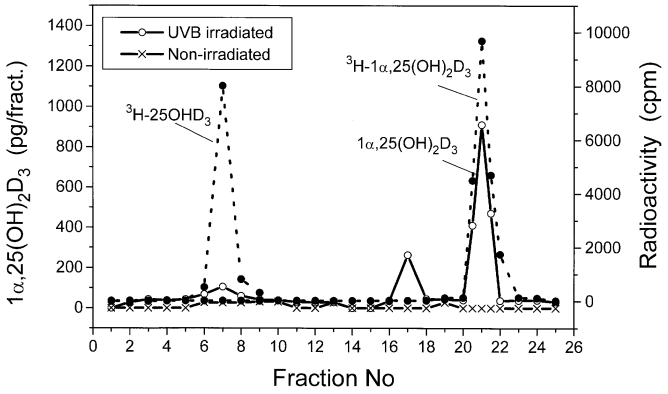

Figure 2. Determination of calcitriol in extracts of irradiated and nonirradiated HSE. HSE supplemented with 25 μM 7-DHC (4.24 nmol per cm^2 HSE) were irradiated at 300 nm (30 mJ per cm^{-2}) or not irradiated (control) and further incubated for 16 h. After extraction and separation by NP-HPLC (eluent 2) fractions 1–25 were analyzed for calcitriol by a radioreceptor assay. Comigration of calcitriol formed with synthetic ^3H-1α,25$(OH)_2D_3$ 116 pg per 0.05 μCi.

fractions of nonirradiated cultures (**Fig 2**) as well as in the other controls (ii) and (iii) described in *Materials* and *Methods* (data not shown). Calcitriol comigrated with synthetic ^3H-1α,25$(OH)_2D_3$ in both NP- and RP-HPLC systems. To exclude the presence of 19-nor,10-keto-25OHD_3, which comigrates with 1α,25$(OH)_2D_3$ in NP-HPLC, 300 pg putative calcitriol was separated by RP-HPLC and the amount of calcitriol recovered was about the same as injected initially, indicating the absence of 19-nor,10-keto-25OHD_3.

Studies of the TMS derivatives of synthetic and *de novo* generated calcitriol by GC-MS demonstrated identical retention times. Two resulting peaks (pyro and isopyro derivative) were identical for synthetic and *de novo* generated 1α,25$(OH)_2D_3$. The full-scan electron impact mass spectra of the pertrimethylsilyl ether derivatives from the reference compound and presumptive 1α,25$(OH)_2D_3$ (pyro peak) are depicted in **Fig 3**(***A***), (***B***). The mass spectra produced by the pyro and isopyro peaks differ only in the relative abundance of the mass fragments. Of note, weak molecular ions [$M^{+\cdot}$] at *m/z* 632 can be observed in both spectra (no mass peaks of intensity greater than 9% of the base peak). The spectra showed the most abundant ion at *m/z* 452 ([M^+ 180]) resulting from the loss of two silanol groups from the ionized molecule. There were two other prominent ions at *m/z* 542 ([M^+ 90]) due to the loss of one silanol group and at *m/z* 362 ([M^+ 270]) arising from the loss of three silanol groups, respectively. The intense ion at *m/z* 501 ([M^+ 131]) may arise from the loss of the C2,3,4-3-silanol fragment. The peak at *m/z* 131 corresponds to a secondary propyl ether trimethylsilyl ion obtained by cleavage of the bond between C24 and C25.

Notably, the GC-MS analysis of pooled fractions collected between 6 and 8 min provided a mass spectrum with identical mass fragments obtained from synthetic 25OHD_3 (**Fig 3*C*, *D***). The most abundant high mass ion was *m/z* 439, resulting from the loss of one silanol group plus one methyl group ([M^+ 90–15]). Only in the standard substance a weak molecular ion [M^+] at *m/z* 544 could be observed (no mass peaks of intensity greater than 4% of the base peak). In unirradiated controls no 25OHD_3 was detectable.

Ketoconazole (1 μM, 5 μM, AND 10 μM) added to HSE immediately after irradiation at 300 ± 2.5 nm and D_{eff} = 30 mJ per cm^2 and further incubation for 16 h in the dark caused a dose-dependent inhibition (**Fig 4**) of 1α,25$(OH)_2D_3$ formation. The antioxidant 1,2-dianilinoethane (10 μM) had only a marginal inhibitory effect (**Fig 4**) on the generation of 1α,25$(OH)_2D_3$.

The time course of UVB-induced generation of VD_3 and calcitriol in HSE containing 4.24 nmol 7-DHC per cm^2 HSE after irradiation at 300 nm with a constant dose of D_{eff} = 30 mJ per cm^2 is shown in **Fig 5**. The maximal synthesis rate of VD_3 is reached after 8–10 h whereas that of calcitriol continuously increases up to 24 h. The viability of keratinocytes decreases with rising incubation time (data not shown in detail).

In HSE preincubated with increasing concentrations of 7-DHC (0.53–5.94 nmol per cm^2 HSE) and irradiated with UVB at 300 nm (D_{eff} = 30 mJ per cm^2) followed by 16 h incubation time a concentration-dependent generation of VD_3 and calcitriol was observed (**Fig 6**).

The UVB dose–response relationship depicted in **Fig 7** indicates increasing VD_3 levels linear with UVB dose after a constant incubation time of 16 h whereas the calcitriol synthesis is maximal near D_{eff} = 30 mJ per cm^2 and declines at higher UV doses. The decrease in viability of cells was dependent on UVB dose (**Fig 7**).

The rate of calcitriol synthesis depends on the UVB wavelength used for irradiation and is very similar to that of VD_3 showing a

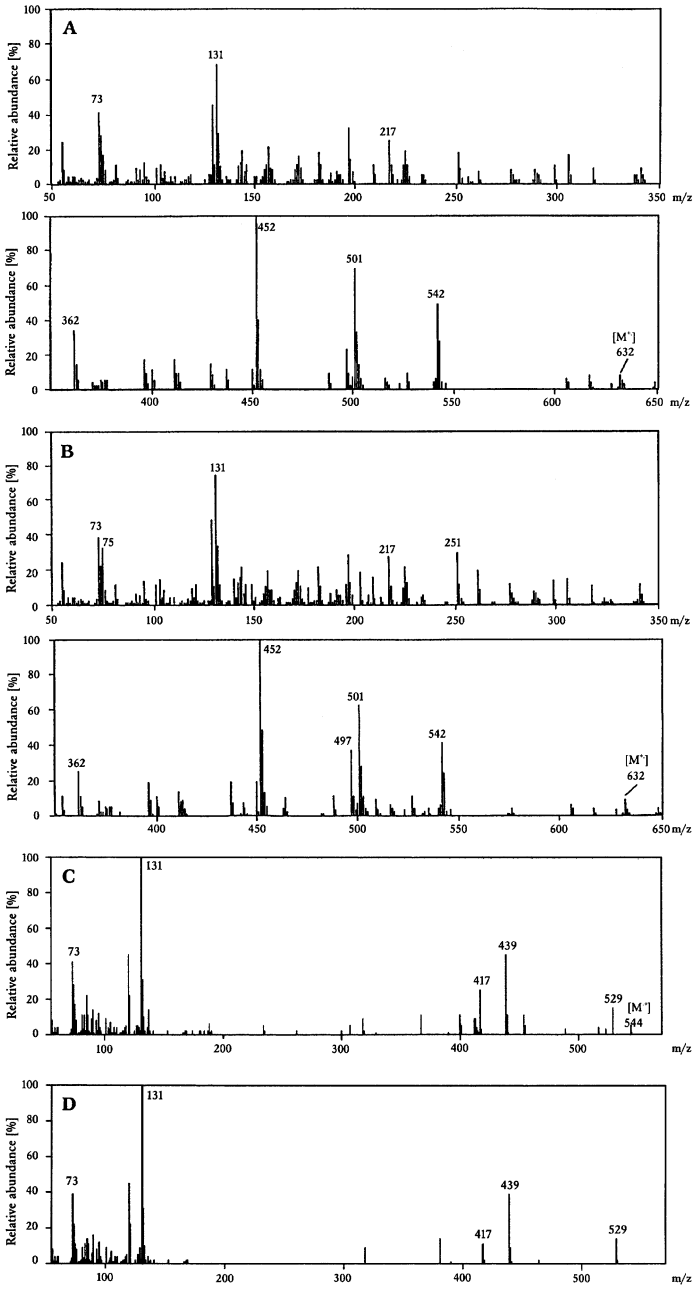

Figure 3. Electron impact mass spectra of chemically synthesized $1\alpha,25(OH)_2D_3$ and presumptive calcitriol as well as of the standard substance $25OHD_3$ and putative calcidiol generated after UVB-induced photolysis of 7-DHC in the presence of HSE. (*A*) Trimethylsilyl derivatives of the synthetic $1\alpha,25(OH)_2D_3$ (M_r 632), (*B*) the corresponding purified metabolite, (*C*) synthetic $25OHD_3$ (M_r 529), and (*D*) the corresponding purified metabolite were compared by GC-MS as described in the text. Spectra represent electron ionization (electron impact, 70 eV) mass spectra.

maximum at around 302 nm (**Fig 8**). No VD_3 and calcitriol were detected at wavelegths > 315 nm.

DISCUSSION

One of the most important sunlight-mediated events in human skin is the photosynthesis of VD_3, the precursor both of calcidiol ($25OHD_3$) and hormonally active calcitriol ($1\alpha,25(OH)_2D_3$) (Holick, 1995). The UVB radiation of sunlight (spectral range 290–315 nm) penetrates into the skin and causes photolysis of 7-DHC to pre-VD_3, which undergoes a rearrangement of its double bond structure to form the thermodynamically more stable VD_3. Both cultured keratinocytes and fibroblasts alone are able to photosynthesize pre-VD_3 and subsequently form, after its thermal isomerization, VD_3 (Nemanic *et al*, 1985).

Previously we have shown that cultured keratinocytes can hydroxylate exogenous VD_3 to $1\alpha,25(OH)_2D_3$ (Lehmann *et al*, 1998; 2000a). In addition, we have recently demonstrated that the complete pathway from 7-DHC to $1\alpha,25(OH)_2D_3$ takes place in the transformed cell line HaCaT (Lehmann *et al*, 2000b). The complete pathway from 7-DHC to $1\alpha,25(OH)_2D_3$ in human keratinocytes under *in vivo*-like conditions has not been demonstrated, however. Furthermore, the question of possible intermediate products ($25OHD_3$ and/or 1α-OHD_3) within the pathway from VD_3 to $1\alpha,25(OH)_2D_3$ has remained unanswered.

In this study we demonstrate the formation of $1\alpha,25(OH)_2D_3$ from VD_3 generated by photolysis of the precursor 7-DHC in an *in vivo*-like HSE model (organotypic culture of human keratinocytes). The UVB-induced nonenzymatic isomerization of 7-DHC via pre-VD_3 to VD_3 in our HSE model is in accordance with several reports showing that UVB radiation (optimum wavelengths between 295 and 300 nm) can photolyze 7-DHC to pre-VD_3 in human skin (Holick *et al*, 1980; MacLaughlin *et al*, 1982) and in cultures of human keratinocytes (Nemanic *et al*, 1985). The identities of generated pre-VD_3 and VD_3 were confirmed by NP-HPLC as well as by spectrophotometry. The time- and temperature-dependent isomerization of pre-VD_3 to VD_3 (Holick *et al*,

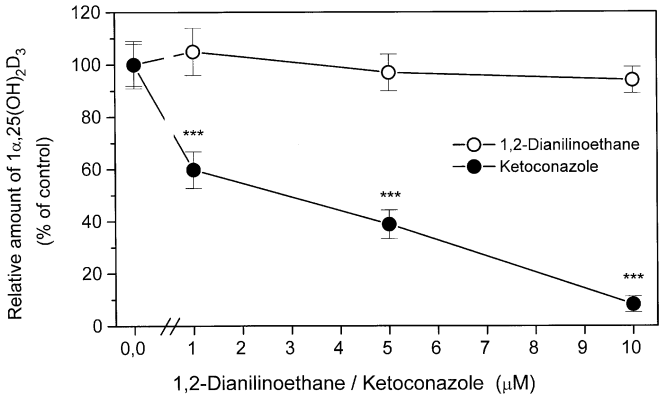

Figure 4. Inhibitory effect of ketoconazole and 1,2-dianilinoethane on hydroxylation of VD_3 created after irradiation with UVB to $1\alpha,25(OH)_2D_3$ in HSE. Cultures containing 25 μM 7-DHC were irradiated at 300 nm (D_{eff} = 30 mJ per cm^2). Immediately after irradiation various concentrations of ketoconazole and 1,2-dianilinoethane (1 μM, 5 μM, AND 10 μM) or ethanol (control) were added to the cultures, and further incubation for 16 h at 37°C was done in the dark. After extraction and separation by NP-HPLC, calcitriol was determined as described. Concentrations obtained are depicted as relative percent ± SD of control (100% ± 9% equal to 564 ± 51 fmol calcitriol per cm^2 HSE) of three independent experiments; ***p < 0.001 compared to control.

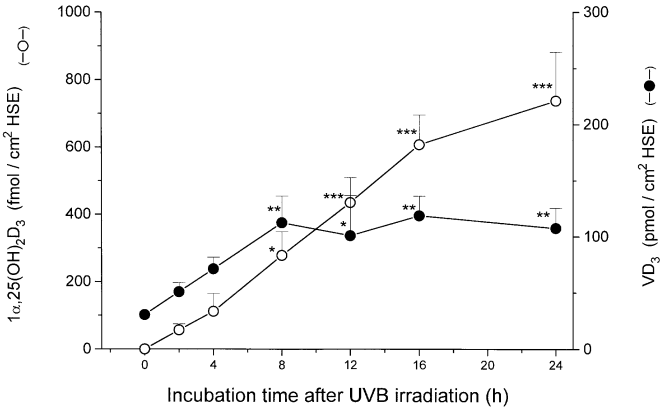

Figure 5. Time course of the levels of VD_3 and calcitriol in cultures of HSE after exposure to UVB. Cultures containing 7-DHC (25 μM) were irradiated with monochromatic UVB at 300 nm (D_{eff} = 30 mJ per cm^2) and further incubated up to 24 h. Concentrations of VD_3 and $1\alpha,25(OH)_2D_3$ are depicted as mean ± SD of three independent experiments; *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the data obtained immediately after irradiation.

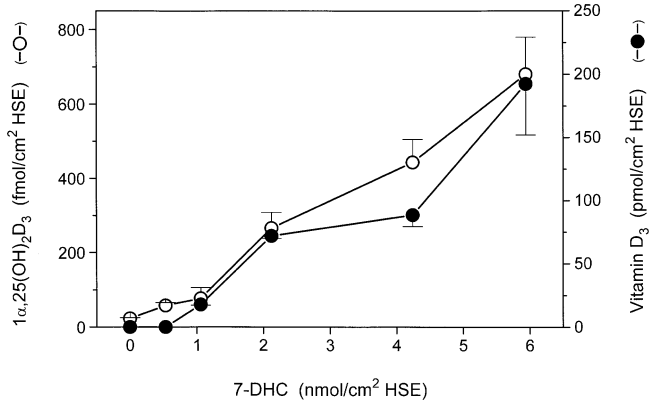

Figure 6. Synthesis rates of VD_3 and calcitriol in UVB-irradiated HSE supplemented with increasing 7-DHC concentrations. HSE containing 0.53–5.94 nmol per cm^2 was irradiated at 300 nm (D_{eff} = 30 mJ per cm^2) and further incubated for 16 h. Data are depicted as the mean ± SD (N = 3).

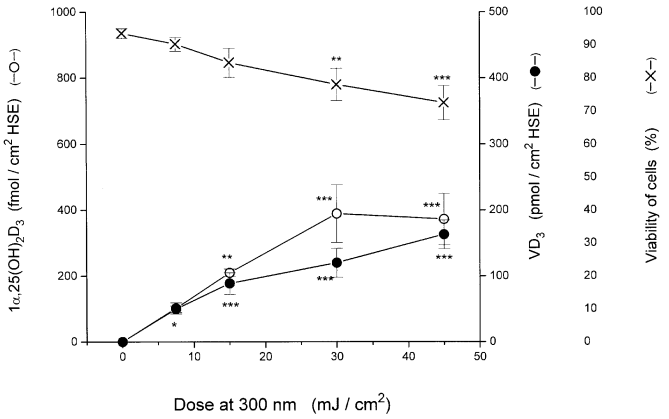

Figure 7. Influence of increasing UVB doses on the production of VD_3 and calcitriol as well as the viability of keratinocytes in HSE. Cultures containing 25 μM 7-DHC in the medium were irradiated at 300 nm at increasing UV doses up to D_{eff} = 45 mJ per cm^2 followed by constant 16 h incubation. Concentrations of VD_3 and $1\alpha,25(OH)_2D_3$ and the cell viability are shown as mean ± SD of three independent experiments; *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the unirradiated control.

1980; MacLaughlin *et al*, 1982; Tian *et al*, 1993) in our HSE model is almost complete after 16 h. This is comparable with findings in human skin where this isomerization was estimated to occur within 20 h (Tian *et al*, 1993).

Our results demonstrate that in normal human keratinocytes VD_3 generated by UVB-induced photolysis of 7-DHC is further metabolized via calcidiol to calcitriol. $1\alpha,25(OH)_2D_3$ was clearly identified by both NP- and RP-HPLC as well as by GC-MS. The mass spectrum was identical to the synthetic reference substance and followed the fragmentation pattern that had been previously described for $1\alpha,25(OH)_2D_3$ (Poon *et al*, 1993; Mawer *et al*, 1994; Schroeder *et al*, 1994). Calcitriol was not detected when medium alone supplemented with 7-DHC was irradiated with UVB or in unirradiated HSE as controls. In UVB-irradiated skin equivalents without the addition of 7-DHC (see **Fig 6**) we found small but detectable amounts of $1\alpha,25(OH)_2D_3$ (≈23 fmol per cm^2 HSE). Hence, the generation of substantial amounts of calcitriol in our HSE model requires a sufficiently high concentration of 7-DHC, the presence of keratinocytes, and UVB radiation. Experiments using HaCaT cells have shown that 7-DHC binds to the cell membrane and/or is transported into the cell when these cells are preincubated with physiologic concentrations of 7-DHC (Lehmann *et al*, 2000b). The cellular uptake of 7-DHC was measured to be approximately 20% of the total amount of 7-DHC (30 nmol) previously added to the cell culture. In these experiments, the corresponding levels of cellular VD_3 and calcitriol after irradiation at 297 nm followed by 16 h incubation at 37°C in the dark were about 37% of the total amount of both VD_3 and calcitriol formed in the original culture.

Two additional metabolites were found in the fractionated eluent of NP-HPLC that showed some cross-reactivity with $1\alpha,25(OH)_2D_3$ in the radioreceptor assay. One of these metabolites (fractions between 6 and 8 min) had in derivatized form (trimethylsilyl ether derivative) a mass spectrum identical to synthetic $25OHD_3$ (Coldwell *et al*, 1989). The identity of the other metabolite (fractions between 16 and 18 min) shown in **Fig 2** is not known at the present time. This finding indicates that $25OHD_3$ is generated as an intermediary metabolite during the

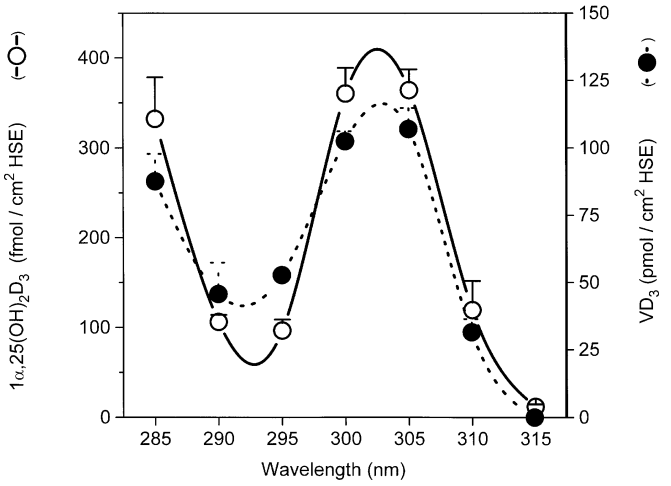

Figure 8. Relationship between wavelengths of UVB light and the generation of VD_3 and $1\alpha,25(OH)_2D_3$ in HSE. Cultures preincubated with 25 μM 7-DHC (4.24 nmol per cm^2 HSE) were irradiated at several wavelengths between 285 nm and 315 nm (D_{eff} = 30 mJ per cm^2) followed by 16 h incubation. Each point represents mean ± SD (N = 3).

UVB-induced metabolic conversion of 7-DHC to $1\alpha,25(OH)_2D_3$. We could not quantitatively determine $25OHD_3$ in the same NP-HPLC step, however, by UV detection at 265 nm. This may be explained by the fact that $25OHD_3$ is located within a coupled equilibrium $VD_3 \leftrightarrow 25OHD_3 \leftrightarrow 1\alpha,25(OH)_2D_3$ and probably with other hydroxylated VD_3 metabolites. The Michaelis constant for the 1α-hydroxylation of $25OHD_3$ [$K_m = 5.4 \times 10^{-8}$ M, determined in human keratinocytes (Bikle *et al*, 1986)] is considerably lower than the Michaelis constant for the 25-hydroxylation of VD_3 [$K_m = 10^{-5}$ M, determined in human liver mitochondria (Saarem *et al*, 1984)]; therefore, the equilibrium should be strongly shifted toward formation of calcitriol, which makes the quantitative determination of $25OHD_3$ impossible with HPLC or by vitamin D binding protein assay (analytical sensitivity 2 ng per ml and 2.2 ng per ml).

Blocking experiments with ketoconazole, a cytochrome P450 enzyme inhibitor (Wilkinson *et al*, 1974), showed the involvement of intracellularly located cytochrome P450 mixed-function oxidases in the formation of $1\alpha,25(OH)_2D_3$. In contrast, 1,2-dianilinoethane, a known radical scavenger and antioxidant (Sietsema and DeLuca, 1982), exerted only a marginal quench effect on the hydroxylations of VD_3. These and our previous results point to the presence of both 1α-and 25-hydroxylase activities in keratinocytes.

The time course of UVB-triggered calcitriol synthesis in HSE shows an almost continuous increase up to 24 h after UVB exposure. Of note, the corresponding time course of UVB-induced calcitriol formation in HaCaT cells obtained after UVB irradiation at 297 nm (D_{eff} = 30 mJ per cm^2) was characterized by a maximum at 16 h after irradiation (Lehmann *et al*, 2000b). This is similar to experiments where when exogenous VD_3 was added instead of being generated by UVB calcitriol reached a peak 6 h after addition of VD_3 and then continuously fell until 24 h (Lehmann *et al*, 1998, 2000a). These differences in the continuity of calcitriol synthesis in keratinocytes are difficult to explain. Based on the fact that calcitriol induces its own catabolism to calcitroic acid (Ray *et al*, 1995) and, in addition, causes inhibition of 1α-hydroxylase (Bikle *et al*, 1986; Lehmann, 1997) we assume that these processes may be differently developed in keratinocytes. Both the catabolism of calcitriol by the 24-hydroxylase and the inhibition by calcitriol of the 1α-hydroxylase are mediated by the vitamin D receptor (VDR) (Chen *et al*, 1994; Takeyama *et al*, 1997). As it has been reported that UVB dose-dependently and potently downregulates the expression of VDR mRNA and protein within a few hours after irradiation in cultured keratinocytes (Courtois *et al*, 1998), a stronger UVB-induced suppression of gene expression of VDR in keratinocytes of our HSE model compared to HaCaT cells might explain our finding of sustained calcitriol synthesis in the former model.

Within 16 h after irradiation at 300 nm (D_{eff} = 30 mJ per cm^2) 0.015% (equal to 636 fmol) of 7-DHC added is converted to $1\alpha,25(OH)_2D_3$. For comparison, the concentration of $1\alpha,25-(OH)_2D_3$ in human serum is 68 ± 27 fmol per ml (Hollis, 1986). The concentration of 7-DHC used in our experiments (30 nmol per 1.2 ml medium equivalent to 4.24 nmol per cm^2 HSE) is comparable to the level of 7-DHC (≈2.7 nmol per cm^2) found in hypopigmented Caucasian human leg skin (Holick *et al*, 1980) and in human neonatal foreskin (≈6.1 nmol per cm^{-2}) (Obi-Tabot *et al*, 2000). In contrast to the HSE model published by Obi-Tabot *et al* (2000), which has a basal 7-DHC content similar to that of human neonatal foreskin, our HSE model showed under basal conditions only marginal concentrations of 7-DHC. The reason why the two HSE models differ in their 7-DHC content is not known. Obi-Tabot *et al* supposed that the interaction between keratinocytes and fibroblasts in their HSE model has a downregulating effect on the 7-DHC reductase that results in a higher intracellular pool of 7-DHC. This hypothesis is supported by the finding that, in cell culture without heterologous cell contacts, both keratinocytes and fibroblasts show barely detectable levels of 7-DHC (Nemanic *et al*, 1985). We assume that the Δ^7-reductase in our HSE model is probably more active than is the enzyme in the HSE model of Obi-Tabot *et al* and in whole skin.

In our experiments the synthesis rate of both VD_3 and calcitriol closely depends on the 7-DHC concentration added before UVB exposure. Also, the rate of synthesis of $1\alpha,25(OH)_2D_3$ from VD_3 showed a close relation to the UVB dose (D_{eff} = 7.5–45.0 mJ per cm^2) with which keratinocytes were irradiated. For comparison, an effective dose of 30 mJ per cm^2 at 297 ± 2.5 nm corresponds to ≈1.5 minimal erythema dose for hypopigmented Caucasian skin.[1] The inhibition of calcitriol synthesis observed with high UVB doses can be explained by UVB-mediated inactivation of intracellular hydroxylases at $D_{eff} \geq 30$ mJ per cm^2, whereas nonenzymatic synthesis of VD_3 remains unaffected.

The rate of formation of $1\alpha,25(OH)_2D_3$ depended on the UVB wavelength used for irradiation and is very similar to that of VD_3, showing a maximum at around 302 nm. This finding demonstrates a close relation between UVB-induced VD_3 synthesis and formation of $1\alpha,25(OH)_2D_3$. It is likely that the concentration of VD_3 determines the synthesis rate of $1\alpha,25(OH)_2D_3$ in our cell system. It cannot be ruled out, however, that the enzymatic activity of the 1α-hydroxylase and/or the 25-hydroxylase shows a dependence on the UVB wavelength used. The UVB wavelength around 302 nm corresponding to maximal VD_3 synthesis is in accordance with observations made in rat skin (Takada, 1983), where the optimum wavelength for the synthesis of VD_3 (305 nm) was longer than in organic solvents (295 nm). It is conceivable that cellular phospholipid/pre-VD_3 interactions (Tian and Holick, 1999) are influenced by wavelength-dependent photodegradation of membrane phospholipids resulting in different rates of VD_3 formation. Such wavelength-dependent processes also may explain changes in the VD_3 production between 285 and 292 nm. In agreement with the literature (MacLaughlin *et al*, 1982), we found no synthesis of VD_3 at wavelengths > 315 nm.

More recently, our finding of an autonomous calcitriol pathway in epidermal cells was indirectly confirmed by experiments demonstrating the UVB-induced expression of 24-hydroxylase, the most sensitive $1\alpha,25(OH)_2D_3$ response gene, in UVB-irradiated keratinocytes pretreated with 7-DHC and a 7-DHC reductase inhibitor.[2] This effect was abolished by preincubation with

[1]Personal communication.

[2]Segeart S, Bouillon R: Epidermal keratinocytes as source and target cells for vitamin D. Paper presented at the 11th Workshop on Vitamin D, Nashville, TN, May 27–June 1, 2000

ketoconazole, and thus a UVB-induced generation of $1\alpha,25(OH)_2D_3$ in cultured keratinocytes was demonstrated. In contrast, our study directly demonstrates the generation of $25OHD_3$ and $1\alpha,25(OH)_2D_3$ in an HSE enriched with 7-DHC and irradiated with UVB. Additional experiments should indicate to what extent the UVB-triggered epidermal calcitriol synthesis regulates genomic and nongenomic processes. It is noteworthy in this context that calcitriol has a photoprotective effect against UVB injury of keratinocytes provoked by induction of the synthesis of metallothionein, a protein with antioxidant properties (Karasawa *et al*, 1987; Hanada *et al*, 1995; Lee and Youn, 1998). Furthermore, we ask whether the known antiproliferative and immunomodulatory effects of calcitriol may be connected to the therapeutic effect of UVB in hyperproliferative skin diseases such as psoriasis.

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REFERENCES

Bell E, Ehrlich HP, Buttle DJ, Nakatsuji T: Living tissue formed *in vitro* and accepted as skin equivalent tissue of full-thickness. *Science* 211:1052–1054, 1981

Bikle DD, Nemanic MK, Gee E, Elias P: 1,25-Dihydroxyvitamin D_3 production by human keratinocytes. *J Clin Invest* 78:557–566, 1986

Chen ML, Heinrich G, Ohyama YI, Okuda K, Omdahl JL, Chen TC, Holick MF: Expression of 25-hydroxyvitamin D_3 hydroxylase mRNA in cultured human keratinocytes. *Proc Soc Exp Biol Med* 207:57–61, 1994

Coldwell RD, Trafford DJH, Varley MJ, Kirk DN, Makin HLJ: Measurement of 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, 24,25-dihydroxyvitamin D_2 and 25,26-dihydroxyvitamin D_2 in a single plasma sample by mass fragmentography. *Clin Chim Acta* 180:157–168, 1989

Courtois SJ, Segaert S, Degreef H, Bouillon R: Ultraviolet B suppresses vitamin D receptor gene expression in keratinocytes. *Biochem Biophys Res Commun* 246:64–69, 1998

Hanada K, Sawamura D, Nakano H, Hashimoto I: Possible role of 1,25-dihydroxyvitamin D_3 -induced metallothionein in photoprotection against UVB injury in mouse skin and cultured rat keratinocytes. *J Dermatol Sci* 9:208–208, 1995

Haussler MR: Vitamin D receptors: nature and function. *Annu Rev Nutr* 6:527–562, 1986

Holick MF: Vitamin D photobiology, metabolism, and clinical applications. In: DeGroot LJ, et al, eds. *Endocrinology*. Philadelphia, PA: Saunders, 1995:pp 990–1013

Holick MF, Frommer JE, McNeal SC, Richtand NM, Henley JW, Potts JT: Photometabolism of 7-dehydrocholesterol to previtamin D_3 in skin. *Biochem Biophys Res Commun* 76:107–115, 1977

Holick MF, MacLaughlin JA, Clark MB, Holick SA, Potts JT: Photosynthesis of previtamin D_3 in human skin and the physiologic consequences. *Science* 210:203–205, 1980

Hollis BW: Assay of circulating 1,25-dihydroxyvitamin D involving a novel single cartridge extraction and purification procedure. *Clin Chem* 32:2060–2063, 1986

Hosomi J, Hosoi J, Abe E, Suda T, Kuroki T: Regulation of terminal differentiation of cultured mouse epidermal cells by $1\alpha,25$-dihydroxyvitamin D_3. *Endocrinology* 113:1950–1957, 1983

Isler O, Brubacher G: *Vitamine I. Fettlösliche Vitamine 2. Die D-Vitamine*. Stuttgart, New York: Georg Thieme Verlag, 1982:pp 90–125

Karasawa M, Hosoi J, Hashiba H, *et al*: Regulation of metallothionein gene expression by $1\alpha,25$-dihydroxyvitamin D_3 in cultured cells and in mice. *Proc Natl Acad Sci USA* 84:8810–8813, 1987

Lee JH, Youn JI: The photoprotective effect of 1,25-dihydroxyvitamin D_3 on ultraviolet light B-induced damage in keratinocyte and its mechanism of action. *J Dermatol Sci* 18:11–18, 1998

Lehmann B: HaCaT cell line as a model system for vitamin D_3 metabolism in human skin. *J Invest Dermatol* 108:78–82, 1997

Lehmann B, Pietzsch J, Kämpf A, Meurer M: Human keratinocyte line HaCaT metabolizes 1α hydroxyvitamin D_3 and vitamin D_3–$1\alpha,25$-dihydroxyvitamin D_3 (calcitriol). *J Dermatol Sci* 18:118–127, 1998

Lehmann B, Rudolph T, Pietzsch J, Meurer M: Conversion of vitamin D_3–$1\alpha,25$-dihydroxyvitamin D_3 in human skin equivalents. *Exp Dermatol* 9:97–103, 2000a

Lehmann B, Knuschke P, Meurer M: UVB-induced conversion of 7-dehydrocholesterol to $1\alpha,25$-dihydroxyvitamin D_3 (calcitriol) in the human keratinocyte line HaCaT. *Photochem Photobiol* 72:803–809, 2000b

MacLaughlin JA, Anderson RR, Holick MF: Spectral character of sunlight modulates photosynthesis of previtamin D_3 and its photoisomers in human skin. *Science* 216:1001–1003, 1982

Matsumoto K, Azuma Y, Kiyoki M, Okumura H, Hashimoto K, Yoshikawa K: Involvement of endogenously produced 1,25-dihydroxyvitamin D-3 in the growth and differentiation of human keratinocytes. *Biochim Biophys Acta* 1092:311–318, 1991

Mawer EB, Hayes ME, Heys SE, Davies M, White A, Stewart MF, Smith GN: Constitutive synthesis of 1,25-dihydroxyvitamin D_3 by a human small cell lung cancer cell line. *J Clin Endocrinol Metab* 79:554–560, 1994

McLane JA, Katz M, Abdelkader N: Effect of 1,25-dihydroxyvitamin D_3 on human keratinocytes grown under different culture conditions. *In Vitro Cell Devel Biol* 26:379–387, 1990

Nemanic MK, Whitney J, Elias PM: *In vitro* synthesis of vitamin D-3 by cultured human keratinocytes and fibroblasts: action spectrum and effect of AY-9944. *Biochim Biophys Acta* 841:267–277, 1985

Obi-Tabot ET, Tian XQ, Chen TC, Holick MF: A human skin equivalent model that mimics the photoproduction of vitamin D_3 in human skin. *In Vitro Cell Dev Biol Anim* 36:201–204, 2000

Poon PM, Mak KYT, Pang CP: Gas chromatographic-mass fragmentographic determination of serum $1\alpha,25$ dihydroxyvitamin D_3. *Clin Biochem* 26:461–469, 1993

Prunieras M, Regnier M, Woodley D: Methods for cultivation of keratinocytes with an air–liquid interface. *J Invest Dermatol* 81:28S–33S, 1983

Ray S, Ray R, Holick MF: Metabolism of ^{3}H-$1\alpha,25$-dihydroxyvitamin D_3 in cultured human keratinocytes. *J Cell Biochem* 59:117–122, 1995

Saarem K, Bergseth S, Oftebro H, Pederson JI: Subcellular localization of vitamin D_3 25-hydroxylase in human liver. *J Biol Chem* 259:10936–10940, 1984

Schroeder NJ, Trafford DJH, Cunningham J, Jones G, Makin HLJ: *In vivo* dihydrotachysterol$_2$ metabolism in normal man: 1α- and 1β-hydroxylation of 25-hydroxydihydrotachysterol$_2$ and effects on plasma parathyroid hormone and $1\alpha,25$-dihydroxyvitamin D_3 concentrations. *J Clin Endocrinol Metab* 78:1481–1487, 1994

Sietsema WK, DeLuca HF: Retinoic acid 5,6-epoxidase: properties and biological significance. *J Biol Chem* 257:4265, 1982

Smith EL, Walworth NC, Holick MF: Effect of $1\alpha,25$-dihydroxyvitamin D_3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. *J Invest Dermatol* 86:709–714, 1986

Takada K: Formation of fatty acid esterified vitamin D_3 in rat skin by exposure to ultraviolet radiation. *J Lipid Res* 24:441–448, 1983

Takeyama KI, Kitanaka S, Sato T, Kobori M, Yanagisawa J, Kato S: 25-Hydroxyvitamin D_3 1α-hydroxylase and vitamin D synthesis. *Science* 277:1827–1829, 1997

Tian XQ, Holick MF: A liposomal model that mimics the cutaneous production of vitamin D_3. *J Biol Chem* 274:4174–4179, 1999

Tian XQ, Chen TC, Matsuoka LY, Wortsman J, Holick MF: Kinetic and thermodynamic studies of the conversion of previtamin D_3 to vitamin D_3 in human skin. *J Biol Chem* 268:14888–14892, 1993

Wilkinson CF, Hetnarski K, Cantwell GP, Di-Carlo FJ: Structure–activity relationships in the effects of 1-alkylimidazoles on microsomal oxidation *in vitro* and *in vivo*. *Biochem Pharmacol* 23:2377–2386, 1974