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VITAMIN D_3 PRODUCTION BY CULTURED HUMAN KERATINOCYTES AND FIBROBLASTS

Michael K. Nemanic, JoAnne Whitney, Sara Arnaud,
Su Herbert, Peter M. Elias*

Dermatology Service (190), V.A. Medical Center,
4150 Clement Street, San Francisco, CA 94121

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SUMMARY: We have demonstrated that monolayers of human cultured newborn foreskin keratinocytes and fibroblasts elaborate vitamin D_3 following exposure to UV-B. This *in vitro* system provides a new means to study those factors (hormones, ions, vitamin D_3 metabolites, etc.) that regulate the production of vitamin D_3 by human skin cells. Vitamin D_3 production was enhanced greatly by using cells that were pre-treated with AY-9944, a non-toxic drug that inhibits cholesterologenesis while elevating cellular levels of 7-dehydrocholesterol, the sterol precursor of vitamin D_3. The pre-D_3 formed within viable, irradiated cells is transformed to D_3 within a matter of hours at 37°C, and keratinocytes proved to be more proficient sources of the vitamin and its metabolites than corresponding skin fibroblasts.

Although the skin's ability to generate 7-dehydrocholesterol (7-DHC) (1-5) and vitamin D_3 (D_3) (6-8) has been appreciated for decades, only recently have details of the chemical events that follow ultraviolet UV-irradiation of skin been elucidated (9-12). Although the photoproduction of D_3 is considered to be a passive process, governed largely by the availability of UV light and 7-DHC, recent reports have presented evidence that cutaneous D_3 production may be influenced by circulating D_3 metabolites (13-17). However, studies on the regulation of D_3 production in the skin have been impeded by the lack of a homogeneous *in vitro* system suitable for testing variables that might orchestrate the synthesis and release of D_3 from the skin.

* To whom correspondence should be addressed.

Abbreviations:

7-dehydrocholesterol (7-DHC); vitamin D_3 (D_3); pre-vitamin D_3 (pre-D_3); ultraviolet (UV); fetal calf serum (FCS); phosphate-buffered saline (PBS); high pressure liquid chromatography (HPLC); and N=(nitrogen).

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In order to assess the capacity of different skin cell types to generate D_3, we compared the production of D_3 and its sterol precursors in irradiated first- or second-passage cultured keratinocytes and fibroblasts derived from newborn human foreskins (18). When we found that the small intracellular pool of 7-DHC in the cultured cells precluded photoproduction of significant amounts of D_3 following irradiation, we added AY-9944, a pharmacologic inhibitor of the Δ^7-reduction of 7-DHC to cholesterol (19-21), which resulted in the accumulation of large quantities of 7-DHC and D_3. Using this system we report here : (1) that both cultured keratinocytes and fibroblasts are capable of generating ^{3}H-D_3 via 7-DHC from ^{3}H-acetate after UV-B irradiation; and (2) that keratinocytes generate more D_3 per cell than skin-derived fibroblasts.

METHODS

Materials: (^{3}H) acetic acid (sodium salt) (1.8 Ci/mmol), (^{3}H) D_3 (18 Ci/mmol), (^{3}H) cholesterol (80 Ci/mmol) were obtained from New England Nuclear Corp. Unlabeled sterol esters, lanosterol, D_3, cholesterol, desmosterol, 7-DHC, and lathosterol were obtained from Sigma Chemical Co. Zymosterol was a gift from Dr. L.W. Parks, Oregon State University, Corvallis, OR. Pre-D_3, lumisterol, and tachysterol were prepared from irradiated 7-DHC (10). Tissue culture media and supplies were obtained from the cell culture facility at the University of California, San Francisco. Fetal calf serum (FCS) was purchased from Sterile Systems, Inc. AY-9944 was a gift from Dr. D. Dvornik, Ayerst Pharmaceuticals. HPLC grade n-hexane, 2-propanol, and ethyl acetate were purchased from Burdick and Jackson Chemicals. Samples were stored in 1.5 ml amber specimen vials (teflon-sealed) from Pierce Chemical Co.

Cells: Newborn human foreskin keratinocytes were co-cultivated with mitomycin-C-treated 3T3 feeder cells. Foreskin fibroblasts were grown in medium (DME) supplemented with 10% newborn calf serum.

Cell Treatments: Confluent first or second passage keratinocytes (99% fibroblast-free) or dermal fibroblasts were incubated with ^{3}H-acetate (250 μCi) overnight (37°C, 5% CO_2 in air) in cell culture medium containing 5-20% FCS, either untreated or delipidized (23), or in medium without serum and factors except antibiotics and amphotericin B. Following incubations, the cells were rinsed with ice-cold PBS, and designated dishes, kept at 4°C, were irradiated for 15-20 min. from above with a 100W mercury arc lamp equipped with a 295 nm barrier filter (WG-295, Schott Optical Co., Duryea, PA), or with a narrow pass 290 ± 5 nm filter (Oriel Corp., Stanford, CT) (total dose UV-B in both cases ≃ 0.5 J/cm^2). Following this treatment, irradiated and control cells were scraped, pelleted and the pellets were extracted at 4°C overnight in methanol:diethyl ether (2:7).

Preparation for HPLC: The organic phase of the cell extract was removed, dried under nitrogen gas and resuspended in 0.5 ml of 0.5% isopropanol in n-hexane. An aliquot of each sample was fractionated by HPLC on a Partisil-5 column (Whatman) equilibrated with 0.5% isopropanol-n-hexane (2 ml/min., 500 p.s.i.). Whereas 6% ethyl acetate in n-hexane was used to separate desmosterol from 7-DHC and lathosterol, D_3 and cholesterol co-migrated in this solvent system. D_3 could then be separated

from cholesterol and tachysterol in 0.2% isopropanol-n-hexane (4 ml/min, 1500 psi).

RESULTS

Keratinocytes and fibroblasts, labeled overnight with ^{3}H-acetate, produced radiolabeled compounds from irradiated 7-DHC that separated on HPLC into fractions that co-migrated with authentic sterol esters, lanosterol, zymosterol, pre-D_3, lumisterol, and tachysterol (Fig 1a). The UV spectra for authentic pre-D_3, D_3, tachysterol, and 7-DHC standards

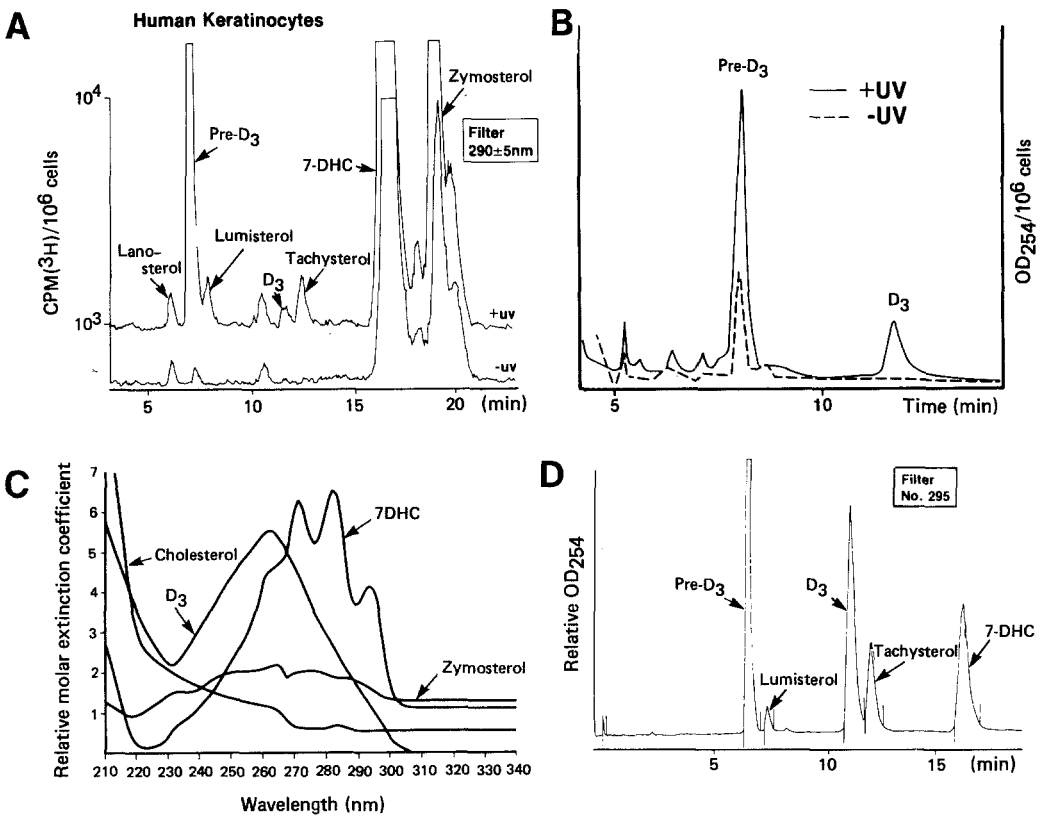

Figure 1: Characterization of the sterols produced by cultured human keratinocytes. (A) Normal phase HPLC separation of lipids extracted from ^{3}H-acetate-labeled keratinocytes with and without prior exposure to UV-B (290 ± 7nm). Pre-D_3, lumisterol, tachysterol and D_3 were generated in the irradiated cultures only. (B) The pre-D_3 regions from (A) with and without UV, were heated to 60°C under N_2 to convert pre-D_3 to D_3 and then they were re-chromatographed on HPLC (A_{254}) as shown. D_3 appeared exclusively from the pre-D_3 region of the irradiated cells. (C) The UV spectra generated by the peaks isolated from (A). The D_3 spectrum was taken from the D_3 isolated from (B). (D) Normal phase HPLC separation of sterols from UV-irradiated 7-dehydrocholesterol (HPLC solvent system for 1a and 1d, 0.2% isopropanol-n-hexane); for 1b, 0.5% isopropanol-n-hexane).

TABLE I: DISTRIBUTION OF RADIOLABELED ACETATE INTO VITAMIN D_3 AND ITS PRECURSORS IN KERATINOCYTES AND FIBROBLASTS: INFLUENCE OF AY-9944 (cpm)*

		CONDITIONS			
		CONTROL		AY-9944	
CELL TYPE	FRACTIONS/REGIONS	(+UV)	(-UV)	(+UV)	(-UV)
Fibroblasts	Pre-D_3	1,770	2,340	5,295	930
	D_3	0	0	735	0
	Cholesterol	42,795	52,680	0	0
	Desmosterol/7-DHC	8,595	12,240	23,190	38,685
	Zymosterol	0	0	5,940	5,415
Keratinocytes	Pre-D_3	3,615	3,825	24,180	2,100
	D_3	0	0	10,410	0
	Cholesterol	136,695	183,540	0	0
	7-DHC	150	150	65,940	71,820
	Zymosterol	0	0	53,595	45,765

*The cells were grown to confluence in DME + 20% FCS + cholera toxin, EFG and hydrocortisone, then incubated with 250 μCi ^{3}H-acetate and AY-9944 (1μg/ml) for 15 hours; results comparable to these were obtained when AY-9944 was used at 0.15 μg/ml. Some cells were irradiated with UV (>290 nm), then all were scraped, extracted and prepared for HPLC. An aliquot of each sample was injected into the HPLC.

were identical to the same HPLC-fractionated compounds generated by the cells (Fig. 1), and all of these compounds were definitively identified by mass spectrometry (24). The D_3 UV-spectrum, shown in Figure 1c, was produced by first heating the pre-D_3 fraction obtained from irradiated cells followed by rechromatography on HPLC to isolate the D_3 fraction.

Whereas small amounts of D_3 were formed by both keratinocytes and fibroblasts in irradiated, non-drug-treated cultures, addition of AY-9944 (0.15-0.3μg/ml), a dose two orders of magnitude below its LD_{50}, completely blocked cholesterol production in both fibroblasts and in keratinocytes, resulting in substantial increases in the levels of both 7-DHC and zymosterol (Fig. 1a, Table I). Lower doses yielded less 7-DHC, but even doses as low as 0.003 μg/ml produced substantial blockade (data not shown). In these experiments higher dose levels were employed in order to maximize the pool size available for photoconversion. In the doses employed here, AY-9944 did not exert toxic effects on the cells as evidenced by unaltered mitotic rates and unaltered rates of synthesis of non-polar lipids, free sterols and total lipid (Tables I & II)

TABLE II: INFLUENCE OF UV-B AND AY-9944 REMOVAL ON ACETATE INCORPORATION INTO VITAMIN D_3 AND OTHER STEROLS IN CULTURED KERATINOCYTES*

TIME(Hrs)	UV-B*	FRACTIONS (cpm) PRE-D_3	D_3	CHOL	7-DHC	ZYMOST	TOTAL COUNTS IN LIPID
(+AY) 0-24	+	17,732	7,634	0	48,356	39,303	8×10^6
	-	1,540	0	0	52,558	33,561	7×10^6
(+AY) (-AY) 0-24 24-48	+	3,916	15,114	0	175,835	110	15×10^6
	-	3,135	3,740	440	486,552	110	31×10^6
(+AY) 0-48	+	3,554	12,848	0	114,499	63,943	15×10^6
	-	4,785	3,091	0	345,059	99,187	32×10^6

*The cells were exposed to UV at 24 hrs while in PBS. All incubations were in DME + 5% FCS + 250 μCi ^{3}H-acetic acid ± AY-9944 (0.3 μg/ml) at 37°C in 5% CO_2/air. An aliquot (5-10%) of the total lipid extract was fractionated on HPLC.

Following UV-B treatment, 7-DHC levels decreased while pre-D_3 and D_3 peaks increased in both keratinocytes and fibroblasts (Fig. 1a, Table I). Keratinocytes produced more 7-DHC, pre-D_3, D_3, lumisterol, and tachysterol per cell than comparably treated fibroblasts (Table I). In the absence of UV-B both cell types produced small amounts of an unidentified compound that co-migrated on HPLC with pre-D_3, but did not yield D_3 upon heating (Fig. 1b). In non-irradiated samples the D_3 region was devoid of significant radioactivity (Fig. 1a, Table I). The efficiency of photoconversion of irradiated cellular 7-DHC to pre-D_3 and D_3 was as high as 70% (Tables I and II).

After removal of AY-9944, the keratinocytes continued to produce 7-DHC without zymosterol, as their main free sterol (Table II), without resumption of cholesterol production for an additional 5 days (data not shown). The pre-D_3 produced in samples irradiated at 24 hours was transformed to D_3 following a second 24 hour incubation at 37°C (nearly all of the D_3 appearing in the first 4-6 hours). Although UV-B irradiation cut the rate of 7-DHC synthesis in half, keratinocytes continued to produce both 7-DHC and zymosterol during the post-irradiation period (Table II).

DISCUSSION

These results demonstrate, for the first time, that both skin keratinocytes and fibroblasts generate vitamin D_3 following *in vitro* exposure to UV-B. The observation that keratinocytes produce more D_3 than fibroblasts can be explained by the fact that the former cells metabolize acetate to cholesterol, via 7-DHC, at a greater rate (Table I). Whereas previous studies of vitamin D_3 production in human skin have been limited to either human volunteers or short-term organ cultures, with the *in vitro* system described here, it will be possible to perform studies on the regulation of vitamin D_3 production by hormones, vitamin D metabolites, ions and other factors in homogeneous cell populations, and under precisely defined conditions. We have shown elsewhere that several factors (calcium, hydrocortisone, EGF), alone and in combination, can modulate 7-DHC levels in cultured human keratinocytes by as much as 30-fold (25), and that cultured keratinocytes generate several, as yet incompletely identified, D_3 metabolites from ^{3}H-25-OH-D_3 (26).

An important observation in these studies relates to the preferential utilization of the Δ^7 vs. Δ^{24} pathway by human keratinocytes. In contrast to other cell types (27-31), keratinocytes *in vivo* (32-34) and *in vitro* (these studies) produce 5-8 times more 7-DHC than desmosterol, indicating that the Δ^7-reductase may be the rate-limiting enzyme in post-lanosterol cholesterol biosynthesis in the epidermis, and pointing to the Δ^7 reductase as a potential control point of both cholesterologenesis and D_3 generation in the skin. The fact that the 7-DHC levels are much higher in epidermis *in vivo* than *in vitro*, further suggests that epidermal cells can adjust Δ^7-reductase activity over a wide range. Future studies will determine whether *in vivo* D_3 (via 7-DHC levels) is regulated not only by the dose of UV-B striking the skin, but also by factors such as those that control the D_3-endocrine system.

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