Biosynthesis of 3,4-didehydroretinol from retinol by human skin keratinocytes in culture

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The uptake and metabolism of radiolabelled retinol was studied in cultivated human skin cells. Normal epidermal keratinocytes in primary culture were able to incorporate unbound [$^{11,12-3}\text{H}$]all-*trans*-retinol from the growth medium and transform it into 3,4-didehydroretinol (dehydroretinol) in a dose- and time-dependent manner. A total of 23% of the radioactive label became cell-associated during a 48-h incubation period when added at 7 nM to differentiated keratinocytes submerged in serum-containing, high-calcium (1.56 mM) culture medium. At that time point, 25–30% of cell-bound radioactive retinol had been converted into dehydroretinol, with no labelled retinal, dehydroretinal, retinoic acid or dehydroretinoic acid being detected in cells or medium. Thus dehydroretinol, which occurs physiologically in mammalian skin tissue *in vivo*, was identified as the predominant neutral retinol metabolite in cultured keratinocytes using h.p.l.c. and anhydro-derivatization procedures.

At least 94% of the product, along with its precursor, was present in the cells in esterified form, with no traces of the compound being secreted into the cell environment. The rate of formation of dehydroretinol from its precursor was significantly lower in keratinocytes grown in serum-free, low-calcium (0.09 mM) culture medium, and in medium pre-incubated with excess unlabelled substrate. Furthermore, the application of 13-*cis*-retinoic acid (isotretinoin), a therapeutic retinoid drug known to markedly reduce dehydroretinol levels in human skin, blocked the biosynthesis of this metabolite in cultured keratinocytes. The 3,4-dehydrogenation pathway observed in this study could not be shown to operate to any significant extent in cultures of human epidermal melanocytes or dermal fibroblasts, supporting the hypothesis that keratinocytes represent the principal cell type involved in dehydroretinol formation from retinol in human skin.

INTRODUCTION

Human skin epidermis represents a stratified squamous epithelium containing tightly packed cells, most of which are keratinocytes. These cells undergo profound morphological and biochemical changes during their migration from the basal, proliferative position at the epidermal–dermal junction to the fully differentiated state at the skin surface. Human keratinocytes, like other mammalian epithelial cells, depend on an adequate supply of retinoids (retinol and derivatives possessing vitamin A activity) for proper maturation and proliferation (reviewed in [1]). The molecular mechanisms underlying the biological effects of these retinoids seem to be mediated through cytosolic retinoid binding proteins and, ultimately, a set of nuclear retinoid receptors (RARs and RXRs), which upon ligand and DNA binding regulate transcription of target genes [2–5]. So far, only all-*trans*-retinoic acid and its 9-*cis* stereoisomer [6,7] have been recognized as likely endogenous ligands for the nuclear retinoid receptors. Considering the multitude of different cellular retinoid receptors expressed, and the diverse cellular responses to individual retinoids, it seems likely that vitamin A-dependent epithelial cells are capable of metabolizing internalized retinol into a wide range of biologically active products.

Quantitative studies of vitamin A in human skin have revealed the presence of two major neutral retinoids, namely all-*trans*-retinol (vitamin A₁) and 3,4-didehydroretinol (referred to as dehydroretinol or vitamin A₂), and their esters [8,9]. Dehydroretinol was originally detected in the livers of fresh-water fish [10], and has not as yet been detected in non-cutaneous human

tissues. This form of vitamin A constitutes 20–25% of the total retinoid content in normal human epidermis, and is less abundant in the underlying dermis (2–10%) and subcutis (<1%) [11]. Dehydroretinol levels are markedly elevated in the skin of humans with hyperproliferative dermatoses such as eczema [12], psoriasis [13] and basal cell carcinoma [14], as well as in human epidermis stimulated to hyperproliferation by tape-stripping [13]. The biological relevance of the compartmentalization of dehydroretinol within normal and diseased skin tissue is not clear. Since dehydroretinol does not appear in the blood, its presence in skin has been attributed to local metabolism of retinol [11]. In confirmation of this, it was demonstrated in organ cultures of human whole skin that retinol is a precursor of dehydroretinol [15]. However, which cell(s) and enzyme(s) of the skin are specifically involved in the biogenesis of dehydroretinol is yet not known. Furthermore, its metabolism and biological role in normal and diseased epithelial conditions remains to be established. In the present study we have demonstrated that cultures of normal human keratinocytes, unlike epidermal melanocytes or dermal fibroblasts, have the potential to generate significant amounts of dehydroretinol from labelled retinol added to the culture medium.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, culture media and supplements were supplied by Gibco (Uxbridge, Middx., U.K.). Joklik's modified Eagle's medium, trypsin, epidermal growth factor (EGF), cor-

Abbreviations used: RAR, retinoic acid receptors; RXR, retinoid X receptor; EGF, epidermal growth factor; FCS, fetal calf serum; DMSO, dimethyl sulphoxide; RBP, retinol-binding protein.

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tisol, cholera toxin, dibutyryl cyclic AMP and geneticin (G-418) were purchased from Sigma (St. Louis, MO, U.S.A.). Melanocyte medium (PC-1) was from Ventrex (Portland, ME, U.S.A.). Basic fibroblast growth factor was from Promega (Madison, WI, U.S.A.). Fetal calf serum (FCS), non-essential amino acids, L-glutamine, penicillin, streptomycin and fungizone were from NordCell (Stockholm, Sweden). Dispase II (neutral protease) and collagenase were from Boehringer-Mannheim (Mannheim, Germany). H.p.l.c.-grade solvents were purchased from Rathburn Chemicals (Walkerburn, Scotland, U.K.).

[11,12-³H]All-*trans*-retinol (sp. radioactivity 47.9 Ci/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA, U.S.A.). Stock solutions of radioactive retinol (1 μ Ci/ μ l) were obtained in ethanol, and working solutions (0.1–0.25 μ Ci/ μ l) were prepared by diluting stock solutions with dimethyl sulphoxide (DMSO).

The authentic retinoid standards all-*trans*-retinol, 13-*cis*-retinol, 3,4-didehydroretinol, 3,4-didehydroretinal, 3,4-didehydroretinoic acid, 4-hydroxyretinoic acid, 4-ketoretinoic acid, 5,6-epoxyretinoic acid, 9-*cis*-retinoic acid and 13-*cis*-retinoic acid, and the internal standards Ro 12-0586 (the aromatic analogue of all-*trans*-retinol) and acitretin, were generously provided by Hoffmann-La Roche (Basle, Switzerland). Unlabelled retinal and all-*trans*-retinoic acid were purchased from Sigma. Before being used, the purity (> 97%) of labelled and unlabelled retinoids was determined by h.p.l.c. as described below.

Cultures of human keratinocytes, melanocytes and fibroblasts

Epidermal keratinocytes were obtained from juvenile foreskins at circumcision or from mammary skin removed during reduction plastic surgery. The specimen was washed thoroughly with PBS and the subcutis, including the lower dermis, was excised. The remaining tissue was minced finely and the epidermis was detached from the dermis using 2 mg/ml dispase for 15–20 h at 4 °C. The epidermal sheets were incubated in trypsin (0.1% in PBS) for 1 h at 36 °C. A suspension of single cells was prepared by vigorous pipetting, and this was centrifuged for 2 min at 300 *g*. The pellet was resuspended in culture medium and inoculated at high plating density (10⁵ cells/cm²) in Nunclon 6-well plastic dishes (Nunc, Roskilde, Denmark) without using a feeder layer of fibroblasts. Culture was routinely performed in 'serum-containing keratinocyte medium', i.e. Dulbecco's modified Eagle's medium plus Ham's F12 medium (3:1, v/v) supplemented with 5% FCS, 0.4 μ g/ml cortisol, 2 mM L-glutamine, 5.3 μ g/ml insulin (Humulin NPH), 0.1 nM cholera toxin, non-essential amino acids, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 2.5 μ g/ml fungizone. The pH of the final medium was 7.2, and the calcium concentration was 1.56 mM as determined by atomic absorption spectrometry. Primary cultures were maintained in a humidified incubator at 36 °C in an atmosphere of 5% CO₂ and 95% air. The growth medium was changed on day 4 and then every 3 days until confluency at day 12. EGF (10 ng/ml) was added to the cultures on day 4 and at subsequent medium replacements. The culture condition promoted proliferation of epitheloid cells, forming within 1 week a semi-confluent monolayer containing numerous islands of stratified, highly differentiated cells displaying electron microscopic features typical of keratinocytes, i.e. desmosomes, keratohyalin granules and tonofilaments.

In one series of experiments, a serum-free growth medium (Gibco's serum-free keratinocyte medium) containing a low calcium concentration (0.09 mM) and no detectable retinol (see Table 1) was used. This medium was supplemented with EGF

(10 ng/ml) and bovine pituitary extract (50 μ g/ml) as recommended by the manufacturer, and is referred to as 'serum-free keratinocyte medium'. In this medium, the cells maintained a less differentiated (keratinized) phenotype, as previously described by Peehl and Ham [16].

Epidermal melanocytes were prepared from finely minced human breast skin obtained at reconstruction surgery [17]. The specimens were incubated for 60 min at 37 °C in Joklik's modified medium containing 0.25% trypsin, 0.10% EDTA, 100 units/ml penicillin and 0.1 mg/ml streptomycin. The epidermis was detached from the dermis and transferred to a test-tube containing PC-1 medium, and then vigorously vortexed for 1 min. The cell suspension was centrifuged and the pellet was resolved in PC-1 medium supplemented with 50 units/ml penicillin, 0.05 mg/ml streptomycin, 2 mM L-glutamine, 5 ng/ml basic fibroblast growth factor and 0.5 mM dibutyryl cyclic AMP ('melanocyte medium'). The cells were seeded into a 75 cm² culture flask (Sigma) and the culture medium was changed every 3 days. At day 10, the medium was supplemented with 45 μ g/ml geneticin in order to selectively inhibit fibroblast growth [18]. When a pure melanocyte culture was obtained at day 14, the cells were trypsin-treated and subcultured in Nunclon 6-well plates in melanocyte medium for another 14 days until they reached semi-confluency prior to experimentation.

Dermal fibroblasts were prepared from human foreskins. Briefly, minced dermal tissue was incubated for 20 h at 36 °C in serum-supplemented keratinocyte medium containing 1 mg/ml collagenase (type V; Sigma). The cell suspension was vigorously pipetted up and down and then centrifuged at 300 *g* for 3 min. The pellet was washed three times with 10 ml of PBS and then inoculated at 10⁵ cells/cm² into 6-well plates. In order to make relevant comparisons with the keratinocyte experiments, the fibroblasts were grown in serum-containing keratinocyte medium for 14 days under conditions identical to those described for keratinocytes.

Incubation of cell cultures with retinoids

The metabolic fate of ³H-labelled retinol was studied in submerged cultures of keratinocytes, melanocytes and fibroblasts at different time points covering a 48-h incubation period. Immediately before the addition of labelled retinol, the conditioned culture medium was replaced with fresh medium (3 ml/well) supplemented with ascorbic acid (50 μ g/ml) to reduce unspecific isomerization (by 5–10%; results not shown) of retinoid alcohols in the medium during the incubation period. To each culture well (containing on average 3 × 10⁵ keratinocytes, 1.4 × 10⁵ fibroblasts or 0.6 × 10⁵ melanocytes per cm² surface area) was added a 1 μ Ci portion of [11,12-³H]all-*trans*-retinol (0.33 μ Ci/ml of medium; 7 nM) dissolved in 10 μ l of DMSO. In some experiments, unlabelled all-*trans*-retinol or 13-*cis*-retinoic acid was added to the cultures 15 min before supplementation with radioactive retinol.

In each set of experiments, cells from the same donor were used to diminish the variability of the results. Controls consisted of cultured cells treated with 35% formalin for 1 min immediately before radioisotope addition.

All operations involving retinoids were carried out in dim yellow light.

Extraction and analysis of retinoids

At various incubation times, ranging from 0.5 to 48 h after [³H]retinol supplementation, the medium was transferred into test tubes. The cells were rinsed three times with 2 ml of ice-cold

PBS containing 1 mg/ml BSA. The washing fluid was then added to the medium previously collected. The cells in each culture well were released using 0.1 % trypsin at 36 °C for 1 min. The cells were transferred to a glass tube and collected by centrifugation for 5 min at 300 g, washed twice with PBS, and then stored under nitrogen at -70 °C for not more than 4 weeks before being analysed.

Neutral and acidic retinoids were analysed separately using a two-step extraction procedure as described previously [19]. Briefly, the cells (or 0.5 ml of culture medium) were spiked with authentic unlabelled retinoids (for analysis of labelled retinoids) or internal standards (for analysis of endogenous retinoids) and then saponified using ethanolic KOH at 80 °C for 15 min. By this procedure, esterified retinoids were hydrolysed into free alcohols, thus facilitating h.p.l.c. separation and improving detectability. Following hydrolysis, the samples were vortex-mixed and the neutral retinoids were extracted twice with n-hexane. The combined organic layers were evaporated at 40 °C under a stream of nitrogen. The residues (containing neutral retinoids) were suspended in 50 µl of methanol, centrifuged for 5 min at 1300 g, and then subjected to reversed-phase h.p.l.c. The remaining aqueous layer was adjusted to pH 5-6 by the addition of HCl and subsequently extracted once with n-hexane. The evaporated hexane layer (containing acidic retinoids) was reconstituted in 50 µl methanol and injected on to the h.p.l.c. column. The protein content of the aqueous residues was determined by the Biuret method [20] using BSA as standard.

Reversed-phase h.p.l.c. separation of retinoids was performed as described [19] using a Nucleosil ODS column containing 5 µm particles (Scandinaviska Genetec, Kungsbacka, Sweden) eluted at 1.2 ml/min with acetonitrile/water/acetic acid (72:18:0.05, by vol.). Non-labelled retinoids were monitored using two u.v. detectors (uvMonitor D; LDC/Milton Roy) connected in series and operating at 325 and 360 nm respectively. The eluate was collected at 20- or 30-s intervals and mixed with 4 ml of Emulsifier scintillant (Packard). The radioactivity of each fraction was determined in a Philips PW 4700 model liquid scintillation counter at 30 % counting efficiency for ³H. The concentrations of unlabelled retinol and dehydroretinol were quantified by relating their peak heights at 360 nm to the internal standard. Normal-phase h.p.l.c. was performed in one experiment using a Nucleosil silica column (Scandinaviska Genetec) eluted with hexane/ethyl acetate/ethanol (85:15:0.05, by vol.) at a flow rate of 1.2 ml/min.

The proportions of esterified versus free radioactive [³H]-retinoids were determined in cultured keratinocytes after a 24 h-incubation with 1 µCi of [³H]retinol. Cells were harvested and disrupted in 0.5 ml of water using a Potter-Elvehjem homogenizer. Spectrographic ethanol (0.5 ml) and 8 ml of n-hexane were added and the mixture was vigorously shaken and centrifuged. An 80 % portion of the upper layer was dried under a stream of nitrogen, redissolved in 50 µl of methanol and subjected to reversed-phase h.p.l.c. and scintillation counting. The remaining 20 % was saponified as described above and then subjected to reversed-phase chromatography. The ratio of radioactivity eluting at the position of dehydroretinol in the unhydrolysed compared with the hydrolysed samples was calculated in order to estimate the proportion of esterified retinol [21]. The corresponding ratio was determined for retinol.

The anhydro derivative of dehydroretinol was obtained by dehydration using ethanolic HCl [22].

The total radioactivity content of keratinocytes was determined by lysing the cell pellet from each well in 0.4 ml of Soluene-350 (Packard) at 40 °C for 2 h, with subsequent scintillation counting as described above.

RESULTS

Contents of neutral retinoids in keratinocyte culture media and cells

The concentrations of neutral retinoids in the two different culture media used for keratinocytes were measured by h.p.l.c. (Table 1). The serum-containing medium (supplemented with 5 % FCS) contained 4.7 ng/ml (17 nM) retinol, i.e. approx. 1 % of the physiological retinol level in human serum. This concentration of the retinol is inadequate to fully suppress keratinization of cultured human keratinocytes. Using the serum-containing medium, the molar concentration of [³H]retinol attained by the standard addition of 1 µCi was 7 nM, i.e. 42 % of the unlabelled retinol in the medium.

The serum-free medium lacked measurable retinol, and neither of the two media used contained detectable dehydroretinol.

The contents of neutral retinoids were determined in two populations of epidermal cells obtained from the same specimens, i.e. cells collected immediately prior to plating (day 0), and cells grown in serum-containing or serum-free medium and collected before retinoid incubation (day 14). Based on protein content, the concentration of retinol in cultured keratinocytes was somewhat higher than that in freshly isolated epidermal cells, despite the relatively low retinol content of the medium. Thus vitamin A derived from 5 % FCS seems to be sufficient to maintain physiological retinol levels in keratinocytes in primary culture. By contrast, the dehydroretinol content of the cells was markedly increased by 14 days of culture, approaching the dehydroretinol/retinol ratio in hyperproliferative human epidermis [13]. Keratinocytes grown in serum-free medium contained no detectable retinol or dehydroretinol.

Uptake of radioactive retinol by primary keratinocytes in culture

The time-dependent distribution of [³H]retinol was studied in keratinocyte cultures by measuring the total radioactivity taken up by the cells during the 48 h incubation period (Figure 1). The tracer was found to be increasingly associated with cells over time. Within 6 h of administration, 16 % of the applied radioactivity had been taken up by the cells. The rapid initial phase of

Table 1 Retinol and dehydroretinol concentrations in human keratinocytes and culture media

Concentrations of neutral retinoids were determined in epidermal cells isolated from human skin by trypsin treatment (i.e. immediately prior to culture), and in keratinocytes after culture in serum-containing or serum-free medium for 14 days. Samples of cells were washed twice in PBS containing BSA, and saponified before being extracted with n-hexane and subjected to reversed-phase h.p.l.c. Values represent means ± S.D. of triplicates. The retinoid concentrations of adult normal human epidermis (shave biopsies) are from [12]. n.d., not detected (< 0.1 ng/mg or ml)

Sample	Medium	Neutral retinoid (ng/ml or mg of protein)	
		Retinol	Dehydroretinol
Medium	Serum-containing	4.7 ± 1.6	n.d.
	Serum-free	n.d.	n.d.
Epidermal cell suspension	Collected before plating	0.9 ± 0.2	0.2 ± 0.1
Keratinocytes in culture	Serum-containing medium	1.6 ± 0.3	1.1 ± 0.2
	Serum-free medium	n.d.	n.d.
Human epidermis		1.6 ± 0.4	0.4 ± 0.2

Figure 1 Time course of the uptake of [^3H]retinol from growth medium into human skin keratinocytes

Epidermal keratinocytes were isolated and cultured in serum-containing medium for 14 days as described in the text. Primary cells were incubated at 36 °C with 1 μCi of unbound [^3H]retinol for the time periods indicated and subsequently washed, harvested and solubilized. Cell-associated total radioactivity was counted and related to radioactivity applied. Results are means $\pm$ S.D.

uptake was followed by a slower rate of incorporation, reaching a plateau by 24–48 h. At the end of the incubation, approx. 23 % of the total radioactivity added was associated with the cells. Based on volume, there was an almost 100-fold accumulation of the label in the cell compartment as compared with the medium.

Identification of esterified [^3H]dehydrorretinol as a major neutral metabolite of [^3H]retinol in cultured keratinocytes

Figure 2 shows a representative h.p.l.c. elution profile of [^3H]retinol and its metabolites. Keratinocytes incubated with the radioisotope were hydrolysed and the neutral and acidic retinoids were separately extracted before reversed-phase h.p.l.c. The neutral extract (Figure 2a) contained predominantly the all-*trans* form of [^3H]retinol, with [^3H]13-*cis*-retinol comprising 10–15 % of the parent compound. The presence of 13-*cis*-retinol, however, did not represent metabolism, but was due to inevitable spontaneous isomerization of the tracer during retinoid analysis [19], since the same extent of isomerization occurred when medium without cells was incubated with the label. In contrast, material not present in the culture medium eluted slightly ahead of 13-*cis*-retinol and baseline-separated from it. This peak (marked 1 in Figure 2a) co-eluted with authentic dehydrorretinol and was present in the neutral extract of cultured keratinocytes, but not in formalin-treated keratinocytes or cultured skin fibroblasts. In primary cultures of human epidermal melanocytes incubated with [^3H]retinal, only minute amounts of [^3H]dehydrorretinal (less than 1.7 % of labelled retinol) could be detected.

A small polar peak eluting at 5 min, and not corresponding to any of the available reference retinoids, appeared in all cell types studied. This peak was not further characterized. Neither [^3H]all-*trans*-retinol nor [^3H]dehydrorretinol were observed as products of the metabolism of radioactive retinol by the cells. Hence, except for the dehydrorretinol found in keratinocytes, no cell-type-specific h.p.l.c. peak appeared.

The acidic cell extract contained only 10 % of the total cell-associated radioactivity. In the three cell types examined, a range

Figure 2 H.p.l.c. elution profile of [^3H]retinol and its metabolites formed in primary cultures of human keratinocytes

Keratinocyte cultures were supplemented with 1 μCi of [^3H]retinol. After a 48 h incubation period, cells were washed, harvested and hydrolysed using ethanolic KOH. Neutral (a) and acidic (b) retinoids were extracted separately with n-hexane and monitored by reversed-phase h.p.l.c. using a Nucleosil ODS 5 μm column followed by liquid scintillation counting. The eluent (1.2 ml/min) was 18 % water and 0.05 % acetic acid in acetonitrile. Authentic retinoids eluted from the column as marked. Standards: 1, dehydrorretinol; 2, 13-*cis*-retinol; 3, all-*trans*-retinol; 4, retinal; 5, 4-ketoretinoic acid plus 4-hydroxyretinoic acid; 6, 5,6-epoxyretinoic acid; 7, dehydrorretinoic acid; 8, 13-*cis*-retinoic acid; 9, 9-*cis*-retinoic acid; 10, all-*trans*-retinoic acid.

of polar h.p.l.c. peaks of low radioactivity appeared (Figure 2b). The h.p.l.c. pattern was similar in intact and formalin-exposed cells. With a relative detectability of about 0.1 % of the amount of [^3H]retinol added, we were not able to observe the formation of labelled retinoic acid, 13-*cis*-retinoic acid or dehydrorretinoic acid in any cell type or culture medium under study. A small peak co-eluting with both 4-hydroxy- and 4-keto-retinoic acid appeared irregularly and was not chemically characterized.

The reversed-phase h.p.l.c. peak co-eluting with unlabelled dehydrorretinol (Figure 2a, peak 1) was further investigated by re-chromatography and derivatization. One portion of the h.p.l.c. fraction collected was dried, redissolved in n-hexane and then subjected to normal-phase re-chromatography and scintillation counting. The eluate contained a single major radioactive peak co-eluting with unlabelled dehydrorretinol (Figure 3a). A second portion of the putative dehydrorretinol peak was prepared for anhydro-derivatization by reaction with ethanolic HCl. The product obtained was redissolved in methanol, and then subjected to re-chromatography and liquid scintillation counting as described above. The eluate contained two distinct radioactive components; one minor peak co-eluting with unlabelled dehydrorretinol, and one major peak co-eluting with the unlabelled anhydro-derivative of dehydrorretinol (Figure 3b).

Figure 3 Identification of [^3H]dehydroretinol by normal-phase re-chromatography, and by anhydro derivatization and subsequent reversed-phase h.p.l.c.

The neutral h.p.l.c. peak marked 1 (see Figure 2a) was collected and further characterized by two different procedures. One-half of the sample was lyophilized, redissolved in 50 μl of hexane and then rechromatographed using a Nucleosil silica column eluted with n-hexane/ethyl acetate/ethanol (85:15:0.05, by vol.) at a flow rate of 1.2 ml/min. The peak eluting at 8.2 min (a; peak I) co-eluted with that of authentic dehydroretinol. The second part of the sample was mixed with non-labelled authentic dehydroretinol and the corresponding anhydro derivative was prepared by reaction with ethanolic HCl as described in the text. The mixture was extracted with n-hexane and evaporated. The sample was redissolved in 50 μl of methanol, and then injected on to reversed-phase h.p.l.c. using the same chromatographic conditions as described in the legend to Figure 2. The two radioactive peaks eluting at 6.8 min (peak II) and 14.8 min (peak III) co-eluted with non-labelled dehydroretinol and the anhydro derivative of dehydroretinol respectively.

By comparing saponified and non-saponified keratinocyte extracts, it was found that most of the labelled retinol and dehydroretinol was present as esters. The proportions of retinyl and dehydroretinyl esters formed after a 48-h incubation period with [^3H]retinol were 96.0 and 94.4% respectively of the total (esters plus free alcohols). These values are higher than the percentages of esterified retinoid present in human skin tissues *in vivo* [8].

Time- and concentration-dependence of the conversion of [^3H]retinol to [^3H]dehydroretinol by keratinocytes in culture

The time course of [^3H]dehydroretinol production by cultured keratinocytes was studied during a 48-h incubation period. Trace amounts of dehydroretinol were formed from [^3H]retinol within

Figure 4 Time-dependent biosynthesis of [^3H]dehydroretinol from [^3H]retinol by cultures of human skin cells

Keratinocytes (■), melanocytes (○) and fibroblasts (▲) were cultured separately for 14 days. Cells were incubated with 1 μCi of [^3H]retinol at 36 $^{\circ}\text{C}$. After the indicated periods of time (1–48 h), cells were washed, harvested and hydrolysed. Unlabelled reference compounds were added to the hydrolysate, and the retinoids were extracted with n-hexane and monitored by h.p.l.c. analysis and scintillation counting for quantification of [^3H]dehydroretinol content. Values represent means $\pm$ S.D. of duplicates; melanocytes were assayed in single experiments.

3 h, and increasing concentrations of the product appeared in cells over time, levelling off by 24–48 h (Figure 4). At the end of the incubation period, the proportion of labelled dehydroretinol amounted to 25–30% of the [^3H]retinol recovered from the cells, which is slightly higher than that of the endogenous counterparts previously observed in normal human epidermis [11]. Over the same time period, and at the same concentrations of added [^3H]retinol, no significant conversion to [^3H]dehydroretinol could be observed in either melanocytes or fibroblasts.

The concentration-dependence of [^3H]dehydroretinol production by keratinocytes was studied by incubating cells for 48 h with 0.01–10 μCi of [^3H]retinol, corresponding to 0.07–70 nM. Over this concentration range there was a nearly linear relationship between the amounts of substrate added and product formed (Figure 5). Saturation was not attained with the trace amounts of radioactive isotope applied.

Dose-response inhibition of [^3H]dehydroretinol biosynthesis from [^3H]retinol by pre-incubation with unlabelled substrate

In a series of experiments, unlabelled retinol was added at increasing concentrations (0–6 μM) to keratinocyte cultures 15 min prior to incubation with a standard dose (1 μCi , 7 nM) of [^3H]retinol. Cells were harvested after co-incubation with the two retinoids for 24 h. Figure 6 displays a biphasic response of cell-associated [^3H]retinol radioactivity following the addition of increasing concentrations of unlabelled substrate. At the lower concentration range of retinol there was a reduction in cell-bound [^3H]retinol, whereas higher concentrations of unlabelled retinol were accompanied by increasing [^3H]retinol levels. The first response may be due to restricted uptake of the labelled substrate as a result of isotope dilution, resulting in decreased levels of intracellular [^3H]retinol, including its dehydro metabolite. The second response is not so readily explained, but may reflect inhibition by excess substrate of enzymes generating

Figure 5 Dose-dependent conversion of $[^3\text{H}]\text{retinol}$ to $[^3\text{H}]\text{dehydroretinol}$ by primary cultures of keratinocytes

Primary cultures of keratinocytes were grown to confluency in serum-containing medium for 14 days and then incubated with trace amounts of $[^3\text{H}]\text{retinol}$ (concentration range 0.07–70 nM). After 48 h the cells were washed, hydrolysed and extracted with n-hexane for reversed-phase h.p.l.c. analysis and scintillation counting.

Figure 6 Effect of increasing concentrations of unlabelled retinol on $[^3\text{H}]\text{dehydroretinol}$ production from $[^3\text{H}]\text{retinol}$ by cultured keratinocytes

Primary cells were grown to confluency in serum-containing medium and then incubated with unlabelled retinol (concentration range 0–6 μM) for 15 min prior to the addition of 1 μCi of $[^3\text{H}]\text{retinol}$ (7 nM). After a 24 h incubation period the cells were washed, hydrolysed and extracted with n-hexane prior to h.p.l.c. analysis of cell-associated $[^3\text{H}]\text{retinol}$ ($\square$) and $[^3\text{H}]\text{dehydroretinol}$ ($\blacktriangle$) radioactivity. An equal volume of DMSO alone was added to the control.

Figure 7 Effect of increasing concentrations of 13-*cis*-retinoic acid on the conversion of $[^3\text{H}]\text{retinol}$ to $[^3\text{H}]\text{dehydroretinol}$ in cultured keratinocytes

Primary keratinocytes were cultured to confluency in serum-containing medium and then exposed to the amount of unlabelled 13-*cis*-retinoic acid indicated for 30 min before addition of 1 μCi of $[^3\text{H}]\text{retinol}$. Cells were collected, hydrolysed and subjected to h.p.l.c. analysis of $[^3\text{H}]\text{retinol}$ ($\square$) and $[^3\text{H}]\text{dehydroretinol}$ ($\blacktriangle$) radioactivity. An equal volume of the retinoid solvent (DMSO) was added to reference cultures.

$[^3\text{H}]\text{dehydroretinol}$ in addition to other retinol-metabolizing pathways. At 50 nM unlabelled retinol (i.e. 3 times the baseline medium retinol level, and 7 times that of its labelled counterpart) there was a 35% reduction in $[^3\text{H}]\text{dehydroretinol}$ production. Cell-associated $[^3\text{H}]\text{dehydroretinol}$ decreased by 60–70% in the presence of 0.25–4 μM retinol. At the final concentration of 6 μM unlabelled retinol, the biosynthesis of $[^3\text{H}]\text{dehydroretinol}$ was almost completely inhibited.

Inhibition of $[^3\text{H}]\text{dehydroretinol}$ production by 13-*cis*-retinoic acid

Since oral 13-*cis*-retinoic acid (isotretinoin) therapy in man is known to affect the cutaneous vitamin A composition, we studied the effect of pre-incubating keratinocytes with non-radioactive 13-*cis*-retinoic acid prior to administration of labelled retinol. As in human epidermis *in vivo*, there was a significant suppression of the dehydroretinol concentration following exposure to unlabelled 13-*cis*-retinoic acid. Compared with unlabelled retinol, this 13-*cis*-retinoic acid was about 40 times more potent in inhibiting dehydroretinol biogenesis. An inhibitory effect on product formation was seen even at the lowest concentration of isotretinoin applied (15 nM; i.e. twice the concentration of labelled retinol in the medium) (Figure 7). The average reduction in cell-associated $[^3\text{H}]\text{dehydroretinol}$ was 50% in the presence of 25 nM 13-*cis*-retinoic acid. At pharmacological concentrations of this drug (1 μM), complete suppression of dehydroretinol synthesis was seen. In contrast, the concentration of cell-bound $[^3\text{H}]\text{retinol}$ increased markedly with increasing amounts of 13-*cis*-retinoic acid. At the highest concentration of 13-*cis*-retinoic acid applied there was a nearly 300% increase in the keratinocyte $[^3\text{H}]\text{retinol}$ concentration relative to control incubations containing vehicle only. The increase in cellular $[^3\text{H}]\text{retinol}$ content was greater than could be explained solely by a concomitant reduction in $[^3\text{H}]\text{dehydroretinol}$ synthesis. Taken together, these findings may suggest several possible interactions of isotretinoin with the uptake and metabolism of retinol in keratinocytes, e.g. (a) stimulating retinol uptake, (b) inhibition of retinol conversion

Figure 8 Effect of different growth media on the production of [^3H]dehydroretinol from [^3H]retinol by cultured human keratinocytes

Primary keratinocytes were cultured in either serum-containing (5% FCS), high-calcium (1.56 mM) medium (□) or in serum-free, low-calcium (0.09 mM) keratinocyte medium (●) for 14 days. Cultures were supplemented with 1 μCi of [^3H]retinol and incubations at 36 °C were terminated after various time periods by collection and hydrolysis of keratinocytes. The [^3H]dehydroretinol production by the cells was determined by reversed-phase h.p.l.c. and scintillation counting. Except for the different media used, experimental conditions were identical. Values represent means $\pm$ S.D. of duplicates.

into dehydroretinol and other metabolites, and (c) enhanced metabolism of dehydroretinol.

Effect of different culture media on [^3H]dehydroretinol biosynthesis

To judge whether the composition of the keratinocyte medium affects the production of dehydroretinol from retinol, cells grown in either serum-containing or serum-free medium were exposed to labelled retinol. Except for this difference, the experimental conditions were identical. The serum-free environment allowed the keratinocytes to grow in a monolayer composed of rather loosely packed, non-stratified cells. As calculated on a cell number basis, the uptake of tracer was similar in both types of cultures. Figure 8 shows that cells grown under serum-free conditions were also able to generate [^3H]dehydroretinol from [^3H]retinol, but at a lower rate than cells cultivated in serum-supplemented medium (Figure 8). Whether this was due to differences in the composition of the medium components *per se* or was related to the differentiated state of the cells remains to be elucidated.

DISCUSSION

Studies on the epithelial cell metabolism of endogenous retinoids have focused mainly on sequential end-chain oxidations of retinol, generating retinal, retinoic acid and other biologically active products. Since retinoic acid is generally believed to be the single most potent retinoid in terms of epidermal differentiation, attempts have been made to identify this particular metabolite in human skin cells or tissues. Siegenthaler et al. demonstrated that both retinal and retinoic acid are produced to some extent from physiological concentrations of radioactive retinol incubated with cytosolic extracts of highly differentiated human keratinocytes in the presence of cofactors [23]. In contrast, these metabolites were difficult to detect when cytosolic extracts of

non-differentiated keratinocytes, e.g. cells cultured in low-calcium medium, were incubated. In the present study, using cultured skin cells growing at either low (0.09 mM) or high (1.56 mM) calcium concentrations, we could not identify with certainty either retinal or retinoic acid production. The difficulty in detecting these species in our study might be a matter of assay sensitivity, since we added only trace amounts (70 nM at most) of the substrate. In addition, the methodology used involved hydrolysis and heating of the sample, which is known to cause isomerizations, thus possibly making minute amounts of these retinoids undetectable. A biological explanation may be that cultured cells metabolize retinol more extensively and diversely than do cytosolic extracts. Hence there may be a faster turnover of retinol intermediates such as retinoic acid by intact cells compared with cell-free systems.

Physiologically, retinol is delivered to target tissues as a complex with a specific carrier protein, retinol-binding protein (RBP), which travels in the blood in association with transthyretin. It is thought that cellular uptake of retinol takes place via a cell-surface RBP receptor, allowing RBP-bound retinol to be translocated into the cell. In human epidermis the presence of a cell-membrane RBP receptor has been suggested on the basis of indirect evidence [24], although a recent study by Hodam et al. [25] using cultured human keratinocytes failed to support the idea of a mandatory role for RBP in the uptake of retinol. Over a 57-h incubation period, these authors found similar total uptake of retinol whether it was added RBP-bound or unbound to the serum-free culture medium. They proposed that retinol is dissociated from the RBP before being associated with the cell membrane and internalized. As was found in our study using labelled free retinol dissolved in DMSO, a rapid uptake of radioactivity by keratinocytes was observed during the first 3–6 h of incubation, with a slower rate of incorporation subsequently. After 24 h, about 22% of the added label was cell-associated in our study, which is similar to the value of 30% found by Hodam et al. [25] using ethanol as the vehicle.

The present study demonstrates that significant metabolism of retinol into dehydroretinol takes place in differentiated human keratinocytes in primary culture. Thus retinol, once incorporated from the extracellular space, seems not only to be subjected to oxidative metabolism and esterification, but also serves, directly or via intermediates, as a substrate for dehydrogenation of the ring. Two metabolic pathways, therefore, may operate in parallel in epidermal keratinocytes; one generating retinoic acid via retinal as demonstrated in the cell-free system [23], and one generating dehydroretinoic acid via dehydroretinal as suggested by Vahlquist and Törmä [26]. In confirmation of this, the conversion of retinol into dehydroretinol was recently shown to occur in a suspension of disaggregated epidermal cells obtained from human skin, particularly in cells originating from the suprabasal (i.e. differentiated) layers of the epidermis [27]. These findings are in agreement with the present observation that, in keratinocytes cultured in the presence of serum and a high calcium concentration (i.e. conditions favouring terminal cell differentiation), the transformation of retinol into dehydroretinol takes place more efficiently than in less differentiated cells. The possibility cannot be ruled out, however, that differences in the components of the serum-containing media other than calcium, natural retinoids and RBP may also affect dehydroretinol biosynthesis. Hence identification of which factors in the culture environment, and what state of cell differentiation, will specifically affect C-3 and C-4 dehydrogenation of retinol requires further investigation.

The subcellular localization of the dehydrogenase activity converting retinol into dehydroretinol is not yet known. The

enzyme(s) may belong to the family of retinol or ethanol dehydrogenases, or represent a specific retinol dehydrogenase. Also, the question remains as to whether the dehydrogenation activity is restricted to keratinizing epithelium exclusively, or operates in other epithelia as well.

In addition to retinol, other precursors should be considered in the biosynthesis of dehydrorretinol. As shown in fresh-water fish, 3-hydroxyretinol (via 3-hydroxyanhydrorretinol) may be a substrate for dehydrorretinol production [28]. Other putative metabolic pathways for dehydrorretinol generation include central cleavage of carotenoids such as anhydrolutein [29].

The biological function and activity of dehydrorretinol in epithelial tissue has yet to be established. Interestingly, its putative metabolite, dehydrorretinoic acid, seems to share with retinoic acid the property of acting as a morphogen in embryonic limb development [30]. Whether dehydrorretinoids exert a corresponding role in the differentiation of epithelial tissue is, however, not known. So far, no specific biological response or selective receptor activity for dehydrorretinoids has been demonstrated to occur in human skin. Dehydrorretinoic acid apparently binds *in vitro* to RARs and is equally as active as retinoic acid in terms of induction of parakeratosis in the reconstructed skin model, and in inhibiting epidermal transglutaminase activity in cultured human keratinocytes (D. Asselineau and H. Törmä, personal communication). High concentrations of its precursor have been found in human skin in disorders of keratinization [9,12–14], indicating that dehydrorretinol accumulation may be related to the pathophysiology of abnormal cell differentiation and proliferation of the epidermis.

Systemic administration of 13-*cis*-retinoic acid is widely used in clinical dermatology as a treatment for keratinizing skin disorders and severe acne. This retinoid, which occurs physiologically at a concentration of 5 nM in human plasma [31], modulates the differentiation of both epidermal keratinocytes and sebocytes. It is not known whether its biological effects are mediated via nuclear retinoid receptors, or by interaction with endogenous vitamin A metabolism, or via other mechanisms entirely separate from the vitamin A pathway. However, the drug interferes markedly with vitamin A homeostasis in human epidermis and sebaceous glands *in vivo* [32,33]. Thus in skin biopsies obtained from acne patients given 13-*cis*-retinoic acid treatment, the concentration of retinol is increased by at least 50% whereas the dehydrorretinol content is decreased to almost undetectable levels. It seems likely that the synthetic retinoid may interfere with cutaneous vitamin A metabolism, since circulating vitamin A status is not significantly affected by treatment with 13-*cis*-retinoic acid, whereas organ-cultured human skin shows the same response as keratinocytes in culture [34]. Interestingly, similar effects on cellular vitamin A composition were observed in the present study when labelled retinol was co-incubated with 13-*cis*-retinoic acid at concentrations that were within and below the therapeutic range of the drug. Possibly the effects on both retinol and dehydrorretinol levels may help to explain the biochemical mechanism by which 13-*cis*-retinoic acid exerts its therapeutic activity. Other synthetic retinoids were not tested in our system, but previous results have indicated that retinoids may vary widely in their capacity to interfere with cutaneous vitamin A metabolism [32,33,35].

Further characterization of the metabolism of retinol by epidermal cells is essential in order to elucidate how biosynthesis of dehydrorretinol and other 3,4-dehydrorretinoids is regulated in normal and abnormal keratinocytes. The keratinocyte culture system may help to explain the mechanisms by which synthetic retinoids interact with endogenous vitamin A, and may serve as a useful model with which to evaluate metabolic effects of new retinoids.

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