Topical Retinaldehyde Increases Skin Content of Retinoic Acid and Exerts Biologic Activity in Mouse Skin

Liliane Didierjean, Pierre Carraux, Denise Grand, Jörn Oliver Sass,* † Heinz Nau,* and Jean-Hilaire Saurat
Department of Dermatology, University Hospital, Geneva, Switzerland; and *Institute for Toxicology and Embryo Pharmacology, Free University of Berlin, Germany

Retinaldehyde, a natural metabolite of β-carotene and retinol, has been proposed recently for topical use in humans. Because retinaldehyde does not bind to retinoid nuclear receptors, its biologic activity should result from enzymatic transformation by epidermal keratinocytes into ligands for these receptors, such as all-trans retinoic acid and 9-cis-retinoic acid. In this study, we analyzed by high performance liquid chromatography the type and amounts of tissue retinoids as well as several biologic activities resulting from topical application of either retinaldehyde or all-trans retinoic acid on mouse tail skin. Biologic activities of all-trans retinoic acid and retinaldehyde were qualitatively identical in metaplastic parameters (induction of orthokeratosis, reduction of keratin 65-kDa mRNA, increase in filaggrin and loricrin mRNAs) and hyperplastic parameters (increase in epidermal thickness, increase in bromodeoxyuridine (BrdU)-positive cells, increase in keratin 50-kDa mRNA, and reduction in keratin 70-kDa mRNA). Some quantitative differences, not all in favor of all-trans retinoic acid, were found in several indices.

Endogenous retinoids in humans are derived from the intake of retinol (ROL) and its esters from food of animal origin and from carotenoids from plants (Underwood, 1994). Beta-carotene cleavage enzyme catalyzes the conversion of all-trans-β-carotene to two molecules of retinaldehyde (RAL), while several other carotenoids and apocarotenoids are also cleaved to yield RAL (Lakshman et al, 1989; van Vliet et al, 1991). RAL is also formed from ROL (Napoli and Race, 1990). RAL is converted enzymatically into ROL, retinyl esters, or all-trans retinoic acid (at-RA) by human keratinocytes. The process is dependent on the differentiation stage of keratinocytes, as it is higher in differentiating than in nondifferentiated keratinocytes (Siegenthaler et al, 1990).1

We have therefore proposed that RAL may be used as a topical agent in humans to target multipotential vitamin A activity into distinct compartments of the epidermis. Indeed, topically applied RAL would (i) be handled only by the epidermal cells having enzymatic activities at pertinent stages of differentiation; (ii) be a precursor of ROL, retinyl ester, or at-RA; and (iii) bypass the first, rate-limiting step of ROL oxidation into at-RA. This would result in controlled delivery of vitamin A metabolites to cells. We have found that RAL is well tolerated by human skin and that its topical application results in measurable biologic activity in the epidermis (Saurat et al, 1994).

Because RAL does not bind to retinoid nuclear receptors (Cretaz et al, 1990), its biologic activity upon topical use should result from enzymatic transformation by epidermal keratinocytes into ligands for these receptors, such as at-RA and 9-cis-RA (Allenby et

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The production of ligands for nuclear receptors within target cells has been described as an "intracrine" process (O'Malley, 1989). The aim of this study was to analyze by high performance liquid chromatography (HPLC) the type and amounts of retinoids resulting from topical application of RAL in mouse skin to identify whether ligands for retinoid nuclear receptors are generated. The type and amounts of retinoids detectable after direct topical application of at-RAL, the ligand for retinoic acid receptors, were also analyzed for comparison.

Materials and Methods

Chemicals Reference retinoids were mostly provided by Hoffmann-LaRoche (Basel, Switzerland). Retinyl esters (Collins et al., 1992a) and all-trans-retinol-β-D-glucuronide (Foerster et al., 1996) were synthesized in the Berlin laboratory by Ch. Eckhoff and R. Rühl, respectively. The 14-hydroxy-retinoic HPLC standard was a gift of Dr. J. Buck (Memorial Sloan-Kettering Cancer Center, New York, NY).

Treatment of Mice At-RAL and RAL were used at concentrations of 0.005, 0.01, 0.025, and 0.05% compounded in a same oil-in-water cream (Saurat et al., 1994). Cream samples of 0.5 g were applied daily for 9, 12, or 14 d on the tail. The daily amount of RAL and at-RAL delivered thus corresponded to 25, 50, 125, or 250 μg. Groups of three or six adult (≈3 mo old) C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were tested per dose/time point. Twenty hours after the last application, the animals were killed by decapitation. Tail skin was stripped off the bone and cut longitudinally, and one part, always from the same tail region, was fixed in Dubosq-Brasil liquid, embedded in paraffin, and processed for histology and morphometry. The remaining specimens were frozen in liquid nitrogen and kept at −80°C. For retinoid analyses, tail skin received 14 d of vehicle, 0.05% at-RAL, or RAL, and the entire tail skin was frozen in liquid nitrogen and kept at −80°C until extraction.

Histology and Morphometry Five-micrometer serial sections were stained with hematoxylin and eosin. Measurements were done using a micrometer at a 10x power field. The metaplastic response was studied by measuring the length (μm) of parakeratotic epidermis in 100 interfollicular zones, i.e., from the last granular cell of one zone to the first cell of the next follicular zone. Thickness of the epidermis (μm) was measured at the middle of 100 interfollicular zones from the basal membrane zone to the last nucleated cell layer. All treatment versus vehicle values were assessed with the nonparametric Wilcoxon test.

Measurement of Cell Proliferation We measured the incorporation of BrdU into DNA. Mice were injected intraperitoneally with 250 μg BrdU per g (Sigma Chemie, Buchs, Switzerland) dissolved in sterile physiologic saline. After 2 h, the animals were killed, and one part of the tail skin was fixed in Dubosq-Brasil liquid, embedded in paraffin, and cut in 5-μm serial sections. Sections were dewaxed, placed in 0.05 M Tris(hydroxymethyl)-aminomethane (Tris) buffer, pH 7.4, and then incubated in 1N HCl at room temperature for 15 min and rinsed in Tris buffer. Slides were incubated with anti-BrdU monoclonal antibody (Boehringer Mannheim AG, Rotkreuz, Switzerland) diluted 1:10 in Tris buffer for 1 h at room temperature, followed by staining by the Strept avidin-biotin peroxidase complex (ABC)/horseradish peroxidase (HRP) method according to the manufacturer's instructions (Dako Diagnostics AG, Zug, Switzerland). Diaminobenzidine (Sigma Chemie) was used as HRP substrate. Slides were observed by light microscopy at a magnification of 250×. The number of BrdU-positive cells in epidermis of all interfollicular zones was counted and then expressed as cells per 100 interfollicular zones.

RNA Isolation and Analysis Total RNA was isolated using the CsCl centrifugation method (Chirgwin et al., 1979) and subjected to northern analysis or slot blotting. Hybridizations were performed either with riboprobes as described previously (Siegenthaler et al., 1992) or with cDNAs labeled with [α-32P]deoxyctydine triphosphate to a specific activity of at least 1× 106 cpm/μg by random priming. Autoradiograms were analyzed by scanning using a PhosphorImager (Molecular Dynamics, Kensing, UK).

Results

Activity of Topical RAL Is Similar to That of At-RAL Metaplastic Response The regular, highly ordered, postnatal pattern of parakeratotic scales and orthokeratotic interscale regions is changed to orthokeratosis in adult mouse tail by at-RAL. This metaplastic effect, which corresponds to a return to the neonatal pattern, is a retinoid-specific pharmacologic process that does not occur upon topical application of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (Schweizer et al., 1987). Topical at-RAL induced, as expected, a time- and concentration-dependent reduction of the parakeratotic scale regions, which were entirely replaced by orthokeratotic epidermis by 0.05% at-RAL after 14 d (Schweizer et al., 1987) (Fig 1). A time-dependent response similar
to that of at-RA was present in animals treated with RAL at 0.05% (Fig 1A), although RAL was significantly more metaplastic at 0.005, 0.01, and 0.05% in the dose-response experiments (Fig 1B).

The expression of the murine type II 65-kDa keratin is suppressed by at-RA, parallel to the induction of orthokeratosis, but not by 12-0-tetradecanoylphorbol-13-acetate (Schweizer et al., 1987); topical at-RA indeed induced a concentration-dependent reduction in 65-kDa keratin mRNA as compared with vehicle (Fig 2A). Topical RAL also induced a reduction in 65-kDa keratin mRNA, which was not different from that induced by at-RA except at the lowest concentration (0.005%), at which at-RA was significantly more active (p = 0.01) (Fig 2B).

The induction of orthokeratosis in the mouse tail corresponds to that of a granular layer; this layer expresses proteins such as loricrin and filaggrin. Loricrin is absent in the mouse tail parakeratotic scale (Hohl et al., 1993), and filaggrin mRNAs are stably expressed only when keratinocytes migrate into the granular layer (Rothnagel et al., 1987). The expression of the loricrin gene was, as expected, induced by at-RA but not by vehicle (Fig 2C,D); RAL also induced loricrin mRNA, but to a lesser extent (p = 0.02). Filaggrin mRNA was studied by northern blot to analyze the 17-kb specific band (Rothnagel et al., 1987). Treatment with 0.05% of at-RA and RAL induced an identical increase in filaggrin mRNA expression (14-fold as compared with vehicle). Low doses (0.005% and 0.010%) of RAL resulted in 14- and 25-fold filaggrin mRNA induction, versus 9- and 16-fold, respectively, with at-RA (not shown).

Hyperplastic Response In this model, at-RA induced a hyperplastic response similar to that induced by 12-0-tetradecanoylphorbol-13-acetate (Schweizer et al., 1987); this includes an increase in epidermal thickness, induction of the murine hyperproliferation-associated type I 50-kDa keratin (the murine ortholog of the human keratin K17), and suppression of the murine type II 70-kDa keratin (the murine ortholog of the human keratin K2e) (Schweizer et al., 1987). We analyzed these parameters and also studied BrdU incorporation.

BrdU-positive cells were significantly more numerous in at-RA- and RAL-treated skin than in vehicle, and in RAL as compared with at-RA treated skin, at the highest concentrations (Fig 3A). Topical at-RA increased epidermal thickness as expected; a similar effect was seen in mice treated with RAL, which induced significantly more thickening than at-RA only at 0.05% (Fig 3B,C).

Topical at-RA induced, as expected, an increase in 50-kDa keratin mRNA; the induction by topical RAL was significantly

Figure 2. Expression levels of 65-kDa keratin (A,B) and loricrin (C,D) mRNA are similar in at-RA- and RAL-treated mouse tail skin. Mice were treated for 7 d with 0.005, 0.010, and 0.05% retinoids or with vehicle. (A,C) Triplicate experiments of RNA slot blots of 1 μg of total RNA hybridized by random priming. (B,D) Quantification of RNA bands of at-RA-treated or RAL-treated samples expressed as percentage of vehicle-treated samples (mean ± SD). Statistical analysis: paired Wilcoxon test.
higher than that by at-RA (Fig 4A,B). Topical at-RA induced, as expected, a reduction in 70-kDa keratin mRNA; topical RAL induced a similar reduction in 70-kDa keratin mRNA (Fig 4C,D).

**Cellular Retinoic Acid-Binding Protein Type II and Cellular Retinol-Binding Protein Type I** The expression of these two retinoid-binding proteins has been shown to be induced by at-RA (Hirschel Scholz et al, 1989; Elder et al, 1993; Piletta et al, 1994; Kang et al, 1995); they may be considered as primary responsive genes because, unlike the other parameters analyzed in the present study, their promoters possess retinoid response elements (Smith et al, 1991, Durand et al, 1992). Cellular retinoic acid–binding protein type II mRNA was induced 4-fold by at-RA and 2.5-fold by RAL; cellular retinol-binding protein type I mRNA was induced 1.3-fold by both at-RA and RAL (14 d, 0.05% not shown).

**A Low Amount of At-RA Is Generated in Mouse Skin upon Topical RAL Application** We studied the retinoid content of tail skin by HPLC analysis after 14 d of treatment at optimal dose (0.05%). Compared with vehicle-treated controls, RAL-treated mouse skin contained detectable amounts of at-RA and of 13-cis-RA, whereas 9-cis-RA, 4-oxo-RA, and didehydro-RA were not detectable (Table I, Fig 5). ROL content increased 10-fold after RAL application, which indicates that tail skin had predominantly transformed RAL into ROL.

To put in perspective the data obtained with topical RAL, we studied the retinoid content in at-RA–treated skin. It contained 314-fold more at-RA than RAL–treated skin. Interestingly, 9-cis-RA was detectable at significant levels, but 13-cis-RA was the main isomer formed (Table I). No didehydro-RA was detectable in at-RA–treated skin, and 4-oxo-RA were only very minor metabolites, if present at all. All-trans-retinoyl-β-D-glucuronide was found only after application of at-RA (131 ± 11 ng/g wet weight).

Unlike in RAL–treated skin, in which at-RA should be formed enzymatically from RAL, a significant proportion of products detected in at-RA–treated skin may still be at the surface of the epidermis. We therefore conducted a second series of experiments in which the at-RA–treated tail was washed before sampling. Although at-RA and isomers were significantly decreased as compared with unwashed tail (at-RA by 92%, 13-cis-RA by 85%, and 9-cis-RA by 82%; Table I), these were still much higher than in RAL–treated unwashed skin; thus there was still 24-fold more at-RA (p = 0.05).

ROL increased 2.5-fold in at-RA–treated skin but significantly less than in RAL–treated skin (p = 0.001, Table I). Because, unlike

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**Table I. Retinoids in Mouse Tail Skin After 14 d of Topical Retinoid (0.05%) Application**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>All-trans-RA</th>
<th>13-cis-RA</th>
<th>9-cis-RA</th>
<th>ROL</th>
<th>RAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>NQ</td>
<td>NQ</td>
<td>NQ</td>
<td>NQ</td>
<td>69.8 ± 21.0</td>
</tr>
<tr>
<td>RAL</td>
<td>13.0 ± 6.9</td>
<td>12.6 ± 5.9</td>
<td>NQ</td>
<td>350 ± 99.5</td>
<td>749 ± 82.7</td>
</tr>
<tr>
<td>All-trans-RA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unwashed skin</td>
<td>4080 ± 432</td>
<td>409 ± 13</td>
<td>136 ± 13</td>
<td>NQ</td>
<td>161 ± 2</td>
</tr>
<tr>
<td>Washed skin'</td>
<td>310 ± 150</td>
<td>61.5 ± 27.2</td>
<td>24.4 ± 10.2</td>
<td>NQ</td>
<td>107 ± 18.7</td>
</tr>
</tbody>
</table>

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*Sampling was done 20 h after the last treatment.

*Concentrations of 4-oxo- and didehydro-retinoids were below the limit of quantitation (NQ) of the HPLC method (<3 ng/g wet weight) in all samples except 4-oxo-RAs in all-trans-RA unwashed skin (23 ± 2 ng/g wet weight).

The tail skin was washed with 0.1% Triton, then rinsed with water before sampling.
RAL, at-RA cannot be transformed into ROL, it is likely that loading of the skin with at-RA stimulates mobilization of ROL from endogenous retinyl esters, as has been shown in HeLa cells (Stenström et al., 1996). This is further supported by the limited decrease in ROL detected in washed versus unwashed tail skin (Table I).

DISCUSSION

Previous observations in humans have indicated that topical RAL exerts some biologic activities (Saurat et al., 1994). Our study confirms and extends these observations in the mouse tail. This is a reproducible model (Schweizer and Marks, 1977; Wrench and Didierjean, 1985) for studying the effects of topical at-RA at the morphologic and molecular levels (Schweizer et al., 1987). The data obtained in this study in at-RA–treated mouse are well in accordance with Schweizer’s results (Schweizer and Marks, 1977; Schweizer et al., 1987); therefore, because topical RAL also showed significant effects on all the parameters studied, it can be concluded that it exerts retinoid activities.

Because RAL does not bind to retinoid nuclear receptors (Crettez et al., 1990), its retinoid biologic activity should result from its enzymatic transformation into at-RA by epidermal keratinocytes. We have indeed found that cytosolic extracts of cultured human keratinocytes transformed [3H]RAL into [3H]at-RA (Siegenthaler et al., 1990); furthermore, intact cultured keratinocytes took up [3H]RAL and transformed it into [3H]at-RA. ¹

Our results now demonstrate that murine skin in vivo also transforms RAL into at-RA. This indicates that RAL can be used topically as a precursor for at-RA, the ligand for retinoic acid receptors (Allenby et al., 1993), which is likely to account for the retinoid biologic effects observed. In this context, it is interesting that, unlike topical at-RA, topical RAL did not generate detectable amounts of 9-cis-RA, the ligand for retinoid X receptors (Allenby et al., 1993). Because this was not correlated with major differences in the biologic effects of the two topical agents, it may be that RAL generated biologically sufficient amounts of 9-cis-RA that were below the detection limit of the assay (5 ng/g). Mouse skin has also been shown to transform topical ROL into at-RA (Connor, 1988): a detailed comparison of the metabolites produced from topical ROL and RAL in tail skin is reported elsewhere (Sass et al., in press). ROL–treated skin showed no detectable RA, slightly less retinyl ester, but a significant amount of 14-hydroxy-retinoic acid, a metabolite not previously reported in the skin.

Table II. Summary of the Biologic Activities of Topical RAL and All-trans-RA Analyzed in This Study

<table>
<thead>
<tr>
<th>Activity</th>
<th>At-RA–Specific</th>
<th>Best Activity by:²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction of orthokeratosis</td>
<td>Yes</td>
<td>RAL</td>
</tr>
<tr>
<td>Suppression of keratin-65 kDa</td>
<td>Yes</td>
<td>At-RA</td>
</tr>
<tr>
<td>Induction of loricrin</td>
<td>NT</td>
<td>RAL</td>
</tr>
<tr>
<td>Induction of filaggrin</td>
<td>NT</td>
<td>RAL</td>
</tr>
<tr>
<td>Epidermal thickness</td>
<td>No</td>
<td>RAL</td>
</tr>
<tr>
<td>BrdU incorporation</td>
<td>NT</td>
<td>RAL</td>
</tr>
<tr>
<td>Keratin 50-kDa induction</td>
<td>No</td>
<td>RAL</td>
</tr>
<tr>
<td>Keratin 70-kDa suppression</td>
<td>No</td>
<td>Identical</td>
</tr>
<tr>
<td>CRABP II</td>
<td>NT</td>
<td>At-RA</td>
</tr>
<tr>
<td>CRBP I</td>
<td>NT</td>
<td>Identical</td>
</tr>
</tbody>
</table>

¹ Indicates (yes) that the activity has been shown with topical retinoic acid but not reproduced by topical 12-0-tetradecanoylphorbol-13-acetate (TPA) in the mouse tail model, according to Schweizer et al. (1987). NT indicates that the activity has not been tested with TPA in this model.
² Indicates higher activity in dose-response experiments (see text). This should be considered to be limited to this model and not necessarily applicable to others.

CRABP, cellular retinoic acid binding protein.
CRBP, cellular retinol binding protein.

An important and surprising observation was not only that topical RAL showed both differentiating and proliferative activities qualitatively similar to those produced by at-RA in this model, but also that these activities were not quantitatively lower. Even if there were some quantitative differences in the modulation of some of the parameters studied (summarized in Table II), these were not all in favor of at-RA. This was surprising because we anticipated that when applied at 0.05%, RAL would be unable to provide as much at-RA as that resulting from 0.05% topically applied at-RA. In fact, this prediction was clearly confirmed by our HPLC analysis, showing much lower tissue content of at-RA after topical RAL than after topical at-RA. Such a discrepancy between tissue amounts of at-RA and biologic activities may have at least two explanations, as follows.

1. A low concentration of topical at-RA (resulting in tissue levels in the range of 15 ng/g, i.e., the amount recovered after 0.05% RAL application) may be sufficient for full activity of the parameters studied in this model. This is not likely when considering the dose-response curves of topical at-RA; thus, 0.005% topical at-RA did not induce a full biologic effect.

2. The second possibility is more likely: After topical at-RA, only a small percentage of the at-RA measured by HPLC analysis may correspond to intracellular, biologically significant at-RA. There is no tool to test this hypothesis directly in vivo. It is, however, in agreement with the ED₅₀ of transcriptional activation by retinoic acid nuclear receptors, which necessitates much lower amounts of at-RA than those recovered after topical application (Duell et al., 1992).

These data strongly support the concept of using precursors rather than at-RA itself (Saurat et al., 1994), because topical RAL loads epidermal cells with low amounts of ligands that are as biologically significant as those resulting from much higher tissue loading of at-RA.

Together our data indicate that the intracrine concept (O’Malley, 1989) is relevant to the field of topical retinoids. According to this concept, future studies should specifically address the applicability of the observations in the mouse to human skin.

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