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

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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. Cellular retinoic acid–binding protein II and cellular retinol-binding protein I mRNAs were increased by both topical retinaldehyde and all-*trans* retinoic acid. Whereas all-*trans* retinoic acid, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid were not detectable (limit 5 ng/g) in vehicle-treated skin, 0.05% retinaldehyde-treated skin contained 13 ± 6.9 ng/g wet tissue of all-*trans* retinoic acid (mean ± SD), 12.6 ± 5.9 ng/g 13-*cis*-retinoic acid, and no 9-*cis*-retinoic acid. In contrast, 9-*cis*-retinoic acid was detectable in 0.05% of all-*trans* retinoic acid–treated skin, which also contained 25-fold more all-*trans* retinoic acid and 5-fold more 13-*cis*-retinoic acid than retinaldehyde-treated skin. Our results show that topical retinaldehyde is transformed *in vivo* into all-*trans* retinoic acid by mouse epidermis. The small amounts of ligand for retinoic acid nuclear receptors thus produced are sufficient to induce biologic effects similar to those resulting from the topical application of the ligand itself in much higher concentration. ***Key words: retinoids/HPLC. J Invest Dermatol 107:714–719, 1996***

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 (Crettaz *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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Abbreviations: *at*-RA, all-*trans* retinoic acid; RAL, retinaldehyde; ROL, retinol.

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[1] Chattelard-Gruaz D, Jaconi S, Saurat JH, Siegenthaler G: Metabolism of retinaldehyde in living cultured human keratinocytes. *J Invest Dermatol* 102:599, 1994 (abstr.).

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al, 1993). The production of ligands for nuclear receptors within target cells has been defined as an "intracrine" process (O'Malley, 1989).

The aim of this study was first 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*-RA, the ligand for retinoic acid receptors, were also analyzed for comparison. Second, we characterized the type and intensity of retinoid-induced biologic effects that may result from the generation of these ligands upon topical application of RAL. The mouse tail skin model was chosen because it allows one to analyze at the morphologic and molecular levels many biologic activities of topical *at*-RA (Schweizer and Marks, 1977; Didierjean *et al*, 1983, Wrench and Didierjean, 1985; Schweizer *et al*, 1987). Topical *at*-RA was used as a control.

MATERIALS AND METHODS

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

Treatment of Mice *At*-RA 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*-RA 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 Duboscq-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*-RA, 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 10× 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 Duboscq-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 1 N 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-^{32}P]deoxycytidine triphosphate to a specific activity of at least 1×10^8 cpm/μg by random priming. Autoradiograms were analyzed by scanning using a PhosphorImager (Molecular Dynamics, Kemsing, UK).

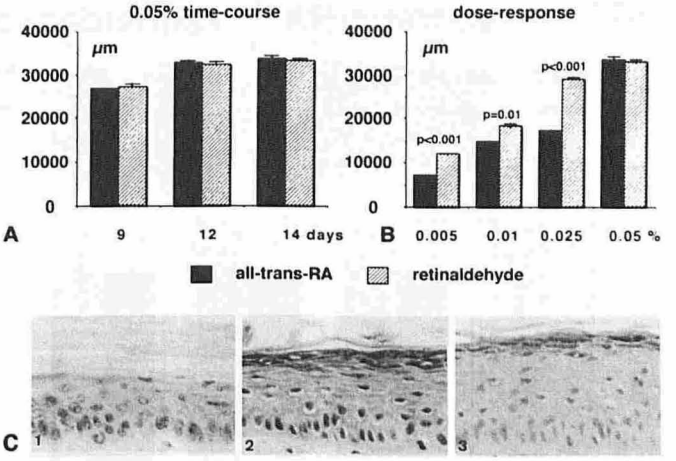

Figure 1. Metaplastic responses to topical RAL and *at*-RA are similar in mouse tail. *(A)* Time course, 0.05% *at*-RA and 0.05% RAL. *(B)* Dose response at 14 d. Values are expressed as the length (mean ± SEM) of interfollicular orthokeratosis induced in 100 interfollicular zones measured in each of three animals per time/dose. *(C)* Hematoxylin and eosin stain of skin showing the metaplastic response (presence of a granular layer) in the interfollicular zone of mouse tail skin treated with 0.05% *at*-RA *(2)* or 0.05% RAL *(3)* for 14 d, as compared with vehicle-treated tail *(1)*.

Hybridization Probes Full-length murine cellular retinoic acid–binding protein type II and cellular retinol-binding protein type I cDNAs were kindly provided by P. Chambon (IGBMC, Illkirch, France). A specific cDNA fragment encoding for mouse loricrin (Mehrel *et al*, 1990) and cDNA fragment encoding for mouse filaggrin (Rothnagel *et al*, 1987) were kindly provided by D. R. Roop (Baylor College of Medicine, Houston, TX); cDNAs encoding for murine 50-kDa (Knapp *et al*, 1987), 65-kDa, and 70-kDa keratin proteins (Schweizer *et al*, 1987) were kindly provided by J. Schweizer (German Cancer Research Center, Heidelberg, Germany).

Retinoid Analyses Retinoids were determined by reversed-phase HPLC with simultaneous detection at 340 and 356 nm using a modification (Tzimas *et al*, 1994; Sass *et al*, 1995) of the method described by Eckhoff and Nau (1990). The modification allows the determination of didehydro-ROL, ROL, RAL, and retinyl esters in addition to polar retinoids. Before analysis, weighed tail skin samples were homogenized in a 4-fold volume of aqueous buffer (50 mM Tris-HCl, 25 mM NaCl, 2.5 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol; pH 7.5). Under permanent cooling with ice, the skin was minced with a pair of scissors and an Ultra-Turrax T25 (Janke and Kunkel, Staufen, Germany). Two hundred microliters of the homogenate were then mixed with 600 μl of isopropanol, submitted to short sonication (Sonifier B-12; Branson Sonic Power Company, Danbury, CT) at setting 2.5, and centrifuged briefly (6500 × *g*). Four hundred microliters of the resulting supernatant were extracted on a solid-phase cartridge as described by Collins *et al* (1992b). HPLC analyses were started with a Varian AASP autoinjector, which inserted the cartridges into the stream of the HPLC eluents. The limit of quantitation for each retinoid was 5 ng/g wet weight of skin. For proper determination of all-*trans*-retinoyl-β-D-glucuronide in skin samples, we used a binary gradient of different HPLC eluents (Sass and Nau, 1994).

RESULTS

Activity of Topical RAL Is Similar to That of *At*-RA

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*-RA. 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*-RA 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*-RA after 14 d (Schweizer *et al*, 1987) **(Fig 1)**. A time-dependent response similar

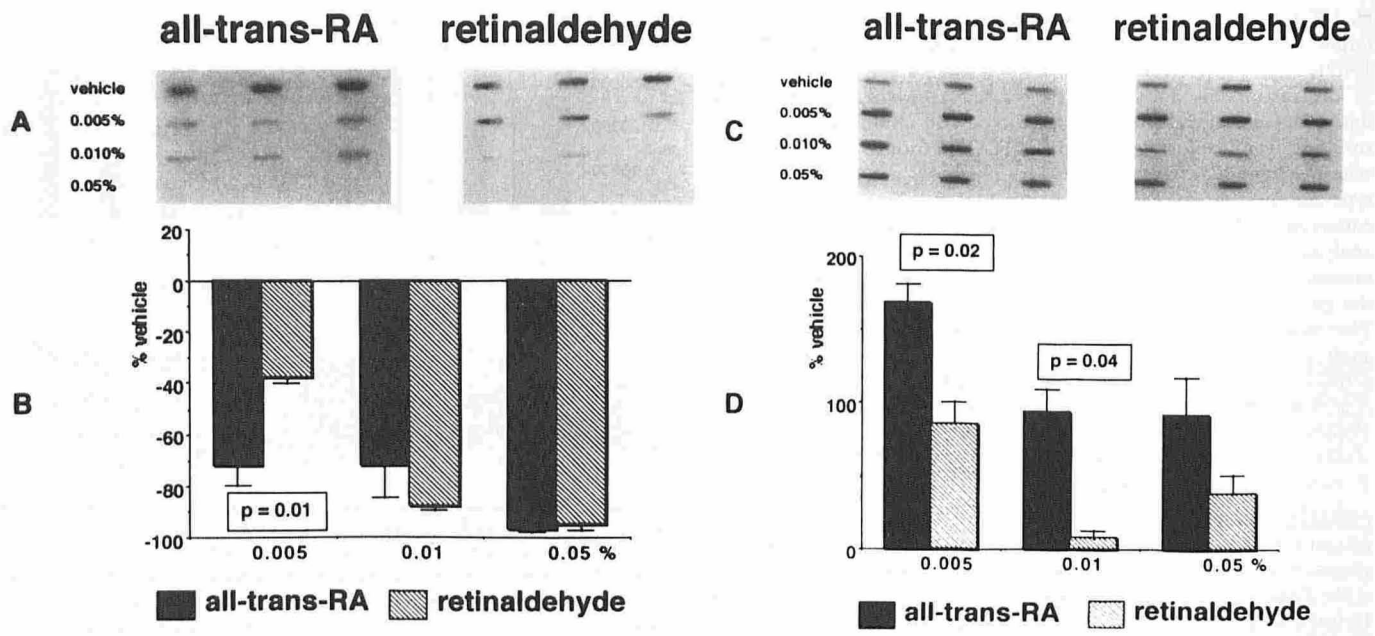

Figure 2. Expression levels of 65-kDa keratin *(A,B)* and loricrin *(C,D)* mRNA are similar in *at*-RA– and RAL-treated mouse tail skin. Mice were treated for 7 d with 0.005, 0.010, and 0.05% retinoids or with vehicle. *(A,C)* Triplicate experiments of RNA slot blots of 1 μg of total RNA hybridized by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

to that of *at*-RA was present in animals treated with RAL at 0.05% **(Fig 1*A*)**, although RAL was significantly more metaplastic at 0.005, 0.01, and 0.025% in the dose-response experiments **(Fig 1*B*)**.

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-*O*-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 2*A*)**. 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 2*B*)**.

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 2*C,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-*O*-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 3*A*)**.

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 3*B,C*)**.

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

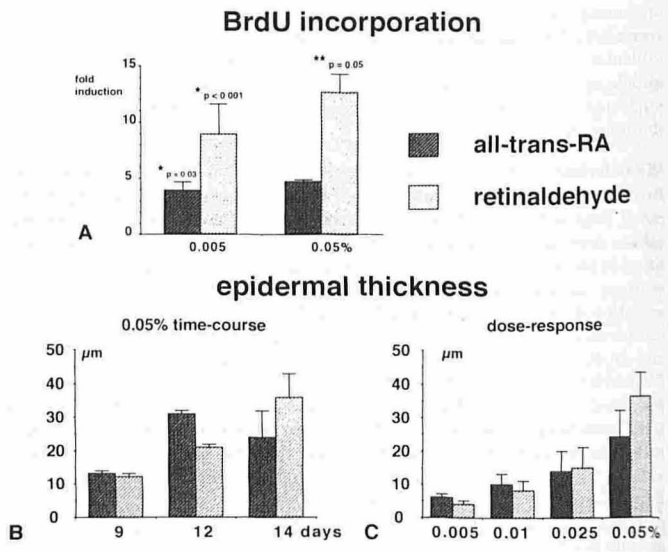

Figure 3. Hyperplastic responses to topical RAL and *at*-RA are similar in mouse tail. *(A)* BrdU-positive cells in 100 interfollicular zones after 9 d of treatment with 0.005 and 0.05% of *at*-RA or RAL (fold induction over vehicle control, mean ± SD, n = 3). *Significant *versus* vehicle; **RAL *versus at*-RA. *(B)* 0.05% time course: thickness of epidermis (μm/100 interfollicular zones, mean ± SD, n = 3 per group). *(C)* dose response at 14 d.

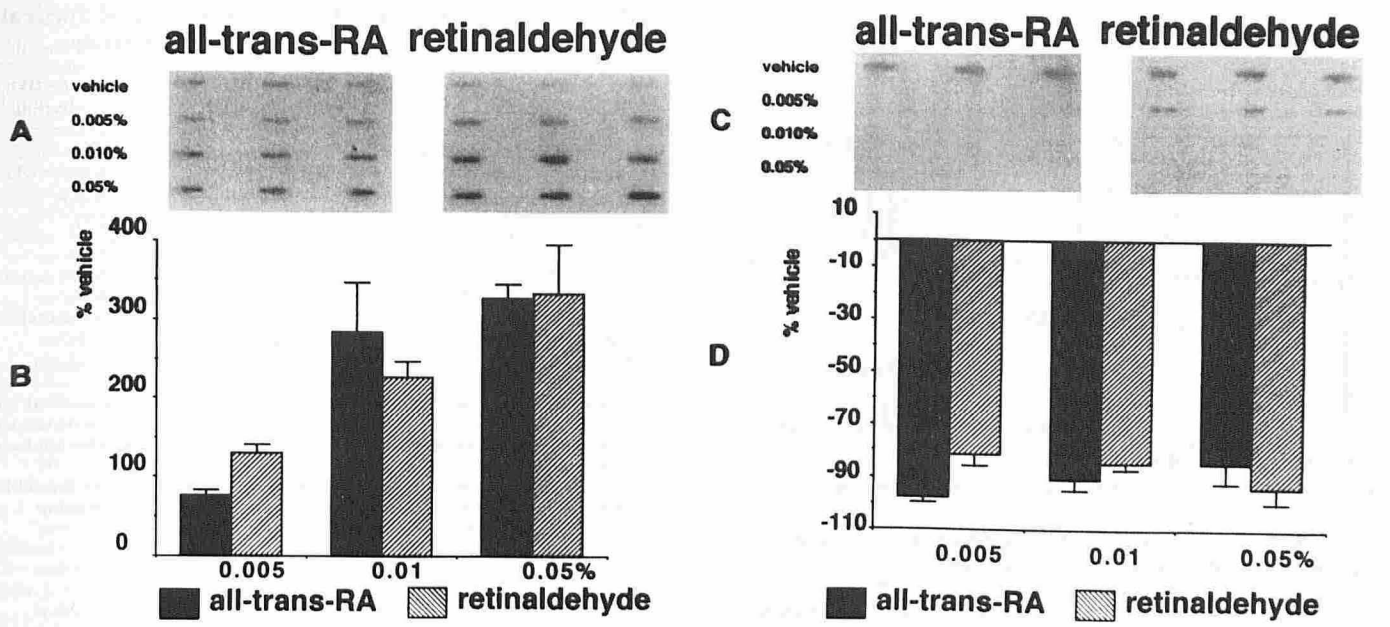

Figure 4. Expression levels of 50-kDa *(A,B)* and 70-kDa keratin *(C,D)* mRNA are similar in *at*-RA– and RAL-treated mouse tail skin. Mice were treated for 7 d with 0.005, 0.010, and 0.05% retinoids or with vehicle. *(A,C)* Triplicate experiments of RNA slot blots of 1 μg of total RNA hybridized by random priming. *(B,D)* Quantification of RNA blots of *at*-RA–treated or RAL-treated samples expressed as percentage of vehicle-treated samples (mean ± SD).

higher than that by *at*-RA **(Fig 4*A,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 4*C,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-RAs 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

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

Treatment	Retinoid Concentration in Tail Skin (ng/g ww)[b]				
	All-*trans*-RA	13-*cis*-RA	9-*cis*-RA	RAL	ROL
Vehicle	NQ	NQ	NQ	NQ	69.8 ± 21.0
RAL	13.0 ± 6.9	12.6 ± 5.9	NQ	350 ± 99.5	749 ± 82.7
All-*trans*-RA					
Unwashed skin	4080 ± 432	409 ± 13	136 ± 13	NQ	161 ± 2
Washed skin[c]	310 ± 150	61.5 ± 27.2	24.4 ± 10.2	NQ	107 ± 18.7

[a] Sampling was done 20 h after the last treatment.
[b] Concentrations of 4-oxo- and didehydro-retinoids were below the limit of quantitation (NQ) of the HPLC method (<5 ng/g wet weight) in all samples except 4-oxo-RAs in all-*trans*-RA unwashed skin (23 ± 2 ng/g wet weight).
[c] The tail skin was washed with 0.1% Triton, then rinsed with water before sampling.

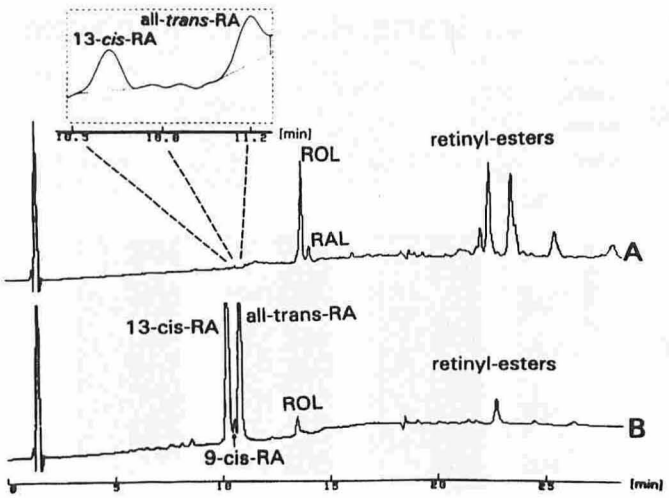

Figure 5. A low amount of *at*-RA is generated in mouse skin upon topical RAL application. Sample HPLC chromatograms of mouse tail skin obtained after 14 d of daily treatment (0.05%) with RAL *(A)* (*insert*, magnified RA peaks) and *at*-RA *(B)*. Detection at 340 nm; 0.0320 AUFS, *insert*, 0.0009 AUFS.

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 (Crettaz *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 [^{3}H]RAL into [^{3}H]*at*-RA (Siegenthaler *et al*, 1990); furthermore, intact cultured keratinocytes took up [^{3}H]RAL and transformed it into [^{3}H]*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-*retro*-ROL, 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

Activity	At-RA–specific[a]	Best Activity by:[b]
Induction of orthokeratosis	Yes	RAL
Suppression of keratin-65 kDa	Yes	*At*-RA
Induction of loricrin	NT	*At*-RA
Induction of filaggrin	NT	RAL
Epidermal thickness	No	RAL
BrdU incorporation	NT	RAL
Keratin 50-kDa induction	No	RAL
Keratin 70-kDa suppression	No	Indentical
CRABP II[c]	NT	*At*-RA
CRBPI[d]	NT	Identical

[a] 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.

[b] 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.

[c] CRABP, cellular retinoic acid binding protein.

[d] 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 ED_{50} 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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