Dermatology

# New Concepts for Topical Use of Natural Retinoids

## Retinaldehyde in Perspective

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Dermatology

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# Topical Natural Retinoids

## The 'Proligand-Non-Ligand' Concept

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Retinoic acid (RA) is widely used for topical therapy of several skin diseases; it also improves the aspect of chronic solar damage. Topical RA induces irritation of the skin, which precludes its use in some skin diseases that respond to systemic retinoids.

Irritation might be explained, in part, by an overload of the RA-dependent pathways with non-physiological amounts of exogenous RA in the skin. The globally attainable concentrations of RA in the different layers from therapeutically efficient formulations have been determined in human skin in vivo. A steep concentration gradient with high concentrations in the epidermis, up to 450 ng/g wet weight (1.5 μ*M*), and relatively low amounts in the dermis (55 ng/g wet weight, 180 n*M*) is achieved 100 min following application of RA 0.1% in isopropanol, corresponding to 15% of the applied dose of 100 mg [1].

Retinoid content analysis in human skin treated with 0.1% RA for 4 days under occlusion showed the following values: RA 824 ng/g (2,750 n*M*), 13-*cis*-RA 745 ng/g (2,480 n*M*) and 4-OH-RA 93 ng/g wet weight (310 n*M*) [2].

A recent study on percutaneous absorption of RA showed that about 2% of a single dose of 100 mg in a 0.05% formulation is absorbed; the same result is obtained after 28 days of daily application [3]. Due to the similarity of chemical structure, a similar pharmacokinetic behaviour is expected for topical 13-*cis*-RA [4]. Such high tissue concentrations of RA are in overexcess of the concentrations needed to saturate nuclear receptors [5, 6].

Although major advances have been made in the analysis of the molecular events resulting from topical application of RA [7], it is still not established if all the therapeutic activities of topical RA are mediated by nuclear receptors, and if irritation is necessary for obtaining some of these activities.

One possibility is that significant biological activity may still be achieved with much lower concentrations of topical RA; in this case, RA is delivered at a low rate from a large epidermal reservoir to its intracellular targets. Alternatively, instead of treating the skin with the ligand of nuclear receptors itself, delivery could be distinctly targeted with 'proligands'.

We have explored the possibility of delivering retinoid activity to human skin topically with a natural retinoid that does not bind nuclear receptors. Such a precursor should be handled by enzymes of keratinocytes in the epidermis and transformed into either RA or storage forms such as all-*trans*-retinol (ROL) and retinyl esters.

Epidermis is a differentiating, non-homogenous tissue, made of keratinocytes that are not yet committed to terminal differentiation (basal cell layer) and a population of differentiating cells (suprabasal cell layers); the need for, and concentration of, RA may not be identical in all layers and a gradient of RA has been considered to be a key event in the maturation of keratinocytes [8]. This is supported by the fact that the conversion rate of ROL into RA by human keratinocytes depends on the state of keratinocyte differentiation, differentiating keratinocytes being able to oxidise ROL at a higher rate than non-differentiating ones [9]. That enzymes transforming the precursors into RA have distinct activities at different stages of differentiation indicates the possibility of targeting RA to epidermal cells in a differentiation related manner. This would be one approach to reduce side-effects. The use of RA precursors, such as ROL, retinyl esters, all-*trans*-retinaldehyde (RAL) and β-carotene, should therefore be considered in this context.

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Validation of the concept would imply to demonstrate that: (i) epidermal cells distinctly metabolise the precursor into RA, (ii) topical application of the precursor results in biological effects and (iii) tolerability of the precursor is better than that of RA.

Human keratinocytes transform ROL into RAL and then into RA by two enzymatic steps involving dehydrogenases. The first step is rate limiting and reversible; RAL can be converted enzymatically into either RA or ROL by human keratinocytes, both in vivo [10, 11] and in vitro [9]. Epidermal cells have a weak capacity to transform ROL into RAL [10, 11]. β-Carotene is not converted into retinoids by epidermal cells [Siegenthaler G., unpubl. observations]. We therefore hypothesised that RAL should be an interesting precursor for topical use because: (i) it bypasses the first limiting step of ROL oxidation into RA and (ii) only the epidermal cells capable of RAL oxidation at a pertinent stage of differentiation would generate active ligand(s). In this supplement issue of *Dermatology*, current knowledge upon RAL has been gathered; most has been presented during a symposium held during the 7th Congress of the European Academy of Dermatology and Venereology in Nice.

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# What Are Natural Retinoids?

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**Key Words**
Vitamin A · Natural retinoids · Retinaldehyde

**Abstract**
Vitamin A (retinol) is the prototype of all other natural retinoids. It is composed of a nonaromatic ring structure, a polyprenoid side chain and a carbonyl end group. These features make it liable to metabolic interconversion and protein interactions but also cause detergent-like properties and a sensitivity to UV irradiation and oxidation. Natural retinoids are present in all living organisms, either as preformed vitamin A or as carotenoids, and are required for a vast number of biological processes, e.g. vision, cellular growth and differentiation and reproduction. Although retinol is the most omnipotent compound, natural retinoids like all-*trans*-, 9-*cis*- and didehydroretinoic acid (RA) are clearly more potent outside the retina and trigger gene expression via binding to nucelar retinoid receptors. Retinaldehyde takes an intermediate position in this respect, with ability to convert to both retinol and RA. Over the years, many natural retinoids have been tried therapeutically against skin disorders with the best effects achieved with retinol, retinaldehyde, 13-*cis*-RA and all-*trans*-RA. The latter compound was the prototype when new, synthetic derivatives of vitamin A were sought, hoping for a better therapeutic index and a higher functional specificity. Inevitably, treatment with such drugs will influence the effects of coexisting natural retinoids. An understanding of the basic principles of these interactions may have major impact on patient outcome.

## Introduction

Natural retinoids are present in all living organisms, either as preformed vitamin A or as carotenoids, some of which are provitamins A. They are required for a vast number of biological processes, e.g. vision, cellular growth and differentiation and reproduction. Vitamin A (retinol), the prototype of all natural retinoids, is essentially a lipid-soluble compound but has also a hydrophilic end group which gives it detergent-like properties. In vivo, retinol and its congeners are usually reversibly bound to proteins or integrated in fatty structures, but hydrophilic conjugates (mainly glucuronides) exist too [for a review, see 1].

It is not self-evident how to distinguish a natural retinoid from a synthetic one. If a broad definition is used, the list of natural retinoids will be very long and overlapping the list

**Table 1.** Some examples of natural retinoids

| |
|---|
| All-*trans*-retinol (vitamin $A_1$ alcohol) |
| All-*trans*-retinal (retinaldehyde) |
| 11-*cis*-Retinaldehyde |
| Retinyl palmitate (the most prevalent retinyl ester) |
| 3-Dehydroretinol (3,4-didehydroretinol; vitamin $A_2$) |
| 4-Hydroxyretinol |
| Anhydroretinol |
| All-*trans*-retinoic acid (vitamin $A_1$ acid) |
| 13-*cis*-Retinoic acid |
| 9-*cis*-Retinoic acid |
| 3,4-Didehydroretinoic acid (vitamin $A_2$ acid) |
| 4-Hydroxyretinoic acid/4-oxoretinoic acid |
| Retinoyl glucuronides (retinoic acid glucuronides) |

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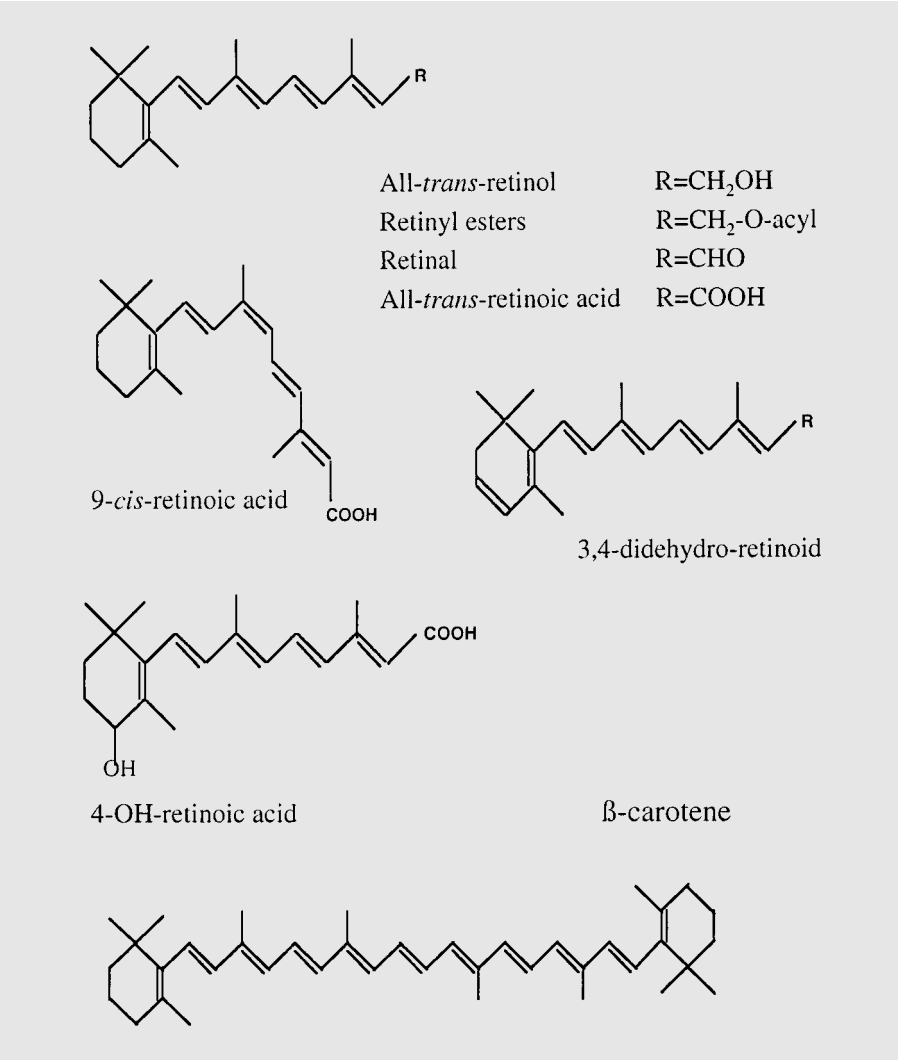


**Fig. 1.** Chemical structures of some natural retinoids and carotenoids.

of compounds that we in daily speaking regard as synthetic retinoids (e.g. isotretinoin). The purpose of this overview is to briefly discuss the chemical nature, metabolism, function and dermatotherapeutic use of natural retinoids, defined here as vitamin-A-related compounds occurring normally in human blood and tissues (table 1). The pivotal role of retinaldehyde in the inter-conversions of natural retinoids and in the biological function of vitamin A is emphasized.

## Chemistry

Retinoids have been defined as a class of compounds consisting of four isoprenoid units [$H_2C = C(CH_3)–CH = CH_2$) joined in a head-to-tail manner. The natural retinoid molecule can be divided into three parts: a nonaromatic ring structure, a polyprenoid side chain and a polar carbon-oxygen functional end group (fig. 1). Taken together these chemical features explain the multifacetted characteristics of natural retinoids both with regard to their physicochemical reactions and with respect to the many biological effects in which they are involved. Typically, vitamin A readily undergoes many chemical and metabolic modifications, and some metabolites are highly reactive. For example, retinylaldehyde easily forms a Schiff base with opsin, and retinoic acid (RA) can cause retinoylation of many proteins such as keratins [2]. An often unwanted characteristic of many natural retinoids is that they exert detergent-like properties in cellular membranes and are extremely sensitive to destruction by UV irradiation and artificial oxidation.

Although natural retinoids are predominantly in the all-*trans* configuration, *cis-trans* isomerizations of the poly-

prenoid side chain are biologically very important and can occur both as a result of tissue metabolism and following UV irradiation. The metabolic formation of 11-*cis*-retinaldehyde in the eye and its UV-mediated conversion to all-*trans*-retinaldehyde are well-known examples. A more recent example of a metabolic isomerization product is 9-*cis*-RA (fig. 1), a natural ligand for the nuclear retinoid X receptor (see below).

## General Metabolism of Natural Retinoids and Carotenoids

Figure 2 presents a general view of the intestinal uptake, the first-passage metabolism of vitamin A and the release of retinol from the liver bound to retinol-binding protein (RBP), which is ultimately degraded in the kidneys [for recent reviews, see 1 and 3]. Vitamin A occurs in food of animal, origin, mainly in the form of preformed vitamin (retinyl esters), and in vegetables and fruits in the form of carotenoids (e.g. β-carotene; fig. 1). Approximately 600 carotenoids have been identified thus far in nature. However, less than 10% of them are vitamin A precursors. Carotenoids are found in different organs and tissues including blood where they are bound to lipoproteins [for a review, see 3]. Soon after ingestion, retinyl esters and carotenoids undergo enzymatic hydrolysis. In humans, most of the absorbed β-carotene is converted to retinol in the enterocytes by the action of specific enzymes. When these enzymes are missing, e.g. due to a genetic error, the uptake of unchanged β-carotene increases dramatically and may result in carotenemia [4, 5].

One of the intermediates in the conversion of β-carotene to retinol is retinylaldehyde; this seems to be the case both in enterocytes during absorption and when cleavage of β-carotene occurs in peripheral tissues (fig. 3). Independent of whether retinylaldehyde is derived from β-carotene or preformed retinol, it can be further oxidized to RA (see below). However, the major bulk of retinylaldehyde is converted to retinyl esters, which are either stored in the peripheral target cells or, in the case of intestinal uptake, are transported by the chylomicrons to the liver and subsequently stored in the stellate cells [for a review, see 3].

## Vitamin A Uptake in the Skin

The transport of vitamin A to the skin under fasting conditions is mediated by RBP, which is bound to transthyretin [for a review, see 6]. After reaching the interstitial fluid of the skin, RBP diffuses freely into the epidermis and delivers retinol against a concentration gradient. It is still debated whether RBP delivers retinol via binding to a cell surface receptor [7–11] or by releasing small amounts of the lipophilic vitamin to the aqueous environment of the cell [12]. Interestingly, Båvik et al. [13] have recently characterized an RBP receptor from retinal pigment epithelium, the isolation of which will hopefully allow a proper elucidation of the receptor mechanism also in the skin.

In vitro experiments on human skin and keratinocytes have shown that the cellular uptake of $^{3}$H-retinol is slower from RBP than from other, less specific carriers, such as albumin [12, 14]. This points to a dual role of the RBP delivery system: (i) to maintain a constant supply of retinol to the target cells that is independent of daily variations in the vitamin A intake and (ii) to avoid excessive delivery of free retinol that might interfere with terminal differentiation of keratinocytes. Although RBP receptors most probably mediate cellular retinol uptake under physiological conditions (see above), retinoids may also enter keratinocytes unspecifically (via the aqueous phase), which is undoubtedly the case in conditions of hypervitaminosis A and during topical treatment with natural retinoids.

The first demonstration of vitamin A in human skin was by Williams in 1943 [15]. Later, Cornblett and Greenberg [16] and Greenberg et al. [17], using fluorescence microscopy, suggested that carotene was converted to vitamin A in sebaceous glands. However, accurate analysis of retinoids

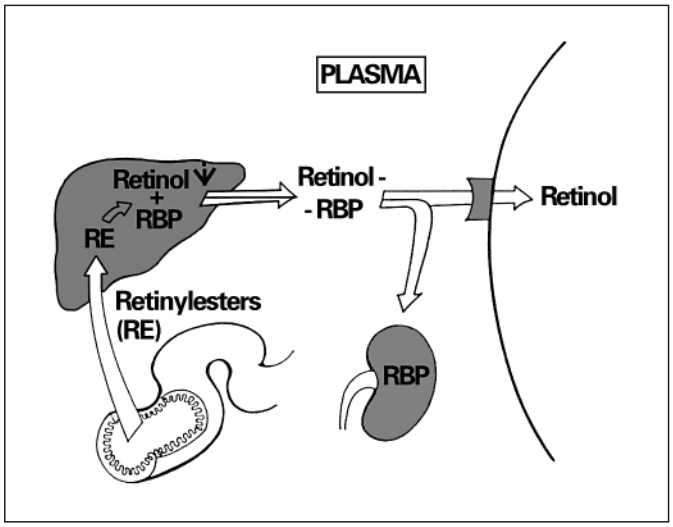


**Fig. 2.** General overview of the vitamin A metabolism in man. RBP = Serum retinol-binding protein.

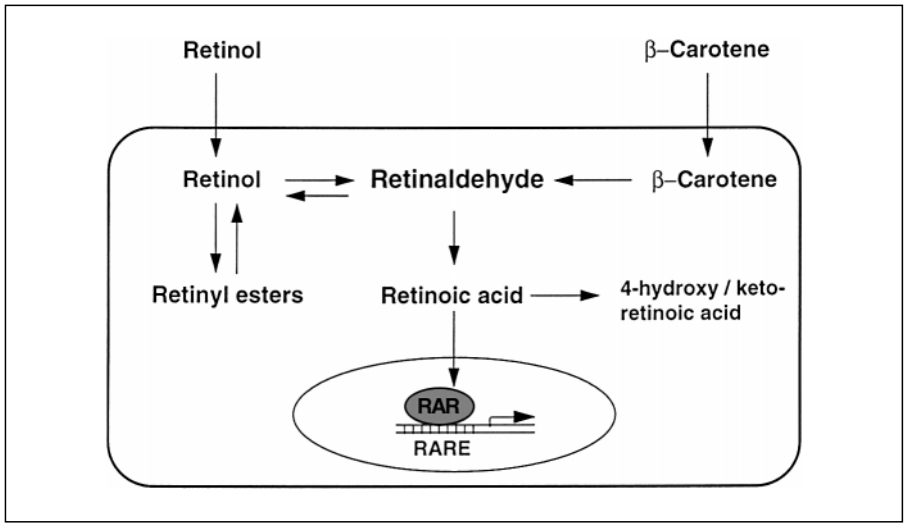


**Fig. 3.** Cellular metabolism of retinol and carotene revolves around retinaldehyde. RAR = RA receptor; RARE = RA response element.

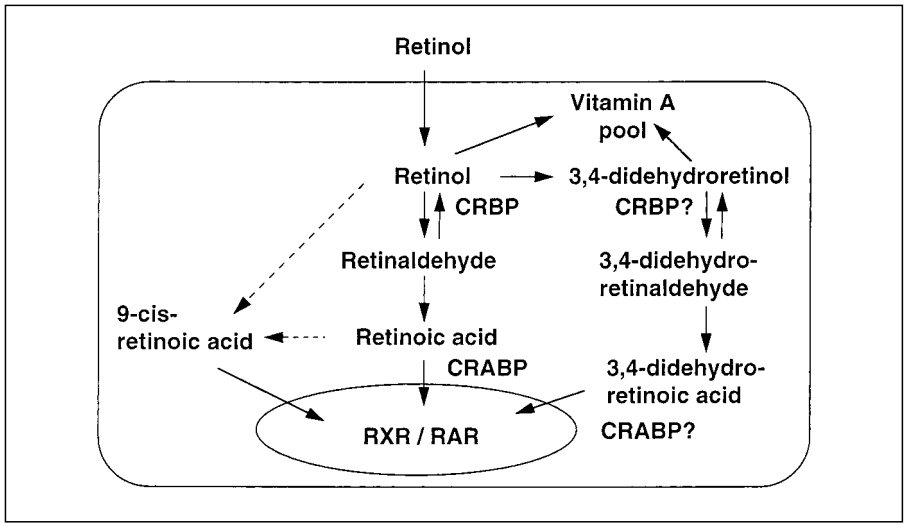


**Fig. 4.** Retinol metabolism and involvement of cellular retinoid-binding proteins in human keratinocytes. CRBP = Cellular retinol-binding protein; CRABP = cellular RA-binding protein; RAR = RA receptor; RXR = retinoid X (9-*cis*-RA) receptor.

in skin biopsies was not possible until the introduction of high-performance liquid chromatography in the late 1970s [18–20].

## Epidermal Vitamin A Metabolism

Figure 4 summarizes the complex metabolic pathways for retinol in human keratinocytes. In contrast to plasma with its predominance of all-*trans*-retinol, human epidermis and cultured keratinocytes contain 4 major vitamin A components, i.e. retinol, 3-dehydroretinol and fatty acyl esters of both these alcohols [19, 21]. 3-Dehydroretinol (3,4-didehydroretinol = vitamin $A_2$; fig. 1), which is abundant in certain amphibians, was first detected in human skin by studies of psoriatic epidermis [18]. It is a major metabolite of retinol in human skin [22] but can probably also be generated by direct cleavage of lutein. The biological activity of 3-dehydroretinol in the whole animal is 30–40% that of retinol, but in reconstructed human skin the activities are equal [M. Darmond, pers. commun.]. Blood serum and tissues other than skin usually contain insignificant or very low amounts of 3-dehydroretinol and its esters [23, 24]. Similarly, with the notable exception of chick embryos [25, 26], the skin of most laboratory animals is devoid of this unusual form of vitamin A [24]. However, cultured human epithelial cell lines (e.g. HeLa cells and keratinocytes) as well as melanoma cell lines contain both retinol and 3-dehydro-

retinol and are able to metabolize tritium-labeled retinol into the latter compound [27, 28].

The concentrations of vitamins A and carotenoids are generally higher in epidermis than in the underlying dermis; this is especially the case for 3-dehydroretinol and its esters [20]. Within the epidermis there is a continuous mass gradient that is characterized by increasing concentrations of neutral retinoids in the differentiating keratinocytes; particularly the fatty acyl esters accumulate in cells from the outermost layers [29].

The extensive esterification of 3-dehydroretinol and retinol in epidermis [23, 29, 30] provides a local store of the vitamin and controls the production of biologically active congeners. The esterifying enzyme acyl CoA retinol acyltransferase (EC 2.3.1.76) has interesting properties in human epidermis [29]; its pH optimum (5.5 instead of 7.4 as in other tissues) seems evolutionary to have adapted to the low pH in stratum corneum. Speculatively, the fall in pH accompanying terminal differentiation of human epidermis could stimulate the acyl CoA retinol acyltransferase activity in stratum corneum and thus explain the high content of retinyl esters near the skin surface. Another possibility is that cells in the upper parts of the epidermis specifically express lecithin retinol acyltransferase, an esterifying enzyme which has not yet been studied in the skin.

## Biologically Active Metabolites

The extensive search for active retinol metabolites has so far not resulted in the demonstration of any compound with higher biological activity than all-*trans*- and 9-*cis*-RA, which display a 100- to 1,000-fold higher activity than their precursors retinol and β-carotene [1]. It is generally believed that the formation of RAs is an essential requirement for vitamin A activity in epithelial cells; however, the endogenous concentration of these compounds in keratinizing epithelia is exceedingly low [19]. The formation of RA from retinol has been demonstrated in mouse epidermis topically treated with vitamin A [31, 32], and enzymatic activity that converts retinaldehyde to RA has been demonstrated in epidermal homogenates [33].

Siegenthaler et al. [34] and Siegenthaler and Saurat [35] have provided evidence that cytosol from undifferentiated human keratinocytes cannot convert retinol into retinal and RA, but cytosol prepared from the differentiated keratinocytes is able to convert retinol into RA. This is consistent with the demonstration of RA formation in adult differentiated tracheal epithelial cells [36] but not in undifferentiated embryonal carcinoma cells [37]. Recently, Châtellard-Gruaz et al. [38] have demonstrated that differentiation of human epidermal keratinocytes is accompanied by increased cellular concentrations of RAs. It is possible that retinol metabolism via 3-dehydroretinol provides an additional source of activated vitamin A [39]. Thus, 3,4-didehydroretinoic acid (ddRA) is a metabolite of retinol in embryonic chick skin [26] and has morphogenetic properties in the limb bud system that are similar to those of RA. It is also equipotent to RA in inhibiting epidermal keratinization in vitro and inhibiting epidermal transglutaminase [40].

The enzymatic basis for the oxidation of retinol to retinaldehyde and RA, as well as the cytochrome-P450-assisted degradation of the latter compound, has been described in detail over the last years [41–51]. However, still many more enzymes await characterization. For example, 9-*cis*-RA levels in human skin are much lower than all-*trans*-RA levels and 9-*cis*-RA applied topically to human skin isomerizes rapidly to all-*trans*-RA, suggesting the existence of an isomerase that preferentially produces all-*trans*-RA [1]. Recently, several other metabolites have been identified whose roles in the skin need to be defined, e.g. 4-oxoretinol [52], 13,14-dihydroxyretinol [53], retroretinoids [54, 55], 9,13-di-*cis*-RA [56] and several other retinol metabolites [57, 58]. Some of these metabolites are biologically active without even binding to the nuclear retinoid receptors, suggesting that they either bind to hitherto unidentified orphan receptors, are further metabolized, or act via nongenomic pathways.

## Epidermal Vitamin A and UV Irradiation

Sun-exposed epidermis contains less retinyl esters than adjacent unexposed skin, indicating that UV irradiation may elicit a focal hypovitaminosis A [for a review, see 59]. In fact, a single UV irradiation may lower the vitamin A content of epidermis by as much as 70–90% and perturb the mass gradient of the vitamin to a depth corresponding to the penetration of the radiation in the skin [60–62]. This is not surprising in view of the notorious sensitivity of retinoids to UV radiation in vitro. Thus the action spectrum of experimentally applied UV radiation shows that the retinol lowering effect in epidermis is optimal around 330 nm [60], i.e. close to the absorption maximum of all-*trans*-retinol. 3-Dehydroretinol (absorption maximum 352 nm) is less affected than retinol when exposed to solar-simulated UV irradiation in the skin [60, 61]. It is an intriguing possibility that the UV-induced depletion of vitamin A in sun-exposed skin is involved in the pathophysiology of extrinsic skin aging and tumor formation. On the other hand, recent animal data suggest that a surplus of vitamin A in the skin can also be detri-

mental during chronic UV irradiation and may enhance tumorigenesis [63]. One explanation to this may be that primary intermediates and free radicals are produced from retinol during irradiation [64]. Interestingly, all-*trans*-RA is less prone to produce these potentially photosensitizing compounds [64].

In the clinical situation too, phototherapy of psoriasis with UVB (280–320 nm) or psoralens + UVA (320–390 nm) reduces the retinol content of the skin [24].

## Metabolic Interactions with Synthetic Retinoids

When synthetic retinoids are administered, they are likely to interact with the pre-existing natural retinoids in the tissues. For example, oral isotretinoin therapy has a marked effect on the epidermal concentrations of retinol and 3-dehydroretinol in acne patients, whereas oral acitretin given to psoriasis patients only marginally affects the vitamin A levels [24, 65]. Neither isotretinoin nor acitretin affects the plasma retinol concentrations in man. In contrast, several studies in rats [66] and man [67] have shown that all-*trans*-RA therapy reduces the retinol level in plasma, and thus could indirectly affect the cutaneous retinoid concentration.

The effect of various synthetic retinoids on the epidermal metabolism of tritium-labeled retinol has been studied in vitro [68]. The production of 3-dehydroretinol by cultured keratinocytes virtually stops in the presence of RA and its isomers [69, 70]. It is presently unknown whether this interaction is caused by feedback inhibition of the vitamin A metabolism or to direct inhibition of the enzyme(s) involved in the desaturation of retinol.

## Biological Function in the Skin

Vitamin A exerts a hormone-like effect on the skin which is believed to be mediated via its conversion to RA and related compounds binding to the nuclear receptor proteins RA receptor (RAR) and retinoid X receptor [for reviews, see 1 and 59]. Until now, no nuclear receptor for retinol – the major naturally occurring retinoid in the skin – has been identified, suggesting that metabolic conversion of retinol to RA is a necessary event in the action of retinoids in vivo. As mentioned above, the skin of some species apparently produces ddRA in addition to RA. Interestingly, RA and ddRA bind to the nuclear receptors (RAR $\alpha$, $\beta$ and $\gamma$) with similar affinity and induce transcriptional activation in cells transfected with RAR and a reporter gene construct [40]. ddRA is an interesting exception to the general rule implying that modification of the cyclohexenyl ring leads to a diminution or loss of binding to RAR. For example, 4-oxo-RA displays only about 5% of the binding demonstrated by RA, and 4-hydroxy-RA (fig. 1) displays no measurable binding whatsoever [71].

The endogenous production of perhaps three or more types of nuclear retinoid receptor ligands (RA, ddRA and 9-*cis*-RA) raises the intriguing possibility that expression of vitamin A activity in the skin depends in part on the relative concentrations of these ligands. How the endogenous ligands orchestrate the RARs in normal and diseased human keratinocytes in the absence or presence of synthetic retinoids remains to be established.

## Retinoid Signaling in Diseased Skin

In psoriasis and certain other hyperpoliferative skin disorders there is an accumulation of vitamin $A_2$ and possibly of other natural retinoids [72, 73]. Hypothetically, this could trigger keratinocyte proliferation. It is also consistent with the fact that several retinoid-regulated genes (e.g. cellular RA-binding protein II and keratin 19) are up-regulated in these disorders, whereas other genes such as keratins 1 and 10 are down-regulated. A more in-depth discussion of these matters is found in a recent overview [59]. To date there are no reports on metabolic errors underlying defective vitamin A signaling in the skin, but follicular hyperkeratosis (phrynoderma) – a classical albeit unspecific sign of hypovitaminosis A – is a common symptom of many skin diseases.

## Clinical Effects

The idea to use vitamin A in the treatment of skin diseases came from the observations in the 1930s that dietary hypovitaminosis A is associated with hyperkeratinization of the skin [74]. The association between hypovitaminosis A and follicular hyperkeratosis has since been reported repeatedly in both man and animals [75–79]. In fact, several clinical trials in the 1940s showed that patients with hereditary disorders of keratinization occasionally responded to high-dose vitamin A regimes [80–83]. However, owing to unpredictable results and serious side effects (hypervitaminosis A), retinol therapy was largely abandoned until the late seventies when several synthetic analogs of vitamin A became available. Synthetic retinoids have since revolutionized the treatment of many skin disorders, such as

ichthyosis, acne and psoriasis. Over the last years, however, there has also been a renewed interest in the effects of natural retinoids, primarily as topical agents. Thus, vitamin A – either in the form of retinol or retinyl esters – is commonly added to cosmetics (although it is a poor prodrug of RA when applied locally on the skin), and retinaldehyde (which is a much more efficient prodrug) attracts increasing interest as a topical treatment for skin aging and other skin disorders [84]. An advantage of using retinaldehyde instead of RA is its more versatile metabolism – a surplus of the drug will be rapidly reduced to retinol and hence inactivated as retinyl esters. This type of 'back-metabolism' is not possible for RA.

## Conclusions and Speculations for the Future

Natural retinoids play a pivotal role in epidermal differentiation and during dermal regeneration and cutaneous inflammation. Physiologically, retinol is the principal source of vitamin A activity in the skin. The mechanisms controlling the cellular uptake of retinol from plasma, the intracellular generation of RA, 9-*cis*-RA and ddRA from retinol, and the intracellular transport and compartmentalization of these ligands for the nuclear receptors are only beginning to be disclosed. Not knowing the etiology of many skin diseases or the exact mechanism of action of retinoids in the skin, the therapeutic rationale for using these drugs is still mostly based on 'trial and error'. As a result of this, however, many unanticipated findings have also emerged that may shed light on the effects of retinoids in healthy and diseased skin. Although high-dose therapy with natural retinoids probably exerts many pharmacological effects unrelated to their physiological role, this may not be the only explanation of their efficacy as remedies of skin diseases. The fact that most of the clinically effective retinoids are derivatives of naturally occurring RA makes it reasonable to hypothesize that in some diseases this therapy compensates preexisting defects in the vitamin A metabolism or in the expression of RA receptors. Indirect evidence for the involvement of vitamin A in the pathogenesis of certain genetic disorders of keratinization has been presented. Hopefully, elucidation of putative errors of the vitamin A metabolism in human epidermis will add further insight into the role played by individual retinoids in the normal function of the skin.

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# Metabolism of Topical Retinaldehyde

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**Key Words**
Retinoids · Retinaldehyde · Epidermis · Metabolism

**Abstract**
***Objective:*** In order to circumvent the tolerance problems encountered with topical application of retinoic acid – a biologically active metabolite of vitamin A – we performed in various models a series of experiments aimed at assessing the bio-availability of topical retinaldehyde and its conversion into either retinoid stores or biologically active metabolites. ***Methods:*** (i) $^{3}$H-retinaldehyde was used as a precursor of either $^{3}$H-retinol or $^{3}$H-retinoic acid in human skin extracts and human cultured keratinocytes; (ii) the concentration of various retinoids resulting from the metabolism of topical retinaldehyde was determined in mouse skin and in human plasma. Retinoids were quantified by reverse-phase HPLC with UV detection. ***Results:*** Human keratinocytes were shown to take up retinaldehyde and to convert it into retinoic acid in a differentiation-dependent manner, differentiating cells oxidising retinaldehyde more efficiently. In vivo models allowed us to demonstrate that retinaldehyde is taken up by the skin and is then predominantly converted into retinyl esters – a storage form of vitamin A – while delivering relatively low amounts of retinoic acid from a large reservoir. ***Conclusion:*** Topical retinaldehyde can be used as a precursor of endogenous retinoids, since it is converted into both storage and bioactive forms of vitamin A.

## Introduction

In spite of the clinical benefits gained by the use of topical retinoic acid isomers in several skin diseases, the erythemogenic properties of these vitamin A metabolites often prevent their use in clinics and promoted a great deal of interest for a new class of vitamin A analogues which would be well tolerated, while preserving the therapeutic potential of natural ligands for nuclear retinoic acid and retinoid X receptors. Since irritation is probably not involved in the beneficial action of retinoids [1, 2], all-*trans*-retinaldehyde (RAL), which was shown to be well tolerated [3], was investigated for its use as an alternative for topical retinoic acid. In particular, we have conducted studies aimed at analysing the two metabolic pathways of RAL, i.e. (i) the oxidation into retinoic acid isomers – the ligands for nuclear receptors – and (ii) the reduction into all-*trans*-retinol (ROL), followed by esterification with long-chain fatty acids, leading to the storage form of retinoids. We have used ex vivo, in vitro and in vivo approaches.

## Results

*Ex vivo: Human Skin Extracts*

Cytosolic extracts were prepared from keratomised normal human skin and incubated with 600 n*M* $^{3}$H-RAL.

The metabolites formed depended on the redox co-factor co-incubated with $^{3}$H-RAL: in the presence of 5 m*M* NADH, $^{3}$H-ROL was formed at a rate of 1.0 ± 0.1 pmol/mg protein/h, whereas in the presence of 2 m*M* NAD, $^{3}$H-all-*trans*-retinoic acid (RA) was formed at a rate of 1.5 ± 0.2 pmol/mg protein/h [4] (table 1).

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**Table 1.** Ex vivo: cytosolic extracts of human skin slices (180 µm)

| Precursor | Analyte | Co-factor | Value pmol/mg protein/h | Reference |
|---|---|---|---|---|
| 600 n*M* $^3$H-RAL | $^3$H-ROL | NADH (5 m*M*) | 1.0 ± 0.1 | 4 |
| 600 n*M* $^3$H-RAL | $^3$H-RA | NAD (2 m*M*) | 1.5 ± 0.2 | 4 |

**Table 2.** In vitro: human cultured keratinocytes

| Fraction | Differentiated | Precursor | Co-factor | Analyte | Value | Unit | Reference |
|---|---|---|---|---|---|---|---|
| Cytosol | yes | 600 n*M* $^3$H-ROL | – | $^3$H-RA | 4.49 ± 0.10 | pmol/mg protein/h | 4 |
| | yes | 600 n*M* $^3$H-RAL | NAD (2 m*M*) | $^3$H-RA | 51.6 | pmol/mg protein/h | 4 |
| | no | 600 n*M* $^3$H-RAL | NAD (2 m*M*) | $^3$H-RA | 14.4 | pmol/mg protein/h | 4 |
| | yes | 600 n*M* $^3$H-RAL | NADH (5 m*M*) | $^3$H-ROL | 8.2 | pmol/mg protein/h | 4 |
| | no | 600 n*M* $^3$H-RAL | NADH (5 m*M*) | $^3$H-ROL | 8.0 | pmol/mg protein/h | 4 |
| Whole cells | yes | 50 n*M* $^3$H-ROL | – | $^3$H-dRA | 6.1 ± 2.2 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-ROL | – | $^3$H-dRA | 1.9 ± 0.5 | pmol/mg DNA | 5 |
| | yes | 50 n*M* $^3$H-ROL | – | $^3$H-RA | 8.1 ± 3.3 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-ROL | – | $^3$H-RA | 7.6 ± 4.3 | pmol/mg DNA | 5 |
| | yes | 50 n*M* $^3$H-ROL | – | $^3$H-dROL | 0.7 ± 0.2 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-ROL | – | $^3$H-dROL | 0.8 ± 0.1 | pmol/mg DNA | 5 |
| | yes | 50 n*M* $^3$H-ROL | – | $^3$H-ROL | 17.2 ± 3.8 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-ROL | – | $^3$H-ROL | 24.4 ± 7.6 | pmol/mg DNA | 5 |
| | yes | 50 n*M* $^3$H-ROL | – | $^3$H-RE | 1,617 ± 695 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-ROL | – | $^3$H-RE | 380 ± 132 | pmol/mg DNA | 5 |
| Whole cells | yes | 50 n*M* $^3$H-RAL | – | $^3$H-dRA | 27.6 ± 7.7 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-RAL | – | $^3$H-dRA | 8.3 ± 2.2 | pmol/mg DNA | 5 |
| | yes | 50 n*M* $^3$H-RAL | – | $^3$H-RA | 59.5 ± 15.4 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-RAL | – | $^3$H-RA | 16.9 ± 5.0 | pmol/mg DNA | 5 |
| | yes | 50 n*M* $^3$H-RAL | – | $^3$H-dROL | 0.8 ± 0.7 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-RAL | – | $^3$H-dROL | 0.2 ± 0.1 | pmol/mg DNA | 5 |
| | yes | 50 n*M* $^3$H-RAL | – | $^3$H-ROL | 11.5 ± 3.2 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-RAL | – | $^3$H-ROL | 5.3 ± 1.3 | pmol/mg DNA | 5 |
| | yes | 50 n*M* $^3$H-RAL | – | $^3$H-RAL | 2.8 ± 0.9 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-RAL | – | $^3$H-RAL | 2.9 ± 1.1 | pmol/mg DNA | 5 |
| | yes | 50 n*M* $^3$H-RAL | – | $^3$H-RE | 914 ± 237 | pmol/mg DNA | 5 |
| | no | 50 n*M* $^3$H-RAL | – | $^3$H-RE | 288 ± 75 | pmol/mg DNA | 5 |

dRA = Dehydro-RA; dROL = dehydro-ROL.

*In vitro: Human Cultured Keratinocytes*

*Cytosolic Extracts.* Human keratinocytes were cultured in low or normal calcium concentrations in order to distinguish between non-differentiating (low calcium) and differentiating (normal calcium) keratinocytes.

In the presence of 5 m*M* NADH, 600 n*M* $^3$H-RAL were reduced into $^3$H-ROL at about 8 pmol/mg protein/h in both culture types; conversely, the oxidation into $^3$H-RA, in the presence of 2 m*M* NAD, was more rapid in differentiating cultures (51.6 pmol/mg protein/h) than in non-differentiating ones (14.4 pmol/mg protein/h) [4] (table 2).

*Whole Cells.* Since some enzymes are found in microsomes rather than in the cytosol, we performed a new series of experiments with whole-cell extracts of differentiating and non-differentiating cultured human keratinocytes. In this study, cultures were incubated with 50 n*M* $^3$H-RAL without addition of exogenous redox co-factor.

**Table 3.** In vivo: topical application of RAL or 9-*cis*-RAL (14 days) on C5BL/6 mouse tail skin, washed or unwashed (analysis of whole skin)

| Application | Washing | Analyte | Value pmol/g wet weight | Reference |
|---|---|---|---|---|
| Vehicle | no | ROL | 244 ± 42 | 7 |
| | yes | ROL | 206 ± 20 | 6 |
| | no | RE | 282 ± 55 | unpublished results |
| | yes | RE | 417 ± 36 | unpublished results |
| | no | RA | <15 | 7 |
| | yes | RA | <15 | 6 |
| RAL | no | ROL | 2,619 ± 118[a] | 7 |
| | yes | ROL | 1,556 ± 81[a, b] | 6 |
| | no | RE | 8,600 ± 2,053[a] | unpublished results |
| | xes | RE | 4,101 ± 218[a] | unpublished results |
| | no | RA | 43 ± 9 | 7 |
| | yes | RA | 25 ± 3 | 6 |
| 9-*cis*-RAL | no | ROL | 916 ± 32[a] | unpublished results |
| | yes | ROL | 958 ± 22[a] | 6 |
| | no | RE | 1,269 ± 243 | unpublished results |
| | yes | RE | 1,507 ± 147[a] | unpublished results |
| | no | RA | 29 ± 1 | unpublished results |
| | yes | RA | 30 ± 1 | 6 |

[a] $p < 0.001$: different from vehicle group.
[b] $p < 0.001$: different from unwashed samples.

Differentiating cultures did metabolise $^3$H-RAL into RA, dehydro-RA, ROL, dehydro-ROL and retinyl ester (RE) at a higher rate than non-differentiating cultures, although RAL uptake was the same in both culture types. When using $^3$H-ROL as retinoid precursor, differentiating cultures were shown to produce more RA and RE than non-differentiating ones, whilst the other metabolites were produced at the same rate in both culture types. Moreover, except for RE in differentiating cultures, the metabolism of $^3$H-RAL was higher than that of $^3$H-ROL [5] (table 2).

These in vitro studies allowed us to demonstrate that RAL can be metabolised into RA, ROL and RE by human cultured keratinocytes; this process is dependent on the differentiation stage of keratinocytes since it is higher in differentiating than in non-differentating cells. We next studied if this occurs in vivo in a mouse model.

*In vivo: Metabolism of Topical Retinoids by Mouse Tail Skin*

*Topical Retinal Isomers in Washed and Unwashed Mouse Tail.* The tail of C57BL/6 mice was treated for 14 days with either excipient, RAL 0.05% or 9-*cis*-RAL 0.05%, a potential precursor of 9-*cis*-RA which was shown to have biological activities in this mouse model [6]. In half of the samples, the skin was washed with a solution of 0.1% Triton X-100; the whole skin of all samples was analysed for retinoid determination.

In unwashed tail skin, topical RAL promoted a 10-fold increase in ROL and a 30-fold increase in RE as compared to vehicle; this increase was more moderate in 9-*cis*-RAL-treated skin. The RA concentration was under the detection limit of 15 pmol/g wet weight in vehicle-treated skin but was measurable following a topical treatment with either RAL or 9-*cis*-RAL. The washing of the skin removed about one half of the retinoid content in RAL-treated skin (ROL, RE and RA), whilst this operation had no effect on the retinoid content of vehicle- and 9-*cis*-RAL-treated skin (table 3).

*Topical RAL on Hairless Mice.* The back of hairless mice was treated for 7 days with vehicle or RAL 0.05%, then the skin was harvested and the epidermis was separated from the dermis by heat. The retinoid content was determined in the epidermis. Topical RAL promoted a 6-fold increase in ROL and a 13-fold increase in RE, as well as a higher concentration of RA in the epidermis (226 pmol/g wet weight) than that of whole tail skin of C57BL/6 mice (43 pmol/g wet weight) (table 4).

**Table 4.** In vivo: topical application (7 days) on hairless mouse back skin (unwashed): analysis of epidermis

| Application | Analyte | Value (pmol/g ww) | Reference |
|---|---|---|---|
| Vehicle | ROL | 152 ± 20 | unpublished results |
| Vehicle | RE | 1,102 ± 85 | unpublished results |
| Vehicle | RA | < 15 | unpublished results |
| RAL | ROL | 947 ± 79*** | unpublished results |
| RAL | RE | 13,761 ± 2,336*** | unpublished results |
| RAL | RA | 226 ± 26*** | unpublished results |

*** Different from vehicle group ($p < 0.001$).

*Systemic Effects of the Skin Metabolism of Topical RAL in Humans*

To see if topical application of a large quantity of RAL on human skin is associated with detectable alteration of constitutive levels of plasma retinoids resulting from metabolism of RAL in the skin, plasma retinoids [ROL, RE (all-*trans*-retinyl oleate + palmitate), RAL, RA, 13-*cis*-RA, 4-oxo-13-*cis*-RA] were analysed by HPLC in 10 healthy male volunteers kept under a poor vitamin A diet before, during and after daily topical application for 14 days of 7 mg (25 µmol) of RAL to 40% of the body surface.

The introduction of a 1-week restricted vitamin A diet before RAL application resulted in a decrease in plasma levels of ROL, RA and RE. Topical RAL did not induce an alteration of the retinoid metabolite plasma levels. No RAL was detectable in any of the plasma samples.

These results indicated that the skin metabolism of topically applied RAL does not result in detectable alteration of constitutive levels of plasma retinoids in humans. Since there was no increase in plasma RE during topical RAL treatment (despite a previous reduction in plasma RE due to the diet), it is likely that the RE produced from the daily applied 7 mg of RAL remained stored in the epidermis. The proportion of RAL transformed into ROL is not likely to be delivered systemically since epidermal enzymes would metabolise it to RE. At any rate, the constitutive levels of plasma ROL (about 2 µ*M*) were not altered by the small amount that may originate from skin. In addition to providing important safety data pertinent to the potential use of RAL as a topical agent in humans, these results supported the concept of targeting vitamin A metabolites in the skin upon topical treatment with RAL.

## Discussion

These observations indicate that RAL would fulfil one criterion in the concept that is to target multipotential vitamin A activity into distinct compartments of the epidermis. Indeed, topically applied RAL would (i) be a precursor of either ROL, RE or RA, (ii) bypass the first, rate-limiting step of ROL oxidation into RA and (iii) be handled only by the epidermal cells having enzymatic activities at a pertinent stage of differentiation resulting in a controlled delivery of vitamin A metabolites into the cells. RAL does not bind to retinoid nuclear receptors [8, 9]. Therefore its biological activity should result from its enzymatic transformation into RA by epidermal keratinocytes and should be qualitatively similar to that of RA. In order to exert a quantitatively comparable activity to that resulting from direct application of RA, topical RAL should generate enough amounts of RA to saturate nuclear retinoic acid receptors. As shown above, this is achieved in mouse skin where RAL concentrations reach 4–20 n*M*, a range of concentrations high enough to saturate nuclear receptors [8, 10], but much less than those (1–10 µ*M*) reached after topical RA [7, 11]. Finally, the modulation of retinoid metabolism induced by a topical RAL application would be restricted to the skin, since a 14-day topical treatment with RAL has no effect on the concentration of plasma retinoids.

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# Biological Activities of Topical Retinaldehyde

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**Key Words**

Biological activities · Citral · Mouse tail model · Myeloperoxidase · Retinaldehyde, topical · Retinaldehyde, 9-*cis* · Retinoid irritation

**Abstract**

***Background:*** We had hypothesised that retinaldehyde (RAL) should be an interesting precursor for topical use. ***Aim:*** We review our observations about its biological activities. ***Methods:*** We performed pilot studies to explore its biological effects and tolerability in human skin and compared the effects of topical RAL to that of all-*trans*-retinoic acid (RA) in the mouse tail test. ***Results:*** The biological activities of RAL were found to be qualitatively identical to that of RA: (i) induction of cellular RA-binding protein type 2 mRNA and protein, (ii) increase in epidermal proliferation (increase in DNA synthesis, epidermal thickness, induction of 50-kD keratin mRNA and reduction in 70-kD keratin mRNA), and (iii) metaplastic effects (induction of orthokeratosis, reduction of 65-kD keratin mRNA, increase in filaggrin and loricrin mRNAs). When associated with RAL, citral (known for its capacity to inhibit the oxidation of retinol to RA in epidermis) counteracted the effects induced by RAL indicating that RAL exerts biological activities through transformation to RA. Hypothesising that keratinocytes would metabolize 9-*cis*-RAL to 9-*cis*-RA, we compared the biological effects induced by topical 9-*cis*-RAL and found that hyperplastic and metaplastic responses were lower than those induced by all-*trans*-RAL or all-*trans*-RA at similar concentrations. This suggests that 9-*cis*-RAL has no advantage over all-*trans*-RAL for specific delivery of natural retinoids into the skin. As in clinical studies conducted in human skin, we also found topical RAL less irritant than RA. ***Conclusion:*** These studies indicate that topical RAL has biological activity and is well tolerated.

## Introduction

We have explored the possibility of delivering retinoid activity to human skin topically with retinaldehyde (RAL) that does not bind nuclear receptors. Validation of the concept implies demonstration that the topical application of RAL results in biological effects. We summarise here the results of studies conducted both in humans and animals.

## Biological Effects of RAL in Human Skin

One parameter of retinoid activity is the induction of CRABP-2 mRNA and protein [1–3]; we have shown that topical RAL induces CRABP-2 mRNA by 10-fold and CRABP-2 protein by 3-fold; the rank order for the CRABP-2 increase was RA > RAL > 9-*cis*-RA > retinol > β-carotene [4]. This suggested that the CRABP-2 induction by topical RAL is a result of transformation by epidermal enzymes into active ligand(s).

In volunteers treated 1–3 months by 0.5, 0.1 and 0.05% RAL (table 1), there was a significant dose-dependent increase in epidermal thickness, keratin 14 immunoreactivity and Ki-67-positive cells. The area of distribution of involu-

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**Table 1.** Biological effects of RAL in human skin

| Duration of treatment | Parameters | Vehicle | 0.05% RAL |
|---|---|---|---|
| 4 days | CRABP-2 mRNA, pixels | 391 ± 72 | 4,410 ± 432 |
| | CRABP-2 protein, pixels | 1,769 ± 200 | 4,593 ± 834 |
| 1 month | Ki-67, cells/mm | 36 ± 11 | 66 ± 62 |
| | epidermal thickness, µm | 44 ± 29 | 59 ± 37 |
| | keratin 4 | absent | induced (focal) |
| | involucrin, µm thickness | 15 ± 1 | 27 ± 11 |
| | filaggrin, µm thickness | 23 ± 1 | 32 ± 13 |
| | transglutaminase, µm thickness | 13 ± 0.5 | 19 ± 6 |
| 3 months | Ki-67, cells/mm | 44 ± 29 | 59 ± 37 |
| | epidermal thickness, µm | 65 ± 17 | 83 ± 27 |
| | keratin 4 | absent | induced (focal) |
| | involucrin, µm thickness | 12 ± 3 | 13 ± 2 |
| | filaggrin, µm thickness | 38 ± 4 | 38 ± 4 |
| | transglutaminase, µm thickness | 11 ± 2 | 11 ± 2 |

crin, transglutaminase and filaggrin immunoreactivities was also increased in a dose-dependent manner, and keratin 4 immunoreactivity was induced in the upper epidermis. Interestingly the induction of filaggrin, involucrin and transglutaminase was no longer seen after 3 months of treatment, indicating an adaptive process. These findings indicate that RAL exerts biological activity when used as a topical agent on human skin.

## Biological Effects of RAL in Mouse Tail Skin

*The Mouse Tail Skin Model*

The mouse tail has been shown to be a normal in vivo model for antipsoriatic drug screening [5]. The adult mouse tail skin possesses a granular layer and exhibits orthokeratosis only in perifollicular areas of the epidermis while epidermal areas between the follicles (scales) do not show a granular layer. It has been observed that repetitive topical application of RA on tail skin induces (i) a granular layer and orthokeratosis in the scale regions accompanied by the suppression of two postnatally acquired 70-kD and 65-kD keratin proteins [6] and (ii) strong epidermal hyperplasia with induction of the murine hyperproliferation-associated 50-kD keratin [7]. We have compared the effects of topical RAL to that of all-*trans*-RA in the mouse tail model [6, 8].

*Hyperplastic Responses*

Table 2 shows the dose and time course experiments; RAL was found to exert similar effects to RA, and at low concentrations such as 0.005%, it was even more active for most of the parameters studied. This may be related to a better availability of ligand to the receptors when delivered through RAL.

*Metaplastic Responses*

All-*trans*-RA and all-*trans*-RAL induced a time- and dose-dependent reduction of the parakeratotic scale regions; the effect was similar for both retinoids at 0.05%, whereas at lower concentrations RAL showed a better metaplastic activity (table 3). Both compounds induced a similar reduction in 65-kD keratin mRNA; loricrin mRNA was significantly more induced by all-*trans*-RA (table 3).

*Effects of Citral on Biological Activities of All-*trans*-RAL*

The monoterpene aldehyde citral (3,7-dimethyl-2,6-octanedial) is a simple partially saturated analogue of RAL. Connor [9] had shown that citral inhibits the oxidation of retinol to RA in epidermis; it was postulated that it can act as a competitive substrate for both the alcohol and aldehyde dehydrogenases involved in this process. Thus, citral provides a tool to study the biological activity of retinoids in vivo. Thus, Connor [9] has shown that citral inhibited the ability of topical retinol but not of RA to induce hyperplasia as well as to inhibit the induction of ornithine decarboxylase activity in hairless mouse epidermis. This was considered as a proof that oxidation to RA accounts for the biological activities of topical retinol.

We have used citral 0.2% (2.6 µmol), half of the minimal dose used in Connor's study [9]. Citral counteracted the induction by RAL of a granular layer in the interfollicular

**Table 2.** Biological effects of RAL in mouse tail skin: hyperplastic responses

| Doses, % | Time, days | Parameters | All-*trans*-RA, % of vehicle | All-*trans*-RAL, % of vehicle |
|---|---|---|---|---|
| 0.005 | 7 | K50 mRNA induction | +77 ± 7 | +129 ± 12 |
| | | K70 mRNA suppression | –98 ± 1 | –88 ± 2 |
| | | epidermal thickness | +9 ± 1 | +38 ± 1 |
| | 9 | DNA synthesis (BrdU) | +374 ± 49 | +664 ± 170 |
| | | epidermal thickness | +21 ± 0.04 | +41 ± 1 |
| | 12 | DNA synthesis (BrdU) | –9 ± 3 | +24 ± 4 |
| | | epidermal thickness | +28 ± 3 | +40 ± 16 |
| | 14 | epidermal thicknesss | +18 ± 4 | +12 ± 1 |
| 0.010 | 7 | K50 mRNA induction | +283 ± 163 | +226 ± 21 |
| | | K70 mRNA suppression | –91 ± 2 | –85 ± 1 |
| | 14 | epidermal thickness | +30 ± 7 | +24 ± 8 |
| 0.025 | 14 | epidermal thickness | +43 ± 15 | +45 ± 15 |
| 0.050 | 7 | K50 mRNA induction | +327 ± 27 | +333 ± 62 |
| | | K70 mRNA suppression | –85 ± 5 | –94 ± 3 |
| | 9 | DNA synthesis (BrdU) | +456 ± 23 | +1,246 ± 108 |
| | | epidermal thickness | +40 ± 5 | +37 ± 1 |
| | 12 | epidermal thickness | +94 ± 3 | +64 ± 4 |
| | 14 | epidermal thickness | +72 ± 20 | +108 ± 15 |

K50 = 50-kD keratin; K70 = 70-kD keratin; BrdU = bromodeoxyuridine.

**Table 3.** Biological effects of RAL in mouse tail skin: metaplastic responses

| Doses, % | Time, days | Parameters | All-*trans*-RA | All-*trans*-RAL |
|---|---|---|---|---|
| 0.005 | 7 | K65 mRNA reduction, % of vehicle | –72 ± 8 | –38 ± 8 |
| | 7 | loricrin mRNA increase, % of vehicle | +168 ± 13 | +86 ± 15 |
| | 14 | orthokeratosis induction/IFZ, µm | 69 ± 1 | 156 ± 37 |
| 0.010 | 7 | K65 mRNA reduction, % of vehicle | –72 ± 13 | –88 ± 13 |
| | 7 | loricrin mRNA increase, % of vehicle | +94 ± 15 | +8 ± 5 |
| | 14 | orthokeratosis induction/IFZ, µm | 147 ± 2 | 183 ± 5 |
| 0.025 | 14 | orthokeratosis induction/IFZ, µm | 173 ± 2 | 291 ± 4 |
| 0.050 | 7 | K65 mRNA reduction, % of vehicle | –97 ± 1 | –95 ± 1 |
| | 7 | loricrin mRNA increase, % of vehicle | +92 ± 25 | +38 ± 13 |
| | 9 | orthokeratosis induction/IFZ, µm | 268 ± 3 | 271 ± 7 |
| | 12 | orthokeratosis induction/IFZ, µm | 326 ± 4 | 322 ± 7 |
| | 14 | orthokeratosis induction/IFZ, µm | 335 ± 7 | 329 ± 6 |

K65 = 65-kD keratin; IFZ = interfollicular zone.

zones of the mouse tail (fig. 1); accordingly, the molecular marker of this induction of orthokeratosis, that is the suppression of keratin 65 mRNA, was also counteracted by citral (fig. 2). After 14 days of treatment, citral also inhibited the RAL-induced epidermal hyperplasia (70 ± 4 vs. 63 ± 5 µm, $p < 0.0001$) and the molecular marker associated with this effect, that is the inhibition of keratin 70 mRNA [6] (fig. 2). Taken together these observations indicate that RAL exerts the biological activities studied in these experiments through transformation to RA.

*Effects of 9-*cis*-RAL versus All-*trans*-RAL*

9-*cis*-RA is the endogenous ligand of retinoid X nuclear receptors (RXRs) and also binds to RA receptors (RARs). Although the epidermis contains five times more RXRs than RARs in humans [11], little is known on the activity of top-

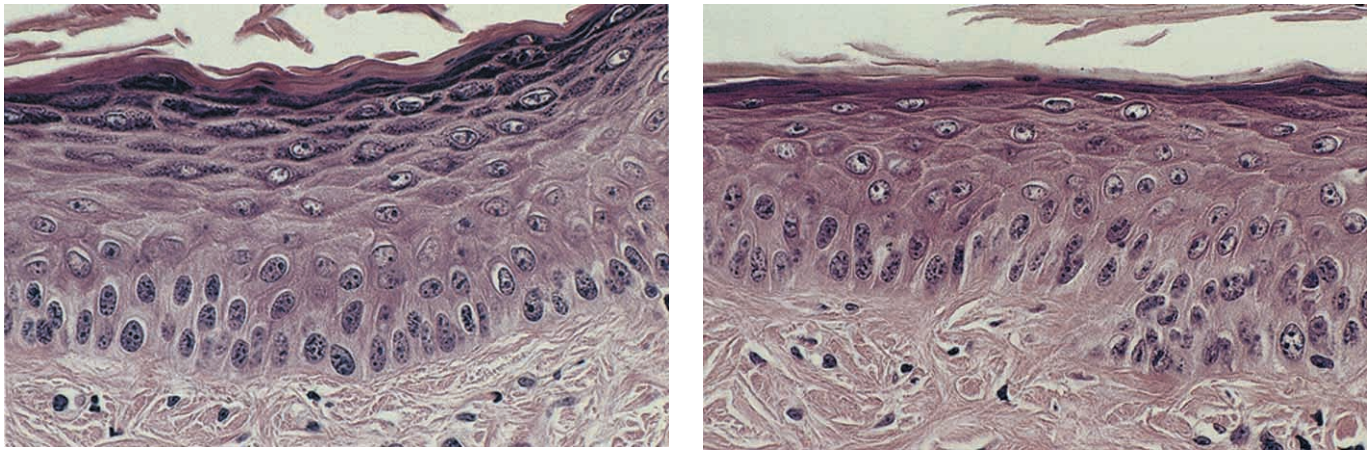


**Fig. 1.** Histology of interfollicular areas of mouse tail skin after 14 days of topical treatments with 0.025% RAL without (**a**) or with 0.2% citral (**b**). Note that citral inhibited both epidermal hyperplasia and induction of granular cell layers. ×250.

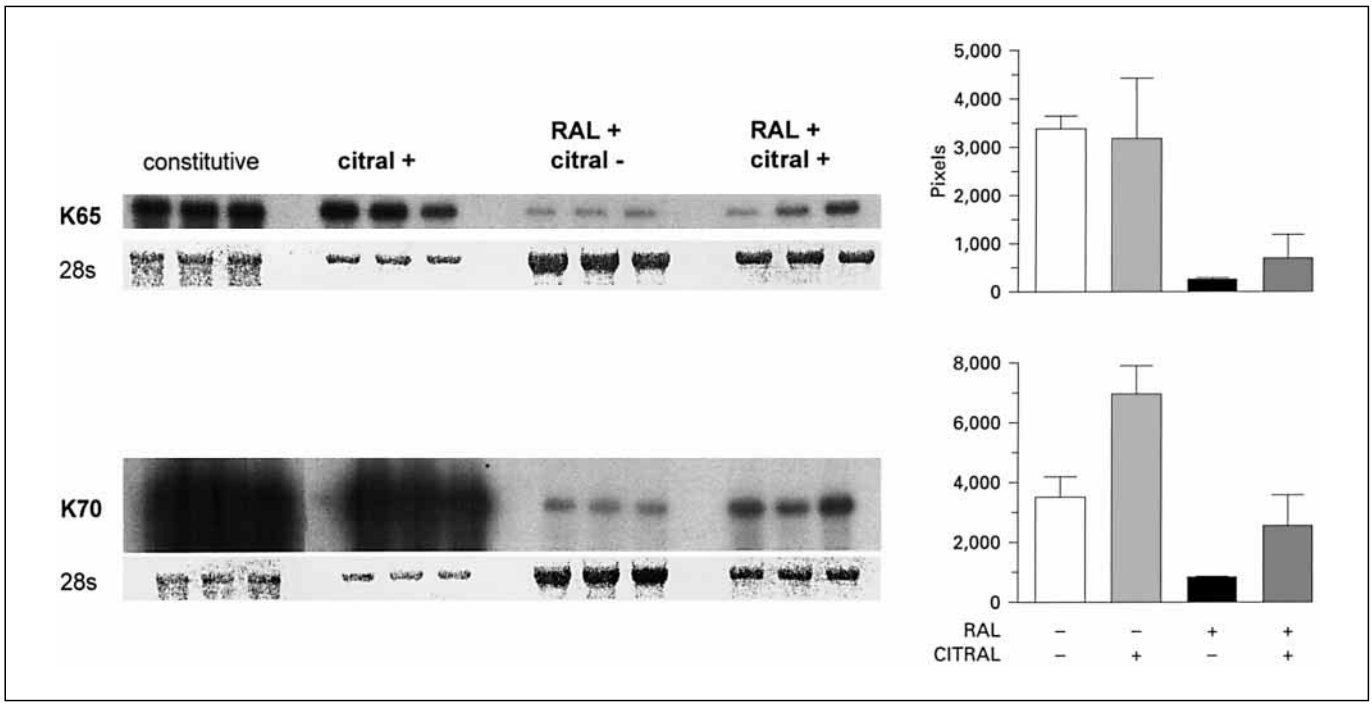


**Fig. 2.** Northern analysis of 65-kD keratin (K65) and 70-kD keratin (K70) mRNAs in mouse tail skin (n = 3 mice) untreated or treated with either 0.2% citral, 0.025% RAL without or with 0.2% citral. The Northern blots were hybridised as described in Didierjean et al. [10]. Ethidium bromide staining of the 28s RNA indicates the loading of each lane. The autoradiograms were scanned using a PhosphoImager (Molecular Dynamics, Kemsing, UK). The mean values (pixels) ± SD of the samples are shown in the graphs.

**Table 4.** Biological effects of RAL in mouse tail skin: effects of citral on biological activities of all-*trans*-RAL

| Doses, % | Time, days | Parameters | All-*trans*-RAL | 9-*cis*-RAL |
|---|---|---|---|---|
| 0.005 | 14 | epidermal thickness induction, µm | 4 ± 1 | 0 |
| | | orthokeratosis induction/IFZ, µm | 156 ± 37 | 192 ± 17 |
| 0.010 | 14 | epidermal thickness induction, µm | 8 ± 3 | 0 |
| | | orthokeratosis induction/IFZ, µm | 183 ± 5 | 205 ± 19 |
| 0.025 | 14 | epidermal thickness induction, µm | 15 ± 6 | 1 ± 3 |
| | | orthokeratosis induction/IFZ, µm | 291 ± 4 | 157 ± 43 |
| 0.050 | 9 | epidermal thickness induction, µm | 12 ± 1 | 10 ± 3 |
| | | orthokeratosis induction/IFZ, µm | 271 ± 7 | 111 ± 28 |
| | 12 | epidermal thickness induction, µm | 21 ± 1 | 15 ± 3 |
| | | orthokeratosis induction/IFZ, µm | 322 ± 7 | 170 ± 20 |
| | 14 | epidermal thickness induction, µm | 36 ± 7 | 13 ± 1 |
| | | orthokeratosis induction/IFZ, µm | 329 ± 6 | 106 ± 16 |

IFZ = Interfollicular zone.

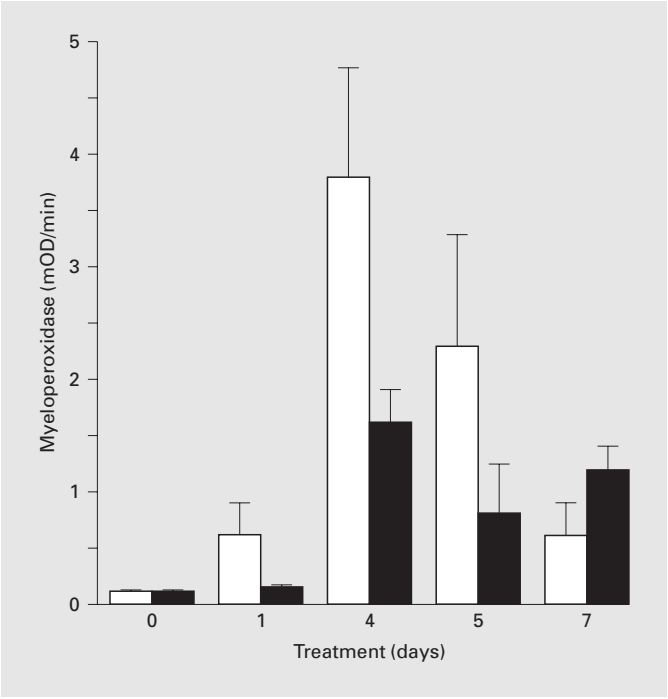


**Fig. 3.** Myeloperoxidase content (mOD/min, mean ± SD) in mouse ears untreated or treated for 1, 4, 5 or 7 days with either 0.005% all-*trans*-RA (□) or all-*trans*-RAL (■).

ical 9-*cis*-RA. In order to circumvent surface isomerisation of topically applied 9-*cis*-RA into all-*trans*-RA, we thought that topical 9-*cis*-RAL might be used as a potential precursor of 9-*cis*-RA, hypothesising that keratinocytes would metabolise 9-*cis*-RAL into 9-*cis*-RA. We investigated if 9-*cis*-RAL generates significant amounts of 9-*cis*-RA in the skin [12, 13] and found that biological activities (hyperplastic and metaplastic responses) were lower than those induced by all-*trans*-RAL or all *trans*-RA at similar concentrations (table 4) and concluded that 9-*cis*-RAL has no advantage over all-*trans*-RAL for specific delivery of natural retinoids into the skin.

*Skin Irritation*

It has been suggested that efficacy and irritation of synthetic retinoids in animal models correlate with RARγ-mediated transcription activation [14]. Topical application of all-*trans*-RA on human skin induces skin irritation as well [15] in spite of similar affinity for the three types of RARs. As RAL does not bind RARs and selectively delivers low concentrations of all-*trans*-RA, we postulated that skin irritation should be reduced. Studies in humans, reviewed in another section of this supplement [16], have clearly confirmed this. We conducted time course experiments on ears of mice where accumulation of neutrophils after topical treatments with either 0.005% all-*trans*-RA or 0.005% all-*trans*-RAL was measured by neutrophil myeloperoxidase content by the assay described by Bradley et al. [17]. The maximal myeloperoxidase activity was found after 4 days of treatment (fig. 3) and was less pronounced upon RAL treatment, showing, in this model, that topical RAL is less irritant than RA.

## Discussion

From these observations we concluded that topical RAL exerts significant biological activity, similar to that of all-*trans*-RA. Experiments with citral indicate that the effects of RAL result from its transformation into RA. Interestingly, the RA tissue levels in RAL-treated skin are much

lower than those in RA-treated skin [12], whereas the biological effects were identical. Several explanations for that have been considered:

a low cell loading of all-*trans*-RA may be sufficient for full activity on the parameters studied in this model; thus, only a small percentage of the RA recovered from RA-treated skin for HPLC analysis may correspond to intracellular, biologically significant all-*trans*-RA;

the uptake of RAL by murine epidermal cells might be more effective than that of RA; in vitro, the uptake of [$^3$H]RAL by cultured human keratinocytes reached 30% at 8 h [18];

finally a possible explanation of our observations might be that metabolites other than all-*trans*-RA are responsible for some biological effects of RAL, which might not be mediated by RARs. Since the major metabolites produced from RAL, both in vitro and in vivo, are retinol and several retinyl esters, the respective role of these metabolites as well as that of RAL itself, in inducing selective topical activities, remain to be analysed. However, the inhibition by citral of some effects of RAL argues against this.

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Dermatology

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# Inhibitory Effects of Retinoids on Vascular Endothelial Growth Factor Production by Cultured Human Skin Keratinocytes

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**Key Words**

Vascular endothelial growth factor · Retinaldehyde

**Abstract**

***Background:*** Vascular endothelial growth factor (VEGF), a potent angiogenic factor and vasodilator, is strongly expressed by epidermal keratinocytes in many angiogenesis-dependent skin disorders. Retinoids may modulate VEGF in skin and this may be related to an effect on rosacea. ***Aim:*** To investigate the effect of retinaldehyde on VEGF production by human keratinocytes. ***Methods:*** The effects of different concentrations of retinoids (all-*trans*-retinal and all-*trans*-retinoic acid) on VEGF production by cultured human skin keratinocytes in both cell extracts and supernatants were determined. Expression of VEGF was analyzed by enzyme-linked immunosorbent assay (ELISA) and RT-PCR. ***Results:*** The amount of cell-associated and secreted VEGF strongly decreased with retinoid concentration (e.g. 48, 69% inhibition at 0.1 μ*M* all-*trans*-retinal and -retinoic acid, respectively, in the supernatants). In parallel, approximately 25% inhibition of VEGF mRNA expression was obtained in the presence of 0.01 μ*M* all-*trans*-retinal. ***Conclusion:*** The decrease in VEGF expression by keratinocytes on contact with retinoids may prevent skin neoangiogenesis in certain skin diseases.

## Introduction

Keratinocytes are the most abundant cells in the epidermis. They synthesize various active biological mediators in response to different extracellular signals present in their environment, especially angiogenic growth factors which play an important role in stimulating and maintaining skin vascularization. They therefore constitute a good cellular model to determine the in vitro ability of different molecules to modulate angiogenic factor production. Vascular endothelial growth factor (VEGF) is regarded as the most important positive regulator of angiogenesis and vascular permeability [for reviews, see 1–3].

Previous studies showed that cultured normal human keratinocytes synthesize this cytokine [4]. VEGF is strongly expressed in many disorders of the skin [5–7] and seems to be an interesting candidate to determine the effects of retinoids on skin vascularization. Various studies showed that retinoids inhibit angiogenesis in several experimental systems. Retinoids inhibit vessel ingrowth into the chorioallantoic membrane of the chick [8, 9]. Retinoids also abrogate tumor-associated angiogenesis in vivo [10]. In the present study, we used ELISA and RT-PCR techniques to assess VEGF protein synthesis and gene expression in normal human keratinocytes cultured with retinaldehyde or retinoic acid.

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## Materials and Methods

*Immunoassay*

Early-passage (P2) normal human skin keratinocytes were seeded into 6-well plates at a density of $1\times10^6$ cells/ml DMEM/well. The amount of VEGF in centrifuged supernatants and in the cell extracts was evaluated using ELISA kits (R & D systems). This type of assay was used to evaluate the effect of retinoids (Sigma) on VEGF secretion; keratinocytes were incubated for 24 h with various concentrations of retinaldehyde or retinoic acid (0.1, 1, 3 µ*M*) in serum-free medium (Gibco). Simultaneous control incubations of keratinocytes without retinoids were performed. This assay was done using three keratinocyte lines. All assays were carried out in triplicate for each agent.

All-*trans*-retinoic acid and all-*trans*-retinal stock solutions were prepared in dimethylsulfoxide (1 p*M*) and stored protected from light at –20 °C. Working dilutions were prepared with serum-free medium (Gibco-BRL).

An aliquot of the suspension was set aside to measure the protein concentration. Cell proteins were measured according to a Biorad protein assay (Biorad) protocol using bovine serum albumin (Sigma) as a standard. Final results are expressed as the amount of VEGF related to total protein.

*RT-PCR Analysis*

Total RNA (1 µg) from cultured skin keratinocytes was reverse transcribed at 37 °C for 60 min into cDNA using the Access RT-PCR System (Promega) in a 50-µl volume.

Polymerase reactions were performed using oligonucleotides complementary to the 5′ and 3′ ends of the coding sequence of VEGF (CTGCTCTCTTGGGTGCACTGC and CACCGCCTTGGCTTGTCACAT). Amplification was performed for 30 cycles (94 °C for 40 s; 57 °C for 1 min; 72 °C for 1.5 min) in a DNA thermal cycler heat block (Perkin-Elmer, Gene Amp PCR system 2400). The amplified PCR products were separated by 3% agarose electrophoresis.

## Results

*Expression of VEGF Protein Is Inhibited in Keratinocytes after Exposure to Retinaldehyde and Retinoic Acid*

The total content in VEGF was inhibited in a dose-dependent manner by retinaldehyde as well as retinoic acid which induced a higher inhibition (table 1). In cell extracts, as compared with untreated cells, retinaldehyde induced a decrease in VEGF protein content only at the higher concentration of 3 µ*M* (table 1); retinoic acid induced a significant decrease at 1 µ*M*. Decreases in the levels of VEGF protein were similarly observed in cell supernatants where the inhibition upon retinaldehyde treatment was higher at the lowest concentrations; a similar effect was observed upon retinoic acid treatment, although the inhibition was higher than that induced by retinaldehyde.

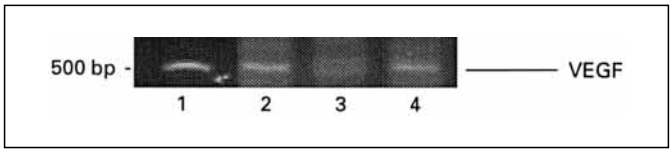


**Fig. 1.** RT-PCR detection of VEGF mRNA in normal human skin keratinocytes. Keratinocytes were treated or not with retinaldehyde or retinoic acid at 0.01 µ*M*. Reverse transcription and amplification were performed as described in the Material and Methods section. Lane 1: PCR marker; lane 2: control; lane 3: retinal, 0.01 µ*M*; lane 4: retinoic acid, 0.01 µ*M*.

**Table 1.** Effects of retinoids on VEGF protein production in cell extracts and supernatants of cultured normal human keratinocytes

| | Supernatants | | Cell extracts | | Total |
|---|---|---|---|---|---|
| | pg/µg proteins ×100 | SD | pg/µg proteins ×100 | SD | |
| Control | | | | | |
| 0 µ*M* | 17.86 | 2.98 | 53.73 | 7.04 | 71.59 |
| Retinal | | | | | |
| 0.1 µ*M* | 9.32* | 2.02 | 64.31 | 10.26 | 73.63 |
| 1 µ*M* | 11.23* | 1.56 | 55.02 | 10.85 | 66.25 |
| 3 µ*M* | 11.31* | 1.6 | 36.32* | 7.73 | 47.63 |
| Retinoic acid | | | | | |
| 0.1 µ*M* | 5.57* | 0.99 | 50.79 | 3.66 | 56.36 |
| 1 µ*M* | 10.6* | 2.89 | 39.87* | 2.04 | 50.47 |
| 3 µ*M* | 10.18* | 1.21 | 13.39* | 1.42 | 23.57 |

VEGF concentrations in the supernatants were determined by ELISA. Keratinocytes were incubated for 24 h in the presence of different concentrations of retinaldehyde or retinoic acid. VEGF production by keratinocytes related to the total cell protein content is expressed as picograms per microgram protein. Values are the mean of 3 assays ± SEM.

* $p < 0.05$: significant difference compared to the control using the Dunnett test.

*VEGF mRNA Expression Is Decreased in Keratinocytes after Exposure to Retinaldehyde and Retinoic Acid*

VEGF mRNA was detected in cultured keratinocytes by RT-PCR (fig. 1). A 25% inhibition of VEGF mRNA was observed after treatment of cells with retinaldehyde 0.01 µ*M*; no significant modification of the VEGF mRNA was observed after treatment with retinoic acid at 0.01 µ*M*.

## Discussion

In this study we confirm and extend our observations upon the inhibition of VEGF expression [11] by all-*trans*-

retinaldehyde and all-*trans*-retinoic acid in primary cultured human normal keratinocytes. Such an inhibition was subsequently confirmed by Weninger et al. [12] who observed that all-*trans*- and 13-*cis*-retinoic acid as well as all-*trans*-retinol inhibited VEGF in normal human keratinocyte supernatants.

Low concentrations of retinaldehyde induced a greater decrease in VEGF in the supernatant than high concentrations, whereas the reverse was seen for the cell-associated VEGF. A similar effect was seen with retinoic acid. This indicates a dose-dependent effect of the two retinoids on the distribution/secretion of VEGF in cultured keratinocytes. As a whole the VEGF protein was inhibited by 33% by 3 μ*M* retinaldehyde, and up to 67% by similar dose of retinoic acid.

The low concentration 0.01 μ*M* of retinaldehyde inhibited VEGF gene expression by 25% after 24 h of incubation which is consistent with the decrease in secreted protein. No inhibition of VEGF mRNA was observed with 0.01 μ*M* retinoic acid at 24 h incubation; this is probably due to a difference in the time course regulation of VEGF between retinoic acid, and retinaldehyde; the latter, comparatively to retinoic acid would act in a delayed manner.

The inhibition of VEGF production by the retinoids tested in the present study points to an effect of retinaldehyde in skin vascularization. The level of VEGF is an important parameter in maintaining balanced skin angiogenesis.

The decrease in VEGF production by keratinocyte cells may prevent skin neoangiogenesis and inflammation developing in rosacea or other angiogenesis-dependent diseases of the skin in which a dense network of microcapillaries is produced and inflammatory cells are present.

The inhibition of VEGF, which is a potent chemotactic agent for inflammatory cells and endothelial cells [13, 14], provides direct evidence that retinaldehyde may contribute to the reduction of inflammation sites by regulating inflammatory mediator synthesis in these diseases. This molecule should play a role in the prevention of uncontrolled microvascularization in skin diseases, but its potential clinical use in such a context needs further investigation in vivo.

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# Antibacterial Activity of Retinaldehyde against *Propionibacterium acnes*

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**Key Words**

Topical retinaldehyde · *Propionibacterium acnes*

**Abstract**

***Background:*** Retinaldehyde has been shown to exert antibacterial activity in vitro. ***Aim:*** This study evaluates the effect of retinaldehyde on *Propionibacterium acnes* both in vivo and in vitro. ***Methods:*** Microbial minimal inhibitory concentrations (MICs) of retinaldehyde and retinoic acid were determined on reference strains of *P. acnes.* In vivo activity of daily topical application of 0.05% retinaldehyde on the *P. acnes* density was evaluated after application in a single-blind randomised study. ***Results:*** MICs of retinaldehyde were 4 mg/l for *P. acnes* No. CIP179 and CIP53119 and 8 mg/l for *P. acnes* No. CIP53117. In contrast, the MICs of retinoic acid were superior to 128 mg/l for these three strains. In vivo, retinaldehyde-treated areas displayed a significant decrease in counts of viable *P. acnes* as compared with the untreated areas with a median decrease of $10^2$ log *P. acnes*/cm$^2$ after 2 weeks of daily application. Vehicle alone had no effect. ***Conclusion:*** The MIC of retinaldehyde against *P. acnes* suggests a direct antibacterial activity. Daily topical application of 0.05% retinaldehyde is associated with a clear reduction of the *P. acnes* density.

## Introduction

Historically, vitamin A or retinol has been considered as an anti-infectious agent [1]. Retinol induces non-specific resistance to infection [2–5], but the mechanisms of these anti-infectious activities remain mostly hypothetical and are probably related to its pleiotropic effects on the immune function [6]. Retinaldehyde is a natural metabolite of retinol, and previous pilot studies have suggested that it has antibacterial activity [7]. Since retinaldehyde is used as a cosmetic ingredient on the face, a zone where *Propionibacterium acnes* is very abundant [8], we wondered if retinaldehyde has a direct action against *P. acnes.* To evaluate this possible antibacterial effect, in vitro and in vivo assays were performed. Minimal inhibitory concentrations (MICs) of retinaldehyde and retinoic acid were determined on reference strains and compared with each other. The in vivo impact of retinaldehyde on *P. acnes* populations was evaluated on the face of volunteers in a single-blind randomised study.

## Material and Methods

*Reagents*

All-*trans*-retinal (Sigma, St. Louis, Mo., USA) was dissolved in a solution of a synthetic triglyceride, silicone and butylhydroxytoluene 0.001% and kept under argon atmosphere at 4 °C for the in vivo study. This preparation does not contain any conservative compound which can interact with the *P. acnes* culture. the final retinaldehyde concentration was 0.05%.

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**Table 1.** MICs (mg/l) of retinaldehyde and retinoic acid against reference strains

| Strains | No. | Retinal-dehyde | Retinoic acid |
|---|---|---|---|
| *Propionibacterium acnes* | CIP 179 | 4 | >128 |
| *Propionibacterium acnes* | CIP 53119 | 4 | >128 |
| *Propionibacterium acnes* | CIP 53117 | 8 | >128 |
| *Escherichia coli* | NCTC 10418 | >128 | >128 |
| *Pseudomonas aeruginosa* | ATCC 9027 | >128 | >128 |

For the in vitro study, retinaldehyde or retinoic acid (Hoffmann-La Roche, Basel, Switzerland) was dissolved in a solution of 50% polyethylene glycol 400 (Sigma) and ethanol (Merck).

*Strains*

Reference strains were obtained from the American Type Culture Collections (ATCC), Rockville, USA, from the National Collection of Type Culture (NCTC), Calindale, London, UK, and the Collection Institut Pasteur (CIP), Paris, France. These strains were frozen at –20°C in peptone water (BioMérieux, Lyon, France) with 10% glycerol (Sigma). Clinical strains were identified according to conventional identification methods.

*In vitro Testing*

Microbial MICs were determined by a microdilution method [9] using an inoculum of $10^5$–$10^6$ CFU/ml, 'medium 20' as growth medium and an incubation time of 3 days in anaerobiosis (Generbag $CO_2$ system, BioMérieux). The MIC was defined as the lowest concentration yielding no growth visible to the naked eye.

*In vivo Protocol*

After randomisation of 22 volunteers, approximately 4 µg of either retinaldehyde with vehicle or vehicle alone were applied daily on a 4-$cm^2$ lateral area of the forehead with a small sterile cotton. The other side of the forehead was left untreated. On day 15, skin flora was sampled on both sides and counts of viable bacteria were performed. Skin bacteria were collected by the cylinder scrub method described by Williamsom and Kligman [10]. Paired results from treated (retinaldehyde or vehicle) and untreated areas were analysed using the Wilcoxon rank sum test. The study has been approved by the institutional ethical committee.

## Results

MIC assays were made on *P. acnes* reference strains (table 1). MICs of retinaldehyde were 4 mg/l for *P. acnes* No. CIP179 and CIP53119 and 8 mg/l for *P. acnes* No. CIP53117. In contrast, the MICs were superior to 128 mg/l with retinoic acid for these three strains. No activity was found with retinaldehyde and retinoic acid against gram-negative *Pseudomonas aeruginosa* ATCC 8027 and *Escherichia coli* NCTC 10418.

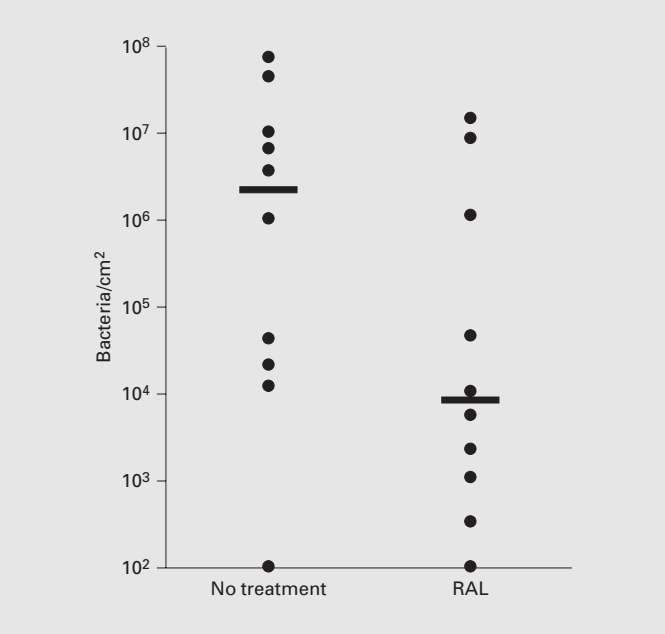


**Fig. 1.** *P. acnes* density after daily topical application of retinaldehyde (RAL) 0.05% during 2 weeks. Horizontal bars indicate the median values.

The daily application of 0.05% retinaldehyde in the experimental vehicle was well tolerated. Twenty-one of the 22 volunteers were evaluated, 1 volunteer was excluded because of lack of compliance. In each volunteer, counts of viable bacteria from treated areas (retinaldehyde in vehicle or vehicle alone) and untreated areas were compared.

In the group treated with retinaldehyde, the treated areas displayed a significant decrease in the counts for viable *P. acnes* as compared to the untreated areas (fig. 1). Of the 10 volunteers of this group, the *P. acnes* density was reduced in 8, stable in 1 and slightly increased in 1. The median decrease in *P. acnes* density was $10^2$ log.

Treatment with the vehicle without retinaldehyde was evaluated in 11 volunteers; the *P. acnes* density did not change. The median density in the area treated with vehicle and the untreated area were $10^7$ *P. acnes*/$cm^2$ on both sides. This result means that no vehicle effect was found. The difference of *P. acnes* density between the vehicle and the retinaldehyde treatment was statistically significant ($p = 0.048$).

## Discussion

In the past, clinical investigations have demonstrated the efficacy of synthetic retinoids in dermatoses in the patho-

genesis of which bacterial agents are implicated such as acne [11] and gram-negative folliculitis [12]. In patients treated with oral 13-*cis*-retinoic acid profound alterations of the skin flora are observed: reduced recovery of *P. acnes* and gram-negative bacteria, increased nasal colonisation by *Staphylococcus aureus* and high incidence of staphylococcal cutaneous infections [13]. However, in vitro experiments with 13-*cis*-retinoic acid [14–16] and acitretin [16] failed to reveal any direct antibacterial activities against either gram-positive *(S. aureus*, *Staphylococcus epidermidis* and *P. acnes)* or gram-negative bacteria. Since retinoic acid does not inhibit bacterial growth by itself [16, this study], changes in skin bacteria likely result from indirect mechanisms, such as reduction of the skin lipid or drying effects. The density of *P. acnes* on the skin depends, among other factors, mainly on the amount of sebum [17]. The reduction of skin lipids induced by oral 13-*cis*-retinoic acid is probably the main cause for the modification of the *P. acnes* population.

The results obtained with retinaldehyde were different. The decrease of *P. acnes* densities observed in vivo can be partially explained by the retinoid activity of retinaldehyde, but in vitro results suggest a second phenomenon. MICs against *P. acnes* were between 4 and 8 mg/l for retinaldehyde and more than 128 mg/l for retinoic acid. The in vitro contact of retinaldehyde with *P. acnes* inhibited its proliferation, whereas this was not observed with retinoic acid. The mechanisms remain to be determined, but these results suggest a direct antibacterial activity of retinaldehyde against *P. acnes.*

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Dermatology

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# Comedolytic Effect of Topical Retinaldehyde in the Rhino Mouse Model

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**Key Words**

Rhino mouse · Retinaldehyde · Topical preparation · Acne

**Abstract**

***Background:*** Retinaldehyde is a key molecule in the metabolism of vitamin A by keratinocytes. In order to evaluate its range of topical activity in acne, its comedolytic effect was compared to that of retinoic acid in the same vehicle, in the rhino mouse model. ***Methods:*** The animals were treated on the back daily for 5 consecutive days per week for 3 weeks. At the end of this period, histological slides were analyzed in order to quantify the features of comedones and epidermal thickness. ***Results:*** Topical treatment with a retinaldehyde (0.05% w/w) and a retinoic acid formulation (0.025% w/w) induced comedolysis and increased the epidermal thickness with the same intensity. ***Conclusion:*** These data indicate that retinaldehyde exerts a significant comedolytic activity.

## Introduction

Topical retinoids are a classic in the treatment of acne; since irritation is a frequent side effect of retinoic acid, new synthetic retinoids have been developed in order to improve the local tolerance [1]. Retinaldehyde, a key intermediate molecule in the metabolism of natural vitamin A by keratinocytes, has been shown to be well tolerated by human skin [2]. The range of its biological activities has been analyzed.

The aim of this study was thus to compare its comedolytic efficacy with retinoic acid in the rhino mouse model, which is acknowledged as an effective model to evaluate anti-acneic products [3, 4].

## Materials and Methods

*Preparations*

All-*trans*-retinoic acid (RA) was obtained from Aldrich and all-*trans*-retinaldehyde (RAL) from BASF. Three O/W emulsions containing paraffin oil, caprylic/capric triglyceride and safflower oil as major emollients, propylene glycol as humectant and phenoxyethanol/parabens as preservative were compounded: RAL preparation (0.05% w/w), RA preparation (0.025% w/w), control preparation.

*Animals*

Rhino mice, aged 4–6 weeks at the start of the experiments, were obtained from the Centre de Production Animale (Olivet, France). Food and water were available ad libitum. The diet used (AO4) was supplied by the UAR (Villemoisson-sur-Orge, France). Four groups of 10 mice were constituted: one group treated by RAL 0.05%, by RA 0.025%, by vehicle and an untreated group. After 4 days of acclimatization, the animals were placed in individual cages in a conditioned room (temperature 22 ± 2 °C).

*Treatment*

50 µl of preparation were applied, with a gloved finger, on the dorsal skin of the animal, once daily for 5 consecutive days per week (excluding Saturday and Sunday) during 3 weeks.

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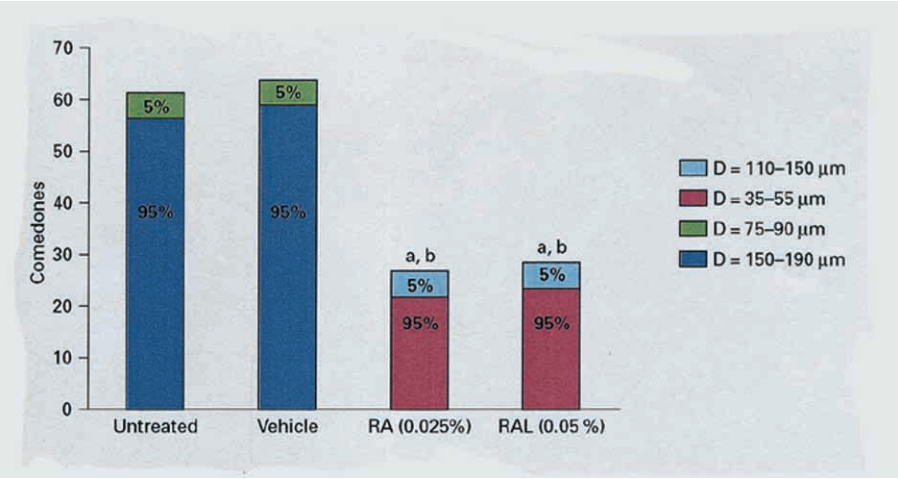


**Fig. 1.** Number of comedones and repartition of the maximal diameter (D) of these comedones. [a]p < 0.05: comparison treated groups versus vehicle; [b]p < 0.05: comparison treated groups versus untreated group. Values are means ± SD.

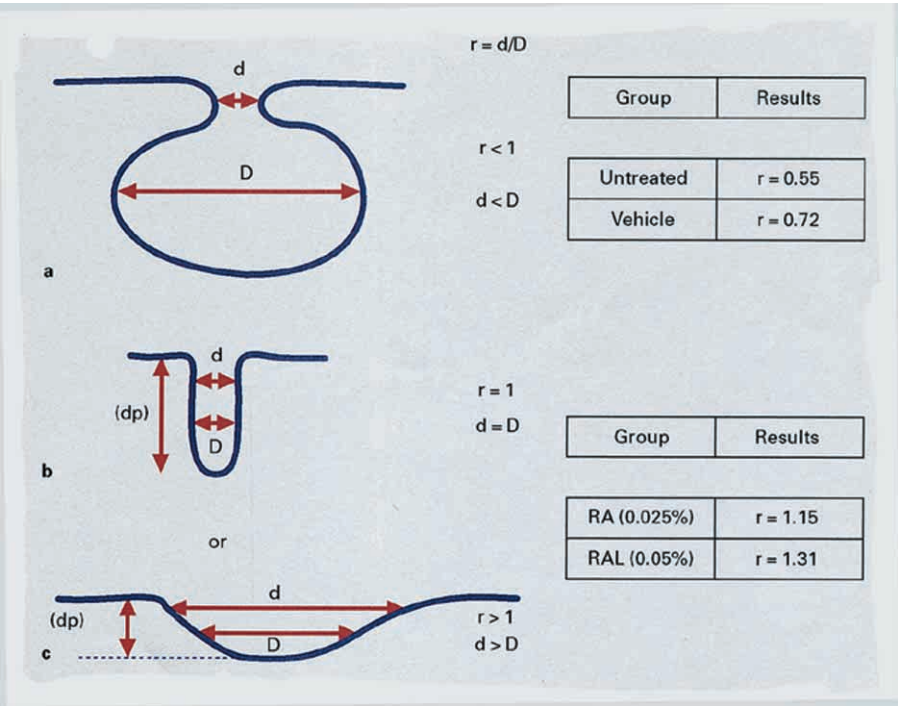


**Fig. 2 a–c.** Comedo profiles (r = d/D). See text for details; dp = Depth.

**Table 1.** Image analysis of epidermal parameters in the rhino mouse skin

| Treatment | n | Comedones (by cm) | Comedo profile (r = d/D) | Epidermal thickness µm |
|---|---|---|---|---|
| Untreated | 10 | 61.12 ± 5.40 | 0.55 ± 0.08 | 25.33 ± 3.85 |
| Vehicle | 10 | 64.14 ± 5.92 | 0.73 ± 0.14 | 29.18 ± 3.12 |
| RA 0.025% | 10 | 26.61 ± 5.99[a, b] | 1.16 ± 0.07[a, b] | 54.15 ± 4.62[a, b] |
| RAL 0.05% | 10 | 28.09 ± 2.76[a, b] | 1.32 ± 0.13[a, b] | 51.64 ± 4.62[a, b] |

[a]p < 0.05: comparison treated groups versus vehicle; [b]p < 0.05: comparison treated groups versus untreated group. Values are means ± SD.

*Skin Biopsies*

At the end of the treatment, the mice were sacrificed by cervical dislocation and the dorsal skin was removed. Skin biopsies (6 mm) were taken from the treated area, fixed in 10% formalin and embedded in paraffin. Three 3-µm-thick slices were prepared from each biopsy at 150-µm intervals. Slices were stained with the Lillie-Pasternak met hod (buffer medium: pH = 4).

*Image Analysis*

An axiomat Zeiss microscope, with a micrometric equipment, was used to determine the following parameters: the number of epidermal comedones reported by centimeters of skin; D = maximal diameter of epidermal comedones or the diameter taken at half depth; r = comedo profile = d/D where d was the diameter of the surface orifice. A semi-automatic image analysis system, Morphomat 10 Zeiss, coupled to the microscope was used for the determination, in the intercomedo areas, of the surface (S) of the epidermis and the length (L) of the corresponding basal layer. The epidermal thickness was evaluated by the ratio S/L.

Statistical analyses between groups were performed using an analysis of variance followed, when useful, by a Bonferroni test (Sigmastat®, Jandel GmbH, Erkrath, Germany).

## Results

The data of the parameters number, r and the epidermal thickness ratio S/L are shown in table 1. The maximal diameter of the comedones is represented in figure 1.

*Number of Comedones*

The two preparations, RAL 0.05% and RA 0.025%, induced a statistically significant reduction of the number of comedones compared to the vehicle and untreated groups. No statistical difference was found between the two active preparations.

*Maximal Diameter of Comedones*

As shown in figure 1, the repartition of the diameter of the comedones was the same for RAL- and RA-treated groups: 95% of comedones at 35–55 µm, 5% of comedones at 110–150 µm. Vehicle and untreated groups showed the following profile: 95% of comedones at 150–190 µm, 5% of comedones at 75–90 µm.

*Comedo Profile*

The r parameter is related to the comedolytic effect of the preparation [3, 4]: if r is equal or superior to 1, the compound is considered as comedolytic (fig. 2). This parameter was statistically modified by the two compounds: RAL and RA induced the transformation of closed comedones into open comedones with an r value equal or superior to 1, demonstrating a comedolytic effect. There was no statistical difference in the intensities of action of the two compounds.

*Epidermal Thickness*

This parameter is increased by the two active preparations with the same intensity and was not affected by the vehicle (table 1).

## Discussion

In this study, RAL 0.05% applied topically exerted the same activity profile than RA 0.025%, namely a comedolytic and an epidermal thickening effect. On all the parameters studied, RAL had the same activity as RA; further studies on dose-dependent activity would be interesting to perform. These effects are those of retinoids, due to an induction of epidermal cell proliferation which is specific to retinoid treatment and well known in the rhino mouse model [5].

These results support the topical use of RAL in the treatment of acne. Actually, RAL is better tolerated than RA and has also, in addition to its retinoid action, an antibacterial profile especially against *Propionibacterium acnes* [6].

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Dermatology

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# Efficacy of Topical 0.05% Retinaldehyde in Skin Aging by Ultrasound and Rheological Techniques

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**Key Words**
Retinaldehyde · Photoaging · Echography · Elasticity · Stiffness · Skin thickness

**Abstract**
***Background:*** The natural precursor of retinoic acid, i.e. retinaldehyde, has been proven to exert retinoid activities. ***Aim and Methods:*** The aim of this prospective instrument study was to determine the effect of topical retinaldehyde 0.05% on the physical properties of aging skin. This was performed using two devices, namely a high-resolution (70–80 μm) ultrasound scanner, which visualizes the thickness of both the epidermis and the dermis, and an echorheometer, which assesses the stiffness and elasticity of the skin by suction. In a 1-year study, 21 patients applied retinaldehyde cream 0.05% on the face, while another group of 19 volunteers were only treated with an emollient (control group). Epidermal and dermal thicknesses were measured on the forehead and temple, and stiffness and elasticity were measured on the forehead only. All the instrumental parameters were assessed at baseline and at the end of treatment. ***Results:*** Compared to the control group, retinaldehyde treatment induced a significant increase in epidermal thickness of the temple, as well as in cutaneous elasticity ($p < 0.01$). Similarly, retinaldehyde treatment tended to increase dermal thickness and reduce cutaneous stiffness, but no statistical difference could be observed between the two groups. ***Conclusion:*** Taken together, the results further suggest that retinaldehyde has counteracting effects on skin aging.

## Introduction

Chronological skin aging and chronic exposure to sunlight induce skin changes that are probably interrelated [1]. These include skin atrophy [2], reduction of elasticity [3] and an increase in cutaneous stiffness [4, 5]. These alterations result in clinical modifications of the skin, including wrinkling, color changes (yellowing, uneven pigmentation) and laxity of the skin.

Topical retinoic acid was the first substance which associates clinical improvement of skin appearance with histological changes [1, 6]. In a recent clinical trial, the natural precursor of retinoic acid, retinaldehyde, has also been proven to be effective in the reduction of facial wrinkles [7]. The aim of this prospective instrumental study was to evaluate the effects of topical retinaldehyde on skin aging and photoaging, using noninvasive techniques such as cutaneous echography and echorheometry.

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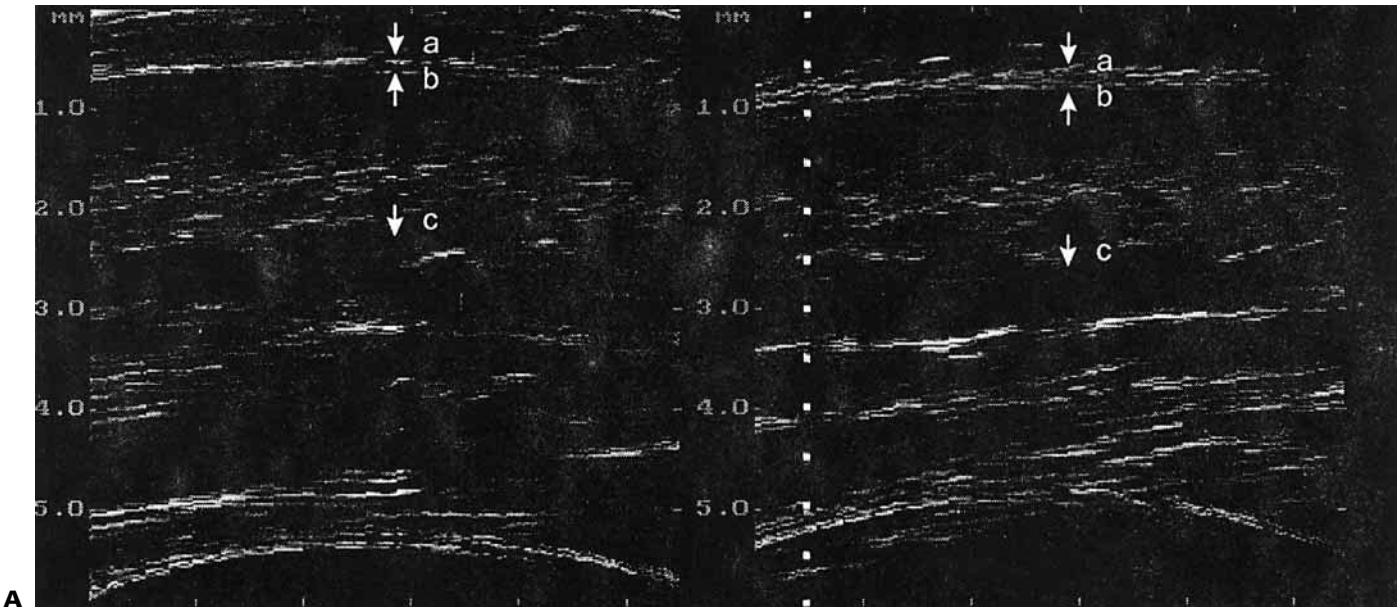


A B

**Fig. 1.** A typical example of the modulation of retinaldehyde 0.05% before (**A**) and after (**B**) the 1-year treatment on the temple, using a 20-MHz ultrasound technique. Three echobands were observed: a = the gel-stratum corneum interface, b = the epidermis-dermis interface and c = the dermis-hypodermis interface. **A** Epidermis = 110 µm; dermis = 1.42 mm. **B** Epidermis = 130 µm; dermis = 1.58 mm.

**Table 1.** Demographic data and baseline values of the physical parameters in the study population (n = 40)

| | Retinaldehyde | Control |
|---|---|---|
| Patients, n | 21 | 19 |
| Age, years | 47.4 ± 4.93 | 47.1 ± 5.47 |
| Weight, kg | 53.9 ± 4.2 | 56.5 ± 6.0 |
| Height, cm | 160.3 ± 3.1 | 160 ± 4.5 |
| Type of skin, n | | |
| Dry | 9 | 6 |
| Normal/mixed | 12 | 11 |
| Greasy | 0 | 2 |
| Instrumental values | | |
| Forehead epidermis thickness, µm | 96 ± 4 | 98 ± 5 |
| Temple epidermis thickness, µm | 106 ± 3 | 97 ± 4 |
| Forehead dermis thickness, mm | 1.4 ± 0.05 | 1.58 ± 0.05 |
| Temple dermis thickness, mm | 1.24 ± 0.05 | 1.41 ± 0.05 |
| Elasticity index, arbitrary units | 0.35 ± 0.01 | 0.32 ± 0.01 |
| Stiffness, kPa | 218 ± 20 | 220 ± 19 |

Results are expressed as means ± SEM.

## Methods

The study population comprised women over 35 years of age, with moderate to severe photodamage. Patients applied either retinaldehyde 0.05% on their faces once daily for 1 year, or were left untreated (emollient only, control group). None of the patients had applied topical retinoids on the treatment areas for more than 4 weeks during the 6-month period before the initiation of the study treatment. None had used chemical peels, exfoliants or any abrasive substance on the face within 45 days before entry into the study. Pregnant and nursing women and patients who planned to use PUVA for tanning were excluded, as well as those with suspected skin cancer or any other con-

dition that could interfere with their evaluation. This study was performed from January 1996 to January 1997. Patients were seen every 3 months for tolerance assessment and product supply.

Instrumental evaluation was performed at baseline and at the end of treatment (1 year). For evaluation visits, patients were asked not to apply any topical product on the face the night before. The test sites (forehead and temple) were precisely located using a transparent plastic film that was positioned according to skin features (moles, hair line and eyebrows). Thicknesses of the dermis and epidermis on the temple and the forehead were measured using a high-resolution B mode 20-MHz ultrasonic device (DermCup 2020®, 2MT, Toulouse, France) [8, 9]. The resolution of the apparatus (axial 70–80 µm, lateral 200–300 µm) allowed observance of the two echos from the gel-stratum corneum and epidermis-dermis interfaces, as shown in figure 1. Thus, the assessment of the epidermal and the dermal thicknesses was made possible by the measurements of the length (µm) between the echos. Stiffness and elasticity of the skin on the forehead were evaluated using an echorheometer, i.e. combining the ultrasound technique (20 MHz) and a deformation system under standard suction conditions. The echorheometer allowed measurement of the kinetics of vertical displacement of the skin under suction. From this kinetics profile, two independent parameters were determined: (i) the stiffness which is Young's modulus, as a reflectance of the resistance of the skin to the suction; (ii) the elasticity index, calculated by an algorithm which reflects the skin recovery of its initial state after suction. These two parameters, which describe the intrinsic mechanical properties of the skin, are independent of its thickness. Full details of the technique and of the mechanical parameters are described elsewhere [9–11]. For each measurement, the relative humidity (mean ± SD: 39 ± 7%) and temperature (23 ± 2 °C) of the room were standardized. All measurements were made by the same investigator.

Results of the study parameters were expressed as means ± SEM. Changes from baseline were analyzed using Student's test and the Wilcoxon test, according to the normal and nonnormal distribution of the values, respectively. Differences between the retinaldehyde and the control group were analyzed using the Bonferroni test.

## Results

A total of 44 women were enrolled in the study. Among the enrolled population, 4 patients were excluded from the study, because they applied nonpermitted products (α-hydroxy acids or retinoic acid). No patient was withdrawn from the study because of adverse events. Accordingly, 40 patients, with 21 patients in the retinaldehyde group and 19 in the control group, constituted the standard analysis population. At baseline, the two study groups were found to be comparable in their demographics and instrumental values (table 1).

Changes from baseline of the instrumental parameters after 1 year (end of treatment) are illustrated in figures 2–5. Globally, epidermal thickness on the forehead was increased in the retinaldehyde group by about 10% ($p = 0.005$) and in the control group by about 4% (nonsignificant, fig. 2).

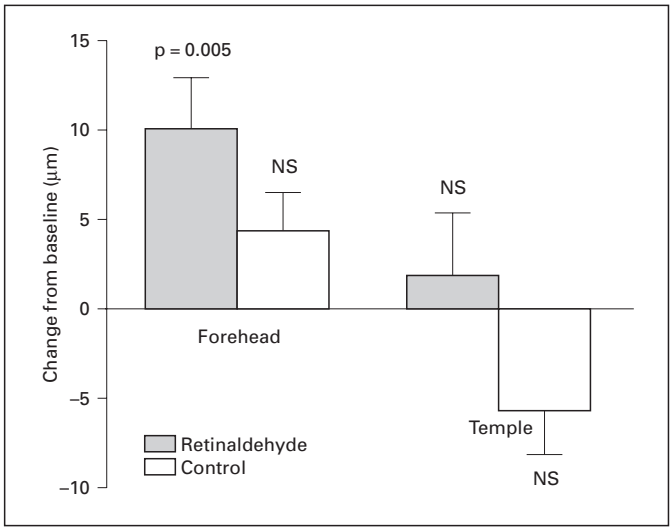


**Fig. 2.** Mean changes from baseline of epidermal thickness (± SEM) on the forehead and the temple, in the retinaldehyde group and the control group. For the intragroup analysis, p values are represented in the graph. For the intergroup analysis, only a statistically significant difference in favor of retinaldehyde was observed on the temple ($p < 0.01$).

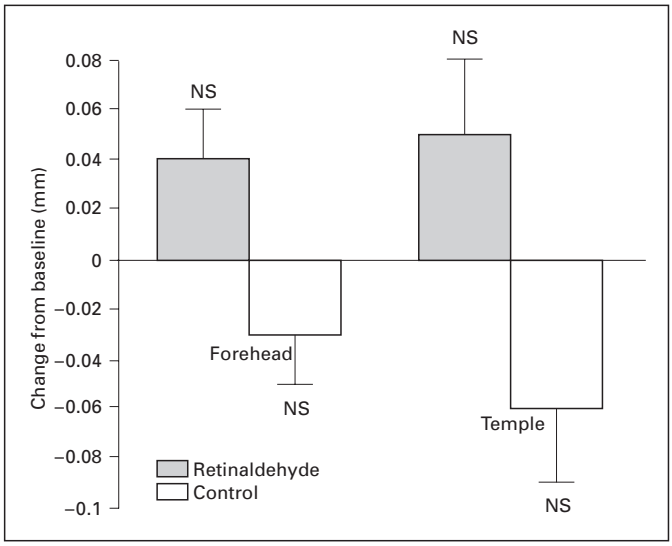


**Fig. 3.** Mean changes from baseline of dermal thickness (± SEM) on the forehead and the temple, in the retinaldehyde group and the control group. For the intragroup analysis, p values are represented in the graph. For the intergroup analysis, no statistically significant difference was observed at both sites.

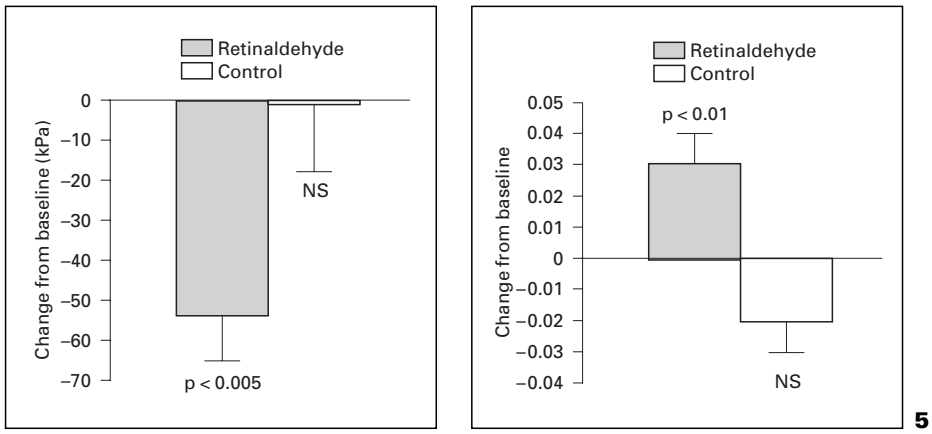


**Fig. 4.** Mean changes from baseline of stiffness (± SEM) on the forehead, in the retinaldehyde group and the control group. For the intragroup analysis (comparison to baseline), p values are represented in the graph. For the intergroup analysis, no statistically significant difference was observed.

**Fig. 5.** Mean changes from baseline of elasticity (± SEM) on the forehead, in the retinaldehyde group and the control group. For the intragroup analysis (comparison to baseline), p values are represented in the graph. For the intergroup analysis, a significant difference in favor of retinaldehyde was observed ($p < 0.01$).

On the temple, epidermal thickness was not significantly changed in each group (fig. 2). By contrast, in the intergroup analysis epidermal thickness on the temple was found to be significantly greater with retinaldehyde compared to the control ($p < 0.01$), but not on the forehead. Similarly, retinaldehyde treatment tended to increase dermal thickness on the forehead and the temple by around 3 and 4%, respectively (nonsignificant, fig. 3). On the contrary, the control group showed a reduction of dermal thickness on both test sites (2% decrease on the forehead, nonsignificant; 4.3% decrease on the temple, nonsignificant). No statistical difference between the groups was observed for this parameter.

Compared to baseline, skin stiffness of the forehead was significantly reduced with retinaldehyde by about 24% ($p < 0.005$, fig. 4). No change was demonstrated in the control group. There was no significant difference between the groups. Similarly, skin elasticity was enhanced with retinaldehyde by about 4% ($p < 0.01$, fig. 5), whereas it tended to be decreased in the control group (3% decrease, nonsignificant). A statistically significant difference between the groups was here achieved ($p < 0.01$).

Local tolerance was shown to be good in all patients. Indeed, only transitory scaling and/or erythema of mild severity were reported in 2 patients of the retinaldehyde group, within the first 3 months of treatment, and no patient of the control group. Consequently, no interference between the instrumental values and the tolerance results could be considered.

## Discussion

Our results indicate that retinaldehyde is able to reverse some physical alterations involved in skin aging and photoaging. Its effects were more pronounced on epidermal thickness and cutaneous elasticity than on dermal thickness and skin stiffness, all four of these being the hallmarks of the skin aging process.

Instrumental investigation of the physical properties of the skin allows the study of its physiological and pathological characteristics [12, 13]. For instance, B mode high-resolution ultrasound was demonstrated to be a rapid and sensitive tool for measuring skin thickness. Its validation and reproducibility were confirmed by parallel measurements of this latter using the histological technique [14, 15]. Similarly, the echorheometer technique was validated in-house on elastic membrane models, the mechanical properties of which had previously been calibrated [9]. Its accuracy and reproducibility were found to be satisfactory [10]. Accordingly, these two techniques appeared to be valuable systems for measuring mechanical properties of the skin.

According to the literature, epidermal and dermal thicknesses [2, 16, 17] as well as elasticity of the skin decrease progressively during the long-term aging process [3], whereas skin stiffness increases [4, 18]. Globally, our data further confirm the literature, as shown in our control group. By contrast, retinaldehyde 0.05% appears to counteract the physical characteristics observed in skin aging and photoaging. In this study, because the mechanical parameters stiffness and elasticity were shown to be independent of the cutaneous thickness, it is likely that the beneficial effects of retinaldehyde are due to some recovery in the quality or density of the structures that are involved in skin suppleness and elasticity (e.g. collagen and elastic fiber network) rather than to its effect on skin thickness only. Taken together, the results confirm that retinaldehyde has restorative effects on skin aging and photoaging.

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# Repair of UVA-Induced Elastic Fiber and Collagen Damage by 0.05% Retinaldehyde Cream in an ex vivo Human Skin Model

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**Key Words**

Retinaldehyde · Normal human skin · Ex vivo model · Organ culture · Collagen · Elastic fibers · Ultraviolet light · Photoaging

**Abstract**

***Background:*** Cellular effects of UV exposure are implicated in cutaneous aging. UV radiations induce structural and cellular changes in all the compartments of skin. ***Aim:*** To study the antiaging efficacy of a cream containing 0.05% retinaldehyde with an ex vivo technique using human skin in order to approximate in vivo metabolic conditions. ***Methods:*** Human skin explants were maintained alive in organ culture for 18 days and subjected to UVA exposure, thus simulating skin photoaging. Retinaldehyde cream was then applied to the surface of the epidermis for 2 weeks and the results were compared with those of nontreated skin explants. Dermal repair was analyzed histologically with quantification of collagen and elastic fibers, and biochemically by the measure of newly synthesized collagen as shown by adding tritiated proline to the culture medium. ***Results:*** UVA exposure induced significant alterations of collagen and elastic fibers as shown by morphometric analysis. In all UVA-exposed and then retinaldehyde-treated skin specimens, collagen and elastic fibers were restored to the level of nonexposed skin. UVA exposure induced a decrease in collagen synthesis, whereas in retinaldehyde-treated UVA-exposed skin the synthesis was similar to that of unexposed skin. ***Conclusion:*** It has been shown that retinaldehyde has many of the properties of tretinoin in its biological and beneficial effects on photoaging. We have verified some of these previous observations, especially on dermal connective tissue, by obtaining significant repair of elastic fibers and collagen alteration induced by UVA exposure.

## Introduction

The effectiveness of topical tretinoin (all-*trans*-retinoic acid) in treating the consequences of photoaging in human skin is now well known, demonstrated by animal, clinical as well as by in vitro studies [1, 2]. Indeed, histological epidermal changes have been described in tretinoin-treated human subjects, i.e. increased epidermal thickness, increased granular layer thickness, decreased melanin content and stratum corneum compaction [3]. At the dermal level, improvement of connective tissue was observed in tretinoin-treated photodamaged skin [4] as well as increased glycosaminoglycans

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[5], new collagen formation (types I and III) [6, 7] and improvement of elastic fibers [4].

Retinaldehyde, an intermediate between retinol and retinoic acid, is known to have biological activity close to that of retinoic acid in mouse [8] and human skin [9]. Retinaldehyde has also been shown in vivo to improve photoaged skin [10] and to achieve this improvement on a par with retinoic acid in a double-blind study but with a better patient tolerance [11].

To analyze some biochemical events that can explain these findings, we have tested a 0.05% retinaldehyde cream in an ex vivo model, thereby avoiding the need for animal testing and reducing the need for further in vivo testing. This model consisted of full-thickness normal human skin fragments obtained from plastic surgery and maintained in long-term organ culture for 21 days and exposed to UVA, thus simulating skin photoaging [12]. With this method, we have induced alterations of elastic fibers and collagen [12] similar to that observed during the acute phase of UV-induced alterations [13].

## Materials and Methods

*Organ Culture of Human Skin Specimens*

Our original culture method is based on previous studies [14, 15]. We adapted these methods to obtain full-thickness skin surviving for 18 days in cultures with both epidermal and dermal structures resembling normal in vivo skin [16]. Eight normal human skin fragments were obtained from plastic surgery in women 35–45 years of age. Skin fragments were cut into 1-$cm^2$ full-thickness pieces and washed three times with antibiotics. Subcutaneous fat and lower dermis were mechanically removed under a stereomicroscope using a surgical scalpel.

Skin explants were placed with the epithelium uppermost, at an air/liquid interface, on culture inserts (filter pore size 12 µm; Costar, Poly-Labo Paul Block, France). These inserts were set on 12-well plates (Costar) for 18 days at 37 °C in a humidified incubator with 5% $CO_2$. Cohesion between skin and insert was obtained with a polysiloxane vinyl seal in such a way that no skin retraction or lateral passage of the cream towards the dermis was possible.

Medium was added to the wells so that the surface of the medium was level with the filter. Organ cultures were performed with Dulbecco's minimal essential medium (Gibco BRL) containing antibotics (100 U/ml penicillin and 100 µg/ml streptomycin; Gibco BRL, USA), 200 µg/ml *L*-glutamine (Gibco BRL), bovine pituitary extract, growth factors and fetal calf serum (DAP, France) [14–18]. All supplements were freshly made at each medium change every 2 days.

*UVA Radiation*

To obtain premature aging of the skin with dermal alterations, we used UVA radiation, known to induce changes in the middle and deep dermis [12]. The source of UV radiation was a Vilbert Lourmat stimulator (France) fitted out with a UVA irradiation source (365 nm) composed of Vilbert Lourmat tubes T-20, L-365 (no UVB and no UVC emission) mercury vapor tubes, low pressure, hot cathodes with a Vilbert Lourmat RMX-365/312 radiometer. The radiometer was associated with a microprocessor programmable in energy (mJ/$cm^2$), with time basis enabling 6 irradiation measurements per second for controlling the energy received by the skin fragment. For skins receiving UVA from day 0 to day 4, 2 irradiations were administered at 12 J/$cm^2$ so that they received in totality 24 J/$cm^2$. This UV radiation is sufficient to induce reproducible alterations in the dermis, as previously described [12].

*Application of 0.05% Retinaldehyde Cream*

From day 4 to day 18 following UVA irradiations, a formulation containing 0.05% retinaldehyde (Ystheal® cream, Pierre Fabre) was applied to the epidermis 5 days a week at the dose of 2 mg/$cm^2$, followed by a slight massage.

*Analysis of Dermal Repair*

After 18 days, skin fragments were removed from the culture inserts and the effects of retinaldehyde cream were studied both histologically and biochemically.

*Histological Study of Elastic Fibers and Collagen Bundles.* Skin fragments were fixed in Bouin's solution and embedded in paraffin. Serial sections of 4 µm thickness were obtained and stained for the elastic fiber and collagen study. Five sections were compared for each skin fragment. The elastic fiber network was revealed with (+)-catechin staining [19]. Collagen was stained with a picric acid solution containing 0.1% sirius red [20].

For a quantitative analysis, a computerized image analysis was made. The stained slides were examined by a microscope (Leitz; magnification × 160) connected with a camera unit (XC-75 CE type) and with a microprocessor (Q520). Approximately, 15–25 fields were analyzed for each skin section. For elastic fiber analysis, two regions were studied: the superficial dermis, reaching to the dermoepidermal basement membrane, containing mainly oxytalan and elaunin fibers, and the middle dermis containing small horizontal reticular elastic fibers, as defined by Cotta-Pereira et al. [21].

The surfaces of elastic fibers and collagen bundles were measured per square micrometer. Then, the relative elastic fiber or collagen content of the dermis was expressed as percentage of surface: area of elastic fibers or collagen per unit area of analyzed dermis [22, 23].

*Collagen Synthesis.* Fibroblastic activity for collagen synthesis was analyzed after 18 days survival of ex vivo cultures. Skin biopsies were removed from inserts, put directly in the wells and 20 µCi/ml of *L*-proline-/2, 3-$^3$H) (Amersham, France, 1 mCi/ml, specific activity 43 Ci/mmol) with 100 µg/ml ascorbic acid and 50 µg/ml β-aminopropionitrile were added in the culture medium for 24 h. Extracellular $^3$H-proline-labeled collagen was extracted by the addition of 1 mg/ml pepsin in 0.5 *M* acetic acid on the biopsies over 48 h at 4 °C. Then, the $^3$H-proline-labeled collagen was purified by Webster's method consisting of successive salt precipitations at acid and neutral pH [24]. Radioactivity in each precipitate was measured in a liquid scintillation counter and expressed in disintegrations per minute (dpm). Total protein concentration was measured by spectrophotometric determination with the Pierce BCA protein Assay Reagent kit and finally results were expressed in dpm per milligram protein.

*Statistical Analysis*

Mean values and standard deviations were calculated for each parameter. The statistical significance of changes recorded in the parameters were determined with paired Student's t test ($p < 0.05$).

Three groups were compared: a normal group (untreated skin), a control group (UVA-exposed skin) and a treated group (UVA-exposed

**Table 1.** Morphometric analysis of elastic fibers

| | Superficial dermis | | Mid dermis | |
|---|---|---|---|---|
| | surface, µm² | % | surface, µm² | % |
| Nonexposed nontreated skin | 4,385 ± 1,315 | 4 | 5,316 ± 1,087 | 4.9 |
| Skin exposed to UVA | 3,065 ± 441 | 2.8 | 4,488 ± 945 | 4.1 |
| Skin exposed to UVA and treated by retinaldehyde | 4,233 ± 1,291* | 3.88 | 5,518 ± 772* | 5 |

*$p < 0.005$: difference statistically significant in comparison with UVA-exposed skin (paired Student's t test). Surface (µm²) and percentage of surface occupied by elastic fibers per square micrometer of dermis ± SD.

skin, then treated with 0.05% retinaldehyde). Comparison was made between normal and control groups to verify the validity of the method. For evaluation of the retinaldehyde cream efficacy, comparison was made between control and treated groups.

## Results

*Computerized Image Analysis of Elastic Fiber Network and Collagen Bundles*

*Elastic Fibers.* As shown in table 1, UVA exposure induced alterations of connective tissue, particularly on elastic fibers. There was a decrease in the elastic fiber network, with fragmentation of elastic fibers (fig. 1). This observation was confirmed by morphometric analysis: after UVA radiation only 2.8% of the dermal area was occupied by elastic fibers, in contrast to 4.0% in nonexposed skin.

In all UVA-exposed and then retinaldehyde-treated skin specimens, elastic fibers stained intensely positive for catechin and tended to be longer and thicker (fig. 2) as compared with UVA-exposed, nontreated specimens. These results were confirmed by morphometric analysis: the surface occupied by elastic fibers in UVA-exposed and retinaldehyde-treated skins was significantly higher (3.88%) than in altered, nontreated skin in the superficial dermis (2.8%; $p < 0.05$). We obtained similar results in the middle dermis, where the elastic fiber network was significantly increased and better organized.

*Collagen Bundles.* As shown in table 2 UVA exposure induced important alterations of collagen bundles (fig. 3). Histologically, they became thinner and disorganized in the dermis. The surface occuppied by collagen decreased after UVA exposure (52.25%) in comparison with normal skin (66.75%; $p < 0.05$). The surface occupied by collagen in UVA-altered and retinaldehyde-treated skins is significantly higher (71.15%) than in UVA-altered skin ($p < 0.05$; fig. 4).

**Table 2.** Morphometric analysis of collagen

| | Surface µm² | Surface occupied by collagen, % |
|---|---|---|
| Nonexposed nontreated skin | 72,750 ± 20,521 | 66.75 |
| Skin exposed to UVA | 56,962 ± 14,493[a] | 52.25 |
| Skin exposed to UVA and treated by retinaldehyde | 77,542 ± 11,810[b] | 71.15 |

[a]$p < 0.05$: difference statistically significant in comparison with nonexposed skin (paired Student's t test); [b]$p < 0.05$: difference statistically significant in comparison with UVA-exposed skin (paired Student's t test). Surface (µm²) and percentage of surface occupied by collagen per square micrometer of dermis ± SD.

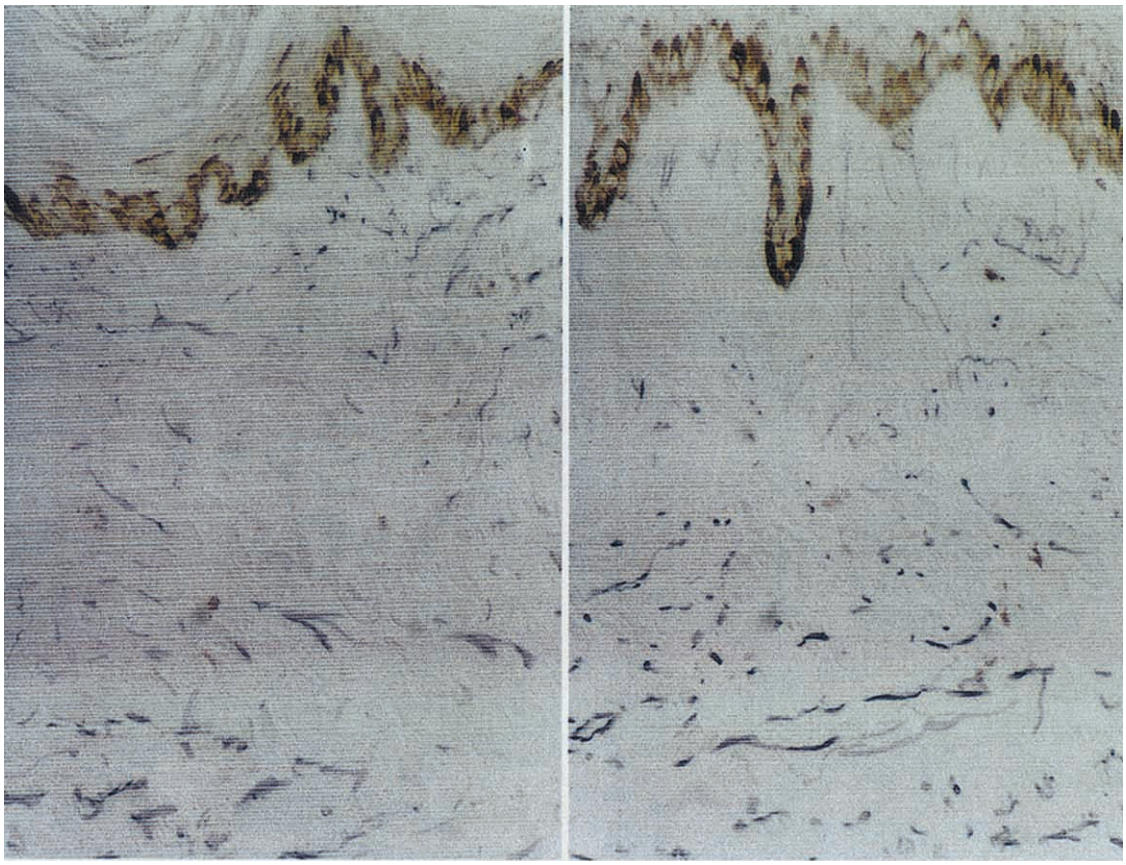
**Table 3.** Collagen synthesis (dpm/mg protein)

| | |
|---|---|
| Nonexposed nontreated skin | 37,128 ± 11,157 |
| Skin exposed to UVA | 25,105 ± 10,866[a] |
| Skin exposed to UVA and treated by retinaldehyde | 38,014 ± 10,182[b] |

[a]$p < 0.05$: difference statistically significant in comparison with normal skin (paired Student's t test); [b]$p < 0.05$: difference statistically significant in comparison with UVA-altered skin (paired Student's t test).

*Collagen Synthesis*

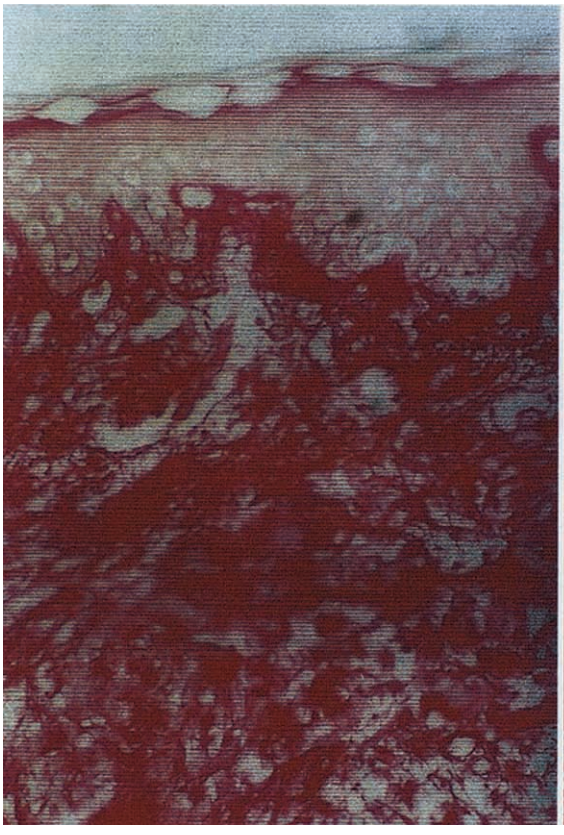
The results of collagen synthesis are given in table 3. Extracellular $^3$H-proline-labeled collagen measured by the Webster method was significantly decreased after UVA radiation in comparison with normal skin. In UVA-exosed and retinaldehyde-treated skin, collagen synthesis reached a higher level than in UVA-exposed, nontreated skin.



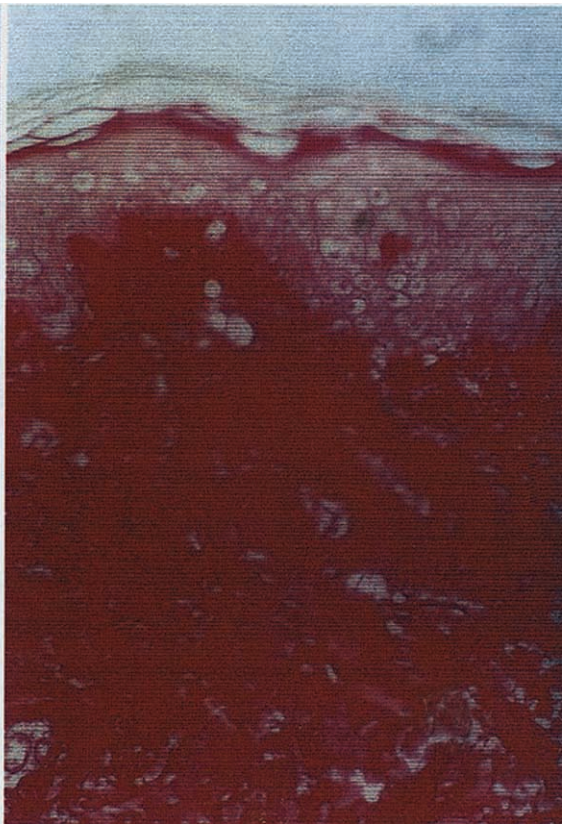
1



2



3



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## Discussion

The ex vivo method of long-term skin culture used in this work offers several advantages. Since this technique is close to the in vivo environment, human biopsies might be avoided and animals spared. Topical formulations can be applied directly on the epidermis, reproducing conditions of application of topical products on human skin in vivo [16].

The most striking UV alterations relate to the fibrous components of the dermis: elastic fibers and collagen degeneration were found to be hallmark events. In our ex vivo human skin model, we have simulated early UVA alterations, obtaining fragmentation and disappearance of collagen and elastic fibers in the superficial and middle dermis, in comparison with unexposed skin [12]. This premature skin aging observed after two UVA exposures could be explained by the increased expression of matrix metalloproteinases. Indeed, these proteinases were reported to be rapidly stimulated (92-kD gelatinase and stromelysin) in the human connective tissue and the outer epidermal layers only 24 h after a single UV exposure [16]. In consequence, a degradation of endogenous type I collagen fibrils was increased by 58% in the irradiated skin, as compared with nonirradiated skin, and which remained elevated for at least 72 h [16].

Moreover, fibroblast metabolism was also modified in skin submitted to UVA irradiations in our model, with a decrease of about 32.4% in tritiated hydroxyproline incorporation in newly synthesized collagen. Our data confirm the immunohistochemical results obtained in a previous clinical study, where collagen type I formation was 56% less in the papillary dermis of photodamaged skin [7].

Although photodamaged skin was believed to be irreversibly altered, studies have found that tretinoin (all-*trans*-retinoic acid) is capable of enhancing the repair of UV-damaged connective tissue in the hairless mouse [4]. Clinical studies have also shown that topically applied tretinoin [25] or isotretinoin [26] are effective treatments of photodamage in humans. Histological changes have confirmed the biological activity of topically applied tretinoin in photodamaged skin [25]. Indeed, animal studies have revealed deposition of new papillary dermal collagen and elastin [4]. A human clinical study using immunohistochemical analysis has shown that 0.1% tretinoin cream topically applied not only restored collagen synthesis but also promoted clinical improvement by repairing dermal collagen [7].

It has been shown that retinaldehyde has many of the properties of tretinoin in its biological and beneficial effects on photoaging [8–11]. We have verified some of these previous observations especially on dermal connective tissue, by obtaining significant repair of elastic fibers and collagen alteration induced by UVA exposure. Moreover, synthesis of collagen was increased as demonstrated by a higher incorporation of tritiated proline by dermal fibroblasts.

This study confirms the reparative efficacy of 0.05% retinaldehyde cream topically applied in a surviving skin model where dermal alterations were induced by UVA radiation. An additional study should be useful for evaluating the efficacy of 0.05% retinaldehyde cream on epidermal damage following UVB irradiation in the same ex vivo skin model, since epidermal damage has previously been shown to improve with tretinoin treatment [3].


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**Fig. 1.** Skin altered by UVA (control group): elastic fibers stained by (+)-catechin. ×160.
**Fig. 2.** Skin altered by UVA, then treated by a 0.05% retinaldehyde cream (treated group): elastic fibers stained by (+)-catechin. ×160.
**Fig. 3.** Skin altered by UVA (control group): collagen stained by sirius red. ×160.
**Fig. 4.** Skin altered by UVA and treated by a 0.05% retinaldehyde cream (treated group): collagen stained by sirius red. ×160.

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# Clinical Use of Topical Retinaldehyde on Photoaged Skin

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**Key Words**
Retinaldehyde · Retinoid · Photoaging

**Abstract**
***Background:*** Retinaldehyde, the natural precursor of retinoic acid, should exert similar effects on photoaged skin. ***Objective:*** To establish the efficacy and safety of topical retinaldehyde on photoaged skin. ***Methods:*** Open and controlled clinical studies using image analysis of silicone skin replicas. ***Results:*** Retinaldehyde proved efficient and safe. ***Conclusion:*** Retinaldehyde is efficient and well tolerated for the improvement of the signs of photoaging.

## Introduction

Kligman et al. [1] first suggested that the application of a retinoid, all-*trans*-retinoic acid (tretinoin), could be effective for the improvement of the clinical consequences of photoaging. Subsequently, Weiss et al. [2] demonstrated in a double-blind, vehicle-controlled study that the use of a cream containing 0.1% tretinoin significantly improves photoaging, including wrinkling and roughness. Unfortunately, in clinical practice few patients are able to benefit from this treatment, due to the high incidence of topical adverse reactions: in the study by Weiss et al., 92% of the patients experienced some degree of dermatitis, with erythema, swelling, xerosis, scaling as well as sensations of burning, tingling and pruritus. Although other tretinoin formulations appear to be better tolerated [3], it is most frequent in daily practice that dermatitis prevents patients from prolonged use and benefit of topical tretinoin.

Retinaldehyde is the natural precursor of tretinoin and its metabolism in the skin has been extensively studied [4]. Saurat et al. [5] showed that the application of retinaldehyde on human skin exerts a biological retinoid effect. In addition, their study suggested that retinaldehyde is well tolerated in a clinical setting.

Thus, it may be hypothesized that the use of topical retinaldehyde may be beneficial to individuals complaining about clinical signs of photoaging. Preliminary open studies have been performed which confirm this hypothesis. Recently, a double-blind vehicle-controlled study has been performed, showing that the microprofile of photoaged skin is improved by retinaldehyde in a way superior to its vehicle [6]. The purpose of this paper is to review these available studies.

## Patients and Methods

*Open Clinical and Instrumental Evaluation of the Effects of a 0.05% Retinaldehyde Cream*

An open trial has been performed to study the effects of a 4-month use of a cream containing 0.05% retinaldehyde on photodamaged skin. Clinical assessments and noninvasive physiological measurements were used for evaluation. The extensive report of this study has already been published [7].

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*Patients*

Thirty-two healthy female volunteers, aged 37–65 years (mean 47 years), have been selected. They presented with one or more of the signs of facial photoaging: elastosis, solar lentigines, wrinkling.

Subjects with facial dermatoses, known allergy to any of the ingredients of the cream or those having used retinoids (topical or systemic) or steroids, as well as those having been submitted to intense sun exposure during the last 12 months were excluded from the study. 0.05% retinaldehyde cream (Ysthéal®; Laboratoires Avène, Boulogne, France) was applied on the face once daily in the evening during a 6-month period. A standardized cosmetic and toilet regimen including the daily morning use of a sun protection factor (SPF) 15 sunscreen was prescribed.

*Methods*

*Clinical Assessment.* Volunteer Self-Assessment. Volunteers were asked to score from 0 (unsatisfactory) to 3 (very satisfactory) each of the following parameters: smoothing of the wrinkles, skin brightness and skin comfort.

Investigator Assessment. A clinical assessment by the investigator was performed at baseline and after 1, 3 and 6 months of treatments for the following parameters: coarse and fine wrinkling, scored according to the intensity and deepness of the wrinkles from 0 (no wrinkle) to 3 (severe wrinkling) and to the number of areas presenting with wrinkles (eyes, forehead, peribuccal area); degree of redness and telangiectasias from 0 (none) to 3 (severe); brightness (dull or radiant) and uniformity of skin color.

Instrumental Measurements. They were performed at baseline and after 1, 3 and 6 months of treatment. The precise area of assessment was strictly defined at each mesurement by means of a flexible plastic strip. These measurements include:

(1) skin surface profilometry with silicone rubber replicas which were evaluated by means of a computerized image analysis system according to the technique described by Grove et al. [8] and Corcuff et al. [9]; skin surfaces were molded using a silicone material; adhesive rings were used to delineate sampling sites and keep track of specimen orientation; the specimens were then analyzed in a randomized fashion by optical profilometry based on digital image processing; the following parameters proportional to the degree of wrinkling or roughness were calculated on these profiles: Rz, which divides the profile into five sections, records the peak-to-valley amplitude within each section and calculates the average of these values to reflect the 'deepness' of the fine wrinkles; Ra, defined as the area above and below the profile mean line mentioned above and reflecting the 'mean roughness'; Rs, which is the developed length of the curve of the generated profile considered as both a wrinkle and roughness parameter;

(2) skin color measured spectrophotometrically with a chromameter for both lesional and normal skin;

(3) hydration measured by means of a corneometer;

(4) lipid indices measured by means of a sebometer.

### *Double-Blind Profilometric Evaluation of Photodamage after 0.05% Retinaldehyde and Vehicle Treatments*

A randomized double-blind, vehicle-controlled multicenter study was conducted in patients with photodamaged skin of the face, comparing the activity and tolerance profile of a 0.05% retinaldehyde cream with the retinaldehyde vehicle. Profilometry was chosen as the main evaluation method, because it allows objective measurements and comparisons of the skin profile, including wrinkle characteristics. Profilometric evaluation was performed in a blind fashion. The extensive report of this study has already been published [6].

*Patients*

Ninety healthy volunteers were included at the end of wintertime (March).

Volunteers were aged 35–70 years and presented with wrinkles on the face of moderate to severe intensity as defined by means of a photograder technique [10].

None of the patients had applied topical retinoids on the treatment areas for at least 1 month during the 6 months before the study. None had used chemical peelings, exfoliants or any abrasive substance on the face within the 45 days before entry into the study. Pregnant and nursing women and patients who planned to use tan or phototherapy were excluded, as well as those with suspected skin cancer or with any other condition that could interfere with the evaluation of the study parameters.

Patients were randomly assigned to one of the two treatment groups: 0.05% retinaldehyde cream (Ysthéal®; Avène Laboratories), the vehicle of retinaldehyde.

The products were supplied in 40-gram tubes which were identical in appearance; both investigators and patients were unaware of the group to which the patients had been assigned.

*Methods*

Patients applied 0.5–0.75 g of cream on the entire surface of the face, daily in the evening for 44 weeks. In the morning, patients were asked to apply an emollient cream (Skin Recovery Cream®; Avène Laboratories) and were also instructed to apply an SPF 20 sunscreen (20B-7A®; Avène Laboratories) on the test area before outdoor activities.

At baseline (wintertime), week 18 (summertime) and week 44 (wintertime), silicone skin replicas of the left crow's feet were studied according to the technique described above. At weeks 4, 10, 18, 32 and 44, signs of local intolerance (erythema, scaling, pruritus and burning) on the test areas were scored by the investigators on a 4-point scale. Patients who experienced significant irritation were allowed to reduce the frequency of the application. This frequency was recorded by the patients on a diary. At each visit, the diaries were collected by the investigators, and an assessment of the maximum intensity of the irritation was performed.

Changes from baseline of wrinkle and roughness parameters (Rz, Ra and Rs) were evaluated by a paired Wilcoxon test. To compare treatment groups, a repeated analysis of variance was used. Local tolerance was evaluated between treatment groups using Fisher's exact test.

## Results

### *Open Clinical and Instrumental Evaluation of the Effects of the 0.05% Retinaldehyde Cream*

#### *Volunteer Assessment*

At the end of the evaluation period, the volunteers expressed a high degree of satisfaction regarding brightness (37% very satisfied), smoothing (53% very satisfied) and skin comfort (55% very satisfied).

*Investigator's Clinical Assessment*

*Coarse and Fine Wrinkling.* During treatment, the intensity of these wrinkles fell, as a mean, from moderate to slight. The extent (number of areas involved) remained unchanged.

*Facial Redness and Telangiectasias.* The number of areas affected as well as the intensity of redness and telangiectasias improved. The number of subjects without areas of redness increased from 9.5 to 42% after treatment. The number of areas affected fell as well. As far as telangiectasias are concerned, 61% of the patients had no telangiectasias after 6 months as compared to 44% initially.

*Skin Colour.* All subjects were deemed to have a shinier, radiant skin after 6 months. Similarly, the skin had become less blotchy after 6 months of treatment.

*Instrumental Assessment*

*Skin Surface Profiles.* Reductions in surface roughness were seen on left and right crow's feet areas with regard to average roughness of the skin surface relief, flattening of the surface profile and reduction in mean peak-to-valley height of the surface profile.

*Skin Color.* Homogenization of the skin color was observed after 1 month and demonstrated by the statistically significant differences in the luminance and redness values for normal and lesional skin.

*Hydration.* Hydration indices grew significantly from month 0 to months 1, 3 and 6.

*Lipid Indices.* The lipid indices fell significantly to lower values at months 1 and 3. The values became normal at month 6.

*Tolerance*

Twenty-nine percent of the volunteers experienced some sensations of stinging and some slight desquamation lasting for 1 or 2 days, mainly during the first month of treatment. These effects became less frequent with the continuation of the treatment and no side effects were reported at month 6 of the treatment.

One subject discontinued the treatment for reasons which were not related with the cream.

*Double-Blind Profilometric Evaluation of Photodamage after Retinaldehyde 0.05% and Vehicle Treatments*

Out of the 90 patients included in the study, 5 were excluded from the profilometric analysis because their skin replicas were technically unusable. So the efficacy analysis population comprised 85 patients (40 patients in the retinaldehyde group and 45 patients in the vehicle group). The 90 patients included in the study constituted the safety population.

**Table 1.** Mean changes (± SEM) of profilometric parameters from baseline to week 18 and week 44, in the efficacy analysis population (n = 85)

| | Week 18 | | Week 44 | |
|---|---|---|---|---|
| | mean | SD | mean | SD |
| Ra: change from baseline, arbitrary units | | | | |
| Retinaldehyde | –4.08 | 1.45 | –2.91 | 1.3 |
| Vehicle | –1.71 | 1.25 | –1.11 | 1.33 |
| Rs: change from baseline, arbitrary units | | | | |
| Retinaldehyde | –118.04 | 39.26 | –77.98 | 35.61 |
| Vehicle | –31.15 | 29.48 | –8.39 | 33.33 |
| Rz: change from baseline, arbitrary units | | | | |
| Retinaldehyde | –17.85 | 5.66 | –12.79 | 5.24 |
| Vehicle | –5.52 | 5.12 | –3.59 | 5.27 |

The profilometric changes from baseline to week 18 and week 44 (end of treatment) of the wrinkle and roughness parameters were assessed (table 1). Patients were found to be more responsive at week 18 than week 44, irrespective of their treatment group: at week 18, retinaldehyde induced a statistically significant reduction of all parameters ($p < 0.01$, paired Wilcoxon test). At week 44, a statistically significant improvement of the 2 parameters Rz and Rs could be maintained with retinaldehyde ($p < 0.05$, paired Wilcoxon test), although to a lesser extent. In contrast, no statistically significant reduction could be showed in the vehicle group. However, comparison of the changes between treatment groups at any assessment time did not show any statistically significant difference. It is likely that this is due to the insufficient sample size of the treatment groups, as reflected by the low power value of the tests (50%). Signs of local irritation were rare in both the retinaldehyde and placebo groups (table 2).

## Discussion

Preliminary open studies allowed to record numerous observations of positive appreciations by patients and investigators on the effects of topical retinaldehyde on the clinical consequences of cutaneous photoaging. In addition to the studies reported here, a large-scale multicenter open study, involving 1,017 patients in dermatological private practice, showed that 77% of patients experienced an improvement in wrinkling and 79% an improvement of their

**Table 2.** Patient distribution according to maximal irritation (erythema, scaling, pruritus or burning) on the face, in the safety analysis population (n = 90)

| Study group | Absent | Mild | Moderate | Severe |
|---|---|---|---|---|
| Retinaldehyde (n = 44) | 34 (77.3%) | 6 (13.6%) | 4 (9.1%) | 0 (0%) |
| Vehicle (n = 46) | 44 (95.7%) | 2 (4.3%) | 0 (0%) | 0 (0%) |

liver spots, and that the topical safety was excellent, with only 2% reporting irritation dermatitis (Avène Dermatological Laboratories, data on file). It was felt however that an objective measurement of the skin profile was necessary to ascertain the beneficial effect of retinaldehyde and also to compare it under double-blind conditions with its vehicle. The reliability and reproducibility of computerized image analysis of skin replicas has previously been established [11]. So we chose to use this method in order to objectivate the changes in cutaneous profile brought about by retinaldehyde use. Using this method we were able to demonstrate significant positive changes. By contrast, no significant change was evident after vehicle cream applications.

## Conclusion

Retinaldehyde, the immediate precursor of retinoic acid, has been shown to exert a retinoid biological activity in the skin. Clinical open and controlled studies demonstrated that the regular use of retinaldehyde improves the cutaneous consequences of photoaging, as evidenced by the profilometric study of skin replicas. Retinaldehyde is well tolerated and irritation dermatitis is infrequent.

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Dermatology

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# Retinaldehyde Alleviates Rosacea

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**Key Words**
Rosacea · Topical retinoids · Retinaldehyde

**Abstract**
***Background:*** Anecdotal observations suggest that retinoic acid may be effective in mild rosacea. ***Aim:*** Our aim was to investigate, by an exploratory clinical and instrumental study, the effects of a topical formulation with the retinoic acid precursor retinaldehyde, in patients with vascular signs of facial rosacea. ***Methods:*** Female patients were treated with a 0.05% retinaldehyde cream that was applied once daily for 6 months. Clinical assessments of persistent erythema and telangiectasia were performed every month, using a 4-point severity score (absent to severe). The clinical response for each parameter was defined as a decrease of at least 1 grade in the severity score. In addition, erythema was further evaluated by measurement of the a* parameter, using a spectrophotometer on lesional and nonlesional areas. ***Results:*** A total of 23 women comprised the study population. At baseline, 10 patients had diffuse erythema, 3 patients had isolated telangiectasia and 10 patients had both. During retinaldehyde treatment, a clinical response was revealed in about 75% of the patients with erythema, after 5 months ($p < 0.05$). Similarly, isolated telangiectasia responded to retinaldehyde, although to a lesser extent and after a longer period of treatment (46% responders after 6 months, nonsignificant). Using the spectrophotometer, the a* parameter diminished in patients with erythema by about 15%, after 2 months of treatment ($p = 0.001$). ***Conclusion:*** This study indicates that retinaldehyde has beneficial effects on the vascular component of rosacea.

## Introduction

Retinoic acid has shown beneficial effects on the vascular component of rosacea [1]. However, the therapeutic response has been found to be delayed, and side effects observed early on in treatment (skin dryness, erythema, burning and stinging) resulted in temporary aggravation of lesions as well as noncompliance.

Retinaldehyde, an intermediate between retinol and retinoic acid in the natural metabolism of retinoids, is known to have a therapeutic activity close to that of retinoic acid and to be well tolerated by the skin [2]. The goal of this pilot study was to investigate the effectiveness of a 0.05% retinaldehyde formulation in the treatment of mild rosacea (erythema and/or telangiectasia).

## Methods

This was a monocentric, open-labeled study, performed in a population of women presenting with facial signs of rosacea, i.e. persistent erythema and/or telangiectasia. Test treatment consisted of daily topical applications of a 0.05% retinaldehyde cream (Ysthéal®; Avène Laboratories, Boulogne, France). Treatment began in March–April for a period of 6 months. No oral antibiotic or corticosteroid and no topical treatment except the use of an emollient cream (Skin Recovery Cream®; Avène Laboratories) and a sunscreen (Total

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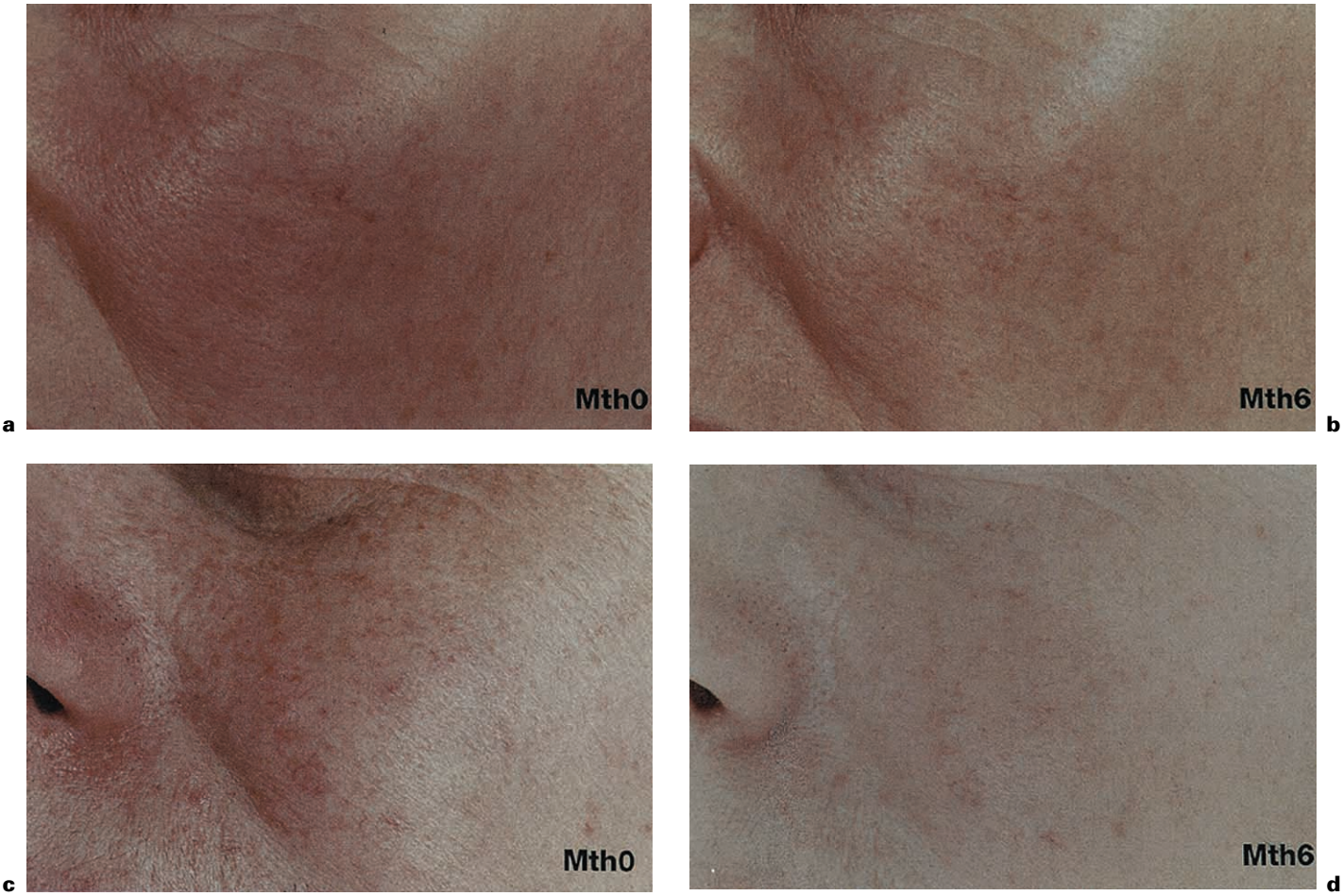


**Fig. 1.** Two examples of clinical response to retinaldehyde 0.05% topical treatment at the end of treatment. **a**, **b** Patient with persistent erythema and telangiectasia. **c**, **d** Patient with isolated telangiectasia. Month 0 = Baseline; month 6 = end of treatment.

**Table 1.** Distribution of the number of responders/nonresponders with erythema and telangiectasia in the study population, at each assessment point

| | Baseline | 1 month | 2 months | 3 months | 5 months | 6 months |
|---|---|---|---|---|---|---|
| Erythema (n = 20) | 0/20 | 4/16 | 12/8 | 10/10 | 15/5* | 14/6* |
| Telangiectasia (n = 13) | 0/13 | 1/12 | 2/11 | 5/8 | 8/5 | 6/7 |

Responders were defined as patients with a reduction of at least 1 grade in the 4-point severity scale (absent to severe). *$p < 0.05$.

Sun Block Cream®; Avène Laboratories) were permitted during the study.

The evaluation parameters were clinical and instrumental. Clinical evaluation was performed by the investigator at baseline, then each month for 6 months, except the fourth (July–August) month. This consisted of an assessment of erythema and telangiectasia, using a 4-point severity scale (absent, slight, moderate and severe). The clinical response was defined as a decrease of at least 1 point in the severity scale during treatment. The instrumental assessment was also performed at each visit by use of a spectrophotometer (Chromameter CR 200; Minolta, Japan) to measure the a* parameter, as the expression of redness. Measurement of skin redness was assessed at 2 sites of the

face: a lesional zone presenting persistent erythema and an adjacent healthy zone. The sites of the measurement were precisely localized at each visit with specially prepared marker masks.

Statistical analysis included verification of data normality, followed by an analysis of variance. Multiple comparisons of significant results were realized using Dunnett's test.

## Results

The study population comprised 23 women (mean age: 47 years, range: 37–55), 10 with erythema alone, 3 with telangiectasia alone and 10 with both clinical signs. Clinical intensity of the 2 signs at baseline was slight to moderate for all patients. No patient was withdrawn because of an adverse event during the study. Accordingly, all patients completed the study period (6 months).

Table 1 represents the number of patients responding to the retinaldehyde 0.05% treatment at each evaluation for erythema and telangiectasia. Globally, around 75% of patients responded to treatment of their erythema after 5 months of treatment ($p < 0.05$). For telangiectasia, they responded at a lower degree and required a longer time of treatment, i.e. 46% responding by 6 months (nonsignificant). Typical examples of responders are given in figure 1.

Figure 2 illustrates values of the a* parameter for redness on the involved and the normal adjacent zones, at each visit. The a* parameter significantly decreased by about 10% in the lesional zone from the first month of treatment onwards ($p < 0.001$, fig. 2a), followed by a further reduction of 14% after 2 months (spring time), then a slightly reduced response around the fifth month (11%, midsummer). Measurements on the nonlesional zones showed a similar pattern through winter to summer, reflecting the treatment-free seasonal variability of the disease ($p < 0.05$, fig. 2b).

In the vast majority of patients, retinaldehyde 0.05% was well tolerated, with no clinical sign of irritation. Only 1 patient reported transitory scaling of mild severity after 3 weeks of treatment, and no patient described exacerbation of rosacea.

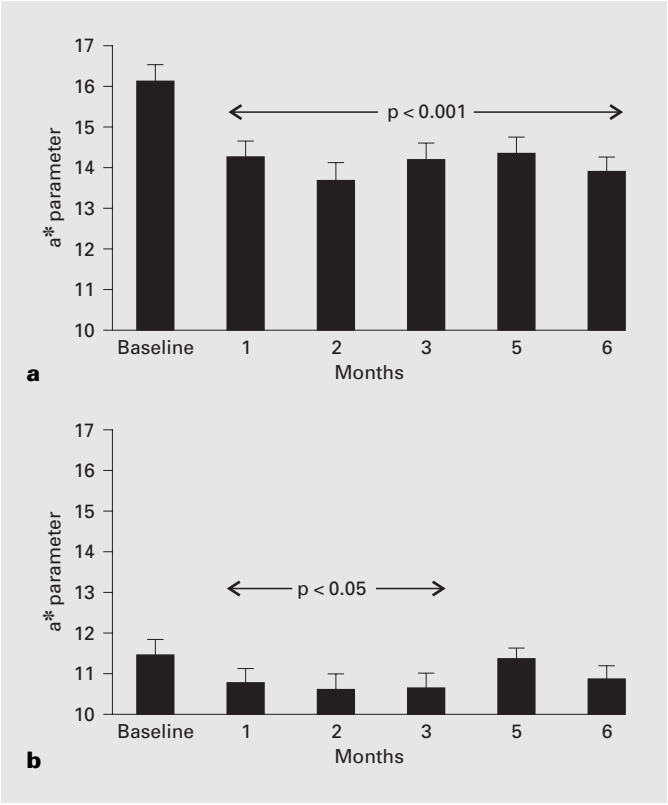


**Fig. 2.** Quantitative measurements of the a* chromametric values in the study population, at each assessment point. The a* values express the reflectance of the severity of redness in involved areas (persistent erythema, **a**) and in noninvolved areas (healthy area, **b**).

## Discussion

Rosacea is a frequent disorder, most often affecting Caucasians from the age of 30 years, and tends to be chronic [for a review, see 3]. It is an unsightly disorder, affecting the nose, cheeks, chin and forehead with persistent erythema, telangiectasia (mild stage), papules and pustules (severer stage). Flushing of the face (episodic erythema) is common at the beginning of the disease. The condition tends to be seasonal with a worsening in the winter (cold, wind) and summer (sun). Tissue overgrowth may occur and, when affecting the nose, is called rhinophyma. Cutaneous lesions may also be associated with ocular signs (ocular rosacea). The cause of rosacea is unknown, but speculations abound. Many etiological factors have been considered, i.e. disorder of vascular regulation, infestation with the *Demodex* mite, bacterial infection *(Helicobacter pylori)* or abnormal cutaneous immune mechanisms.

Treatment of rosacea includes multifaceted management [4]. For instance in mild rosacea, patients are instructed to avoid the sun, to apply sun-protective creams and to avoid facial irritants. They are often told to exclude alcoholic beverages, stimulants such as tea, coffee, hot drinks and spicy foods. At a later stage, drug therapy is often necessary. This includes systemic antibiotics such as tetracyclines, topical antibiotics such as erythromycin and clindamycin, antifungal agents such as metronidazole, azelaic acid and topical and systemic retinoid therapy [5]. Treatment with topical

retinoic acid has been proven to be successful, but patients tended towards noncompliance due to skin irritation [1, 5]. It is shown here that topical retinaldehyde alleviates the redness of rosacea, with fewer side effects and no exacerbation of the lesions, and is more acceptable to the patients.

Suggested mechanisms of action of topical retinaldehyde may be their known beneficial action in photodamaged skin, which often accompanies rosacea [6]. Also, it is well known that in acne, retinoids exert a prime action through their comedolytic activity resulting in the extrusion of comedones. Comedones are not part of rosacea, but perhaps beneficial effects of retinaldehyde in this disease result in the extrusion of the *Demodex* mite known to lodge in the pilosebaceous apparatus of the face, although the etiological role of *Demodex* in rosacea remains to be determined. Another speculation may be that retinaldehyde acts by masking the dermal vasculature, due to the thickening of the overlying epidermal layer induced by the topical retinoid.

Retinaldehyde has already been demonstrated to display a significant retinoid-type activity [2]. As such, these putative mechanisms associated with its good tolerance profile may account for its efficacy in rosacea. Furthermore, retinaldehyde might also exert a more specific action on the vascular tissue, since it inhibits the vascular endothelial growth factor in vitro [7]. Nevertheless, the implication of vascular endothelial growth factor in the pathogenesis of rosacea remains also to be clarified. In conclusion, retinaldehyde should be considered as part of the therapeutic arsenal to alleviate the vascular component of rosacea.

## Acknowledgments

The authors wish to thank C. Masella for his help in the organization of the manuscript.

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Dermatology

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# Tolerance Profile of Retinol, Retinaldehyde and Retinoic Acid under Maximized and Long-Term Clinical Conditions

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**Key Words**

Patch-testing · Local tolerance · Irritation · Retinol · Retinaldehyde · Retinoic acid

**Abstract**

***Background:*** Topical retinoic acid (RA) causes irritation of the skin. To prevent this side effect, natural precursors of RA have been proposed. The aim of the present study was to compare the local tolerance profiles of retinol (ROL), retinaldehyde (RAL) and RA. ***Methods:*** ROL, RAL and RA were studied using repeated insult patch tests for 14 days (n = 6). Similarly, RAL and RA were assessed in long-term clinical use for 44 weeks (n = 355). Clinical scoring on irritation, measurement of transepidermal water loss (barrier function) and laser Doppler blood flow perfusion units (irritation) were performed. ***Results:*** Under maximized conditions, an equally low irritation potential for ROL and RAL and a more pronounced irritant effect with RA could be demonstrated clinically ($p < 0.05$ in the intergroup analysis). Furthermore, RAL and RA induced more scaling than ROL ($p < 0.05$), and ROL and RA tended to induce more burning/pruritus than RAL (nonsignificant). The TEWL values were low with ROL and high with RAL and RA (nonsignificant, intergroup analysis). The laser Doppler measurements confirmed pro-irritating effects of RA and the nonirritating effects of ROL and RAL ($p = 0.001$, intergroup analysis). The long-term clinical study showed that the study population developed a high frequency of erythema (44% of the population), scaling (35%) and burning/pruritus (29%) with RA in the first 4 weeks of treatment, whereas these 3 parameters were significantly less frequent with RAL ($p < 0.0001$ in the intergroup analysis). ***Conclusion:*** The natural retinoids ROL and RAL do have a good tolerance profile, in contrast with the irritating potential of RA.

## Introduction

The irritative potential of retinoic acid (RA) and synthetic retinoids is well known [1]. Irritation may partly be explained by an overload of nonphysiological amounts of exogenous RA in the skin. It has been speculated that natural RA precursors such as retinol (ROL) and retinaldehyde (RAL) may be less irritant in clinical and experimental use [2, 3]. Here, we report the results of 2 studies comparing the tolerance profile of ROL, RAL and RA, either under repeated insult patch testing or under normal long-term conditions.

## Methods

*Subjects*

For the maximization test, 6 male volunteers (mean age ± SEM: 32 ± 1.1 years; range: 29–36) were recruited. The volunteers had no history of allergy against ingredients of the test products and no course of systemic or topical anti-inflammatory treatment. In the clinical study, 355 patients with photodamage of mild to severe intensity (mean

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age ± SEM, 47.72 ± 7.04 years; range: 35–70) were enrolled. None of the patients had applied topical products (e.g. retinoids, chemical peels or abrasive substances) able to interfere with the evaluation parameters before entry into the study. For all subjects, written informed consent was obtained.

*Products*

The 3 following marketed products were tested: 0.07% ROL, 0.05% RAL and 0.05% RA. In the long-term clinical study, RAL, RA and the vehicle of RAL were used.

*Maximization Test*

A hundred milligrams of the test products ROL, RAL and RA were daily applied during 14 days under occlusive conditions (11-mm Finn Chambers; Hermal, Reinbeck, Germany) on the ventral forearms of the volunteers. Test sites were chosen according to randomization schedule. One additional site was left untreated (control site). All test sites were free of nevi, pilosity and skin pathology. A clinical scoring of erythema, scaling and burning/pruritus was performed by a trained dermatologist, at baseline and 1 h after removal of the occlusion dressings at the end of treatment. The intensity of each parameter was graded from 0 to 3 (0 = absent, 1 = slight, 2 = moderate, 3 = severe). The instrumental evaluations of barrier function and irritation were performed by the respective measurements of transepidermal water loss (TEWL, Tewameter TM 210; Courage & Khazaka, Köln, Germany) and perfusion unit (PU) by a laser Doppler PF 2 (Perimed, Jarfally, Sweden). TEWL and PU measurements were performed according to the standard guidelines [4, 5].

*Clinical Study*

Three hundred and fifty-five patients with photoaged skin were enrolled and treated in a double-blind manner for 44 weeks on the entire face, with 500–750 mg of the following test products: RAL, RA and RAL vehicle. A scoring was performed on local signs of erythema, scaling and burning/pruritus after 4, 18 and 44 weeks of treatment. The intensity was scaled from 0 to 3 (0 = absent, 1 = slight, 2 = moderate, 3 = severe). Patients were asked to apply the test creams in the evening and an emollient cream (Skin Recovery Cream®, Avène Laboratories) in the morning. They were also instructed to apply a sunscreen (Total Sun Block Cream®, Avène Laboratories; sun protection factor 20) on their face before outdoor activities.

For the maximization test, direct comparison of data at baseline and at the end of treatment (day 14) was performed using the signed sum rank test. The group comparison (ROL, RAL and RA) of the Δ values (between end of treatment and baseline) was performed with a two-way analysis of variance, followed by MacNemar's test when significance was reached. In the clinical study, the parameters of tolerance were evaluated between treatment groups using Fisher's exact test.

## Results

*Maximization Test*

The results of the clinical scoring at the end of treatment are given in figure 1. Erythema was rather low with ROL and RAL and significantly higher with RA ($p < 0.05$ between treatment groups). The scaling (exfoliative power)

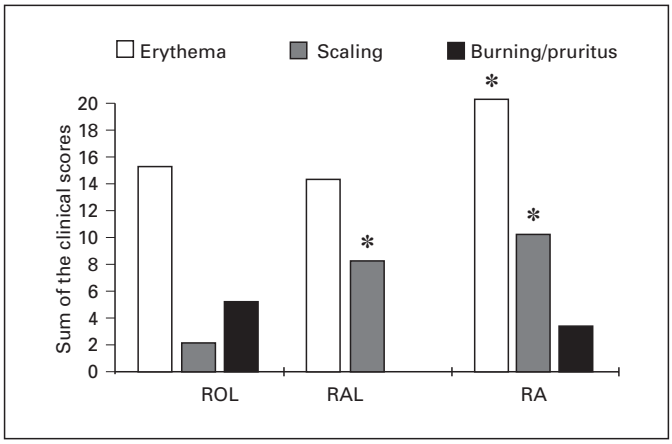


**Fig. 1.** Sum of the clinical scores for each parameter (erythema, desquamation and burning/pruritus) with ROL, RAL and RA, in the study population of the maximization test (n = 6); *p < 0.05, in the intergroup analysis.

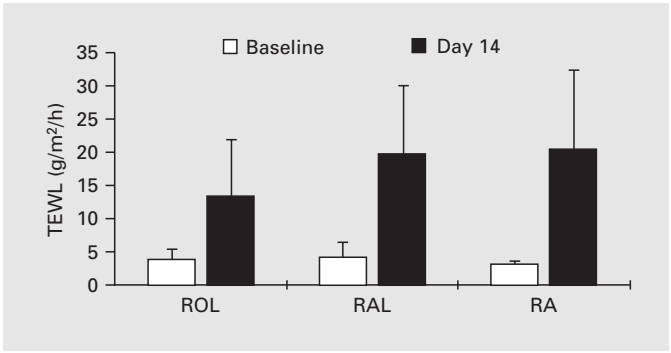


**Fig. 2.** TEWL values (means ± SEM) for ROL, RAL and RA treatment groups, at baseline and at the end of treatment (day 14).

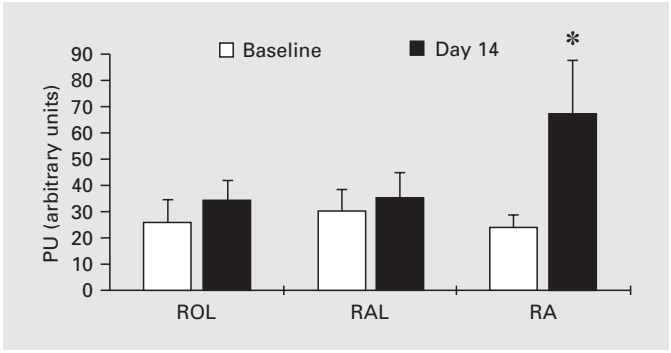


**Fig. 3.** PU values (means ± SEM) for ROL, RAL and RA treatment groups, at baseline and at the end of treatment (day 14); *p = 0.001, in the intergroup analysis.

was comparable with RAL and RA, whereas ROL showed lower effects on this parameter ($p < 0.05$ in the intergroup analysis). RAL induced no burning/pruritus, whereas ROL and RA tended to induce this latter more frequently (non-significant). By evaporimetry, the TEWL values tended to be increased with RAL and RA (fig. 2). With ROL, little change was seen. By laser Doppler flowmetry, the increases in PU values were significantly higher with RA, and no significant increase was observed with ROL and RAL. In the intergroup analysis, the difference between RA on the one hand and ROL and RAL on the other hand was statistically significant ($p = 0.001$; fig. 3).

*Clinical Study*

One hundred and nineteen patients in the RAL group, 117 patients in the RA group and 119 patients in the RAL vehicle group were enrolled in the study. In each group, the distributions of patients according to the clinical severity of the tolerance parameters at each visit are given in figure 4. Globally, signs of local intolerance developed more frequently with RA and occurred within the first 4 weeks in 44% of the patients for erythema, 35% for scaling and 29% for burning/pruritus. By contrast, signs of local irritation were rare in both the RAL and RAL vehicle groups ($p < 0.0001$ in the intergroup analysis). In a majority of patients, RA irritation necessitated modification in the treatment regimen. This resulted in fewer total days of treatment in the RA group (272 ± 8 days) than in the RAL group (300 ± 4 days) and the RAL vehicle group (309 ± 2 days, $p < 0.01$).

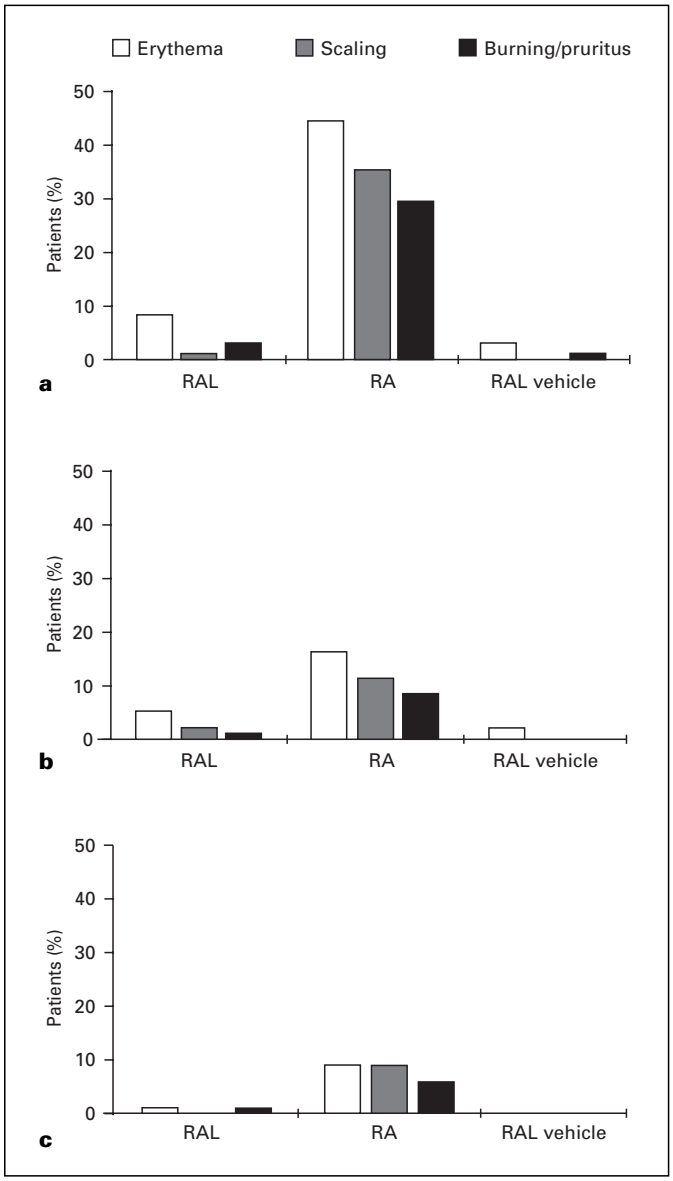


**Fig. 4.** Patient distribution (n = 355) according to the clinical severity of each irritation parameter (erythema, scaling, burning/pruritus) on the face with RA, RAL and RAL vehicle, after 4 weeks (**a**), 18 weeks (**b**) and 44 weeks (**c**) of treatment.

## Discussion

Retinoids are widely used in the treatment of acne and photodamage [6–10]. However, the frequent irritating effects of RA may alter the compliance of the patients. Because the conversion of the precursors ROL and RAL into RA is controlled by rate-limiting enzymatic steps within the cells [3, 11], their pharmacological use may avoid an overload of RA that is putatively incriminated in the RA-induced irritation [12, 13]. Under occlusive conditions, we could demonstrate an equally rather low irritation potential with ROL and RAL, which was by contrast more pronounced with RA. Interestingly, the low irritation potential of RAL, as demonstrated by the clinical scoring and the laser Doppler measurement, was associated with an exfoliative activity comparable to RA, as shown by the clinical scoring of scaling and the TEWL. Since this exfoliative action is one of the hallmarks of a retinoid activity, RAL seems to combine both the low irritation potential of ROL and the activity of RA on barrier function.

Under normal clinical long-term conditions, RAL confirms its good tolerance profile compared to RA. This resulted in a better compliance of the patients with RAL than that with RA. Taken together, our findings further demonstrate the good tolerance of ROL and RAL, as well as

the retinoid-type exfoliative activity of RAL and RA. Accordingly, RAL appears to display a favorable activity/tolerance ratio among natural topical retinoids.

## Acknowledgments

The authors wish to thank C. Masella, for his help in the organization of the manuscript.

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# Tolerance of Topical Retinaldehyde in Humans

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**Key Words**

Retinaldehyde · Retinoic acid · Retinol · Tolerance · Safety

**Abstract**

***Background:*** Retinaldehyde (RAL) has been used as a topical agent in many countries since 1994. ***Aim:*** To review current data on the tolerance of retinaldehyde and to report the results of a long-term pilot study. ***Methods:*** Data from published and on-file studies have been compiled. Forty-five patients who had applied RAL on the face for 12–89 months were specifically examined for side-effects. ***Results:*** Studies in humans demonstrated an excellent tolerance of topical RAL on human skin. It was much better tolerated than retinoic acid and could be used even on sensitive facial skin. It does not have phototoxic or photo-allergic properties. No side-effects were associated with long-term use. ***Conclusion:*** Current data indicate a good topical tolerance of RAL in humans.

## Introduction

The efficacy of retinoic acid (RA) in the treatment of several skin diseases has largely been shown [1]. It is also well known that this molecule often causes irritation of the skin eventually explained by an 'overload' of the RA-dependent pathways with non-physiological amounts of exogenous RA in the skin [2]. However, it is not well established yet if this cutaneous irritation limiting the clinical use of RA is partly necessary for the therapeutic activity of this molecule or if it is only an undesirable side-effect.

It has been demonstrated that retinaldehyde (RAL), a natural metabolite of vitamin A and direct precursor of RA, has a similar biological activity to RA [3, 4] and postulated that it might have weaker side-effects on the skin as differentiating keratinocytes are capable of oxidizing RAL to RA at a higher rate than non-differentiating keratinocytes resulting in a targeted and controlled delivery of ligand [5].

To investigate the local tolerance of RAL numerous studies have been performed, first in animals, later in humans. We review current data upon the tolerance of RAL and report the results of a long-term pilot study.

## Open Clinical Studies of Tolerance

In the first pilot study of biological effects and tolerance [3], 229 volunteers with various skin problems were treated with RAL 1.0, 0.5, 0.1 or 0.05% once daily for 1–3 months (mean 3.7 months). Tolerance was graded on a scale from 0 to 3. The 1% preparation was tolerated by up to 69% of the treated subjects, mostly psoriatics treated on the trunk and limbs; grade 2–3 irritation leading to cessation of treatment was observed in 31%. Tolerance of the 0.5% preparation was slightly better, whereas both 0.1 and 0.05% preparations, used mostly on facial skin, were much better tolerated and allowed prolonged use on facial skin in patients with inflammatory dermatoses.

In another study with 32 female healthy volunteers [6] applying RAL 0.05% on the face once daily during a 6-month period, 29% of the volunteers experienced some sensations of stinging and some slight scaling lasting for 1 or 2 days, mainly during the first months of treatment. These effects became less frequent with the continuation of the treatment, and no side-effects were reported at month 6 of treatment.

An open multicentric study including 130 cases [7] has been conducted in 10 dermatology clinics. These patients

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presented with erythrosis (42 patients), rosacea (42 patients) or seborrhoeic dermatitis (46 patients). Assessment of topical tolerance indicated that RAL gel 0.05% was well tolerated by the irritated skin of the face. Only 3.8% of the patients had to stop the treatment after 4 months due to the occurrence of side-effects like erythema, scaling, burning and/or pruritus.

## Phototoxicity and Photosensitivity

A study investigating the phototoxic potential of RAL included 10 volunteers of phototypes 2 and 3. RAL was applied on the forearm under occlusion (Finn Chambers) for 24 h. The sites were then irradiated with either 0.75 minimal erythema dose (MED) of UVB or 0.75 MED of UVA and examined 24, 48 and 72 h after irradiation. No reaction as compared to control sites was observed, indicating lack of phototoxic potential of RAL in this vehicle and at this concentration [8].

Another study explored the possibility that RAL may influence UVB and UVA MED after either short- or long-term application [9]. A single application of 0.05% RAL in 20 volunteers did not result in any sun protection activity since the sun-protective factor for UVA-UVB (290–390 nm) was 1.02 ± 0.1 with the RAL cream 0.05% and, as expected, 3.64 ± 0.5 with the control sunscreen product. After 10 weeks of daily application of RAL cream 0.05%, the MED ($J/cm^2$) was 2.92 ± 0.4 in untreated skin and 3.00 ± 0.5 in RAL-treated skin, again indicating lack of sun protection after long-term use of RAL. It is noteworthy however that there was no increase in photosensitivity after 10 weeks of RAL treatment.

## Comparative Studies of Topical Tolerance

*Tolerance of Repeated Application under Occlusion*

Six healthy volunteers applied retinol (ROL), RAL and RA under occlusion (Finn Chambers) on the ventral side of the forearm every day for 14 days at a concentration of 0.05% (RAL and RA) and 0.075% (ROL) [10]. One additional site was left untreated (control site). Clinical signs of side-effects (erythema, scaling, infiltration, vesicles, burning, pruritus) were evaluated according to a scale ranging from 0 to 3 (0 = absent, 1 = slight, 2 = moderate, 3 = severe). Erythema was rather low with ROL and RAL and significantly higher with RA ($p < 0.05$ between treatment groups). The scaling (exfoliative power) was comparable with RAL and RA, whereas ROL showed lower effects on this parameter ($p < 0.05$ in the intergroup analysis). RAL induced no burning/pruritus in contrast to ROL and RA. Instrumental evaluations of barrier function and irritation were performed by measurements of transepidermal water loss (TEWL) and perfusion unit laser Doppler flowmetry. The TEWL level tended to be significantly increased with RA (17.4) and RAL (15.6). With ROL, little change was seen (9.7). The increase in perfusion unit values was significant ($p = 0.0313$) with RA (43.3); no significant increase was observed with RAL (12.1) and ROL (8.3). In the intergroup analysis, the difference between RA on the one hand and ROL and RAL on the other hand was statistically significant ($p = 0.001$). These results demonstrated an equally rather low irritation potential with ROL and RAL, which was by contrast more pronounced with RA. Moreover, RAL combined the low irritation potential of ROL and the exfoliative activity of RA (one of the hallmarks of retinoid activity) on barrier function [10].

*Tolerance of Repeated Application without Occlusion*

Twelve volunteers applied RAL 0.05%, RA 0.05% or the vehicle on the inner side of the forearm on well-defined zones, once a day during 5 days running for 4 weeks. The tolerance was evaluated by measuring the cutaneous blood flow by laser Doppler velocimetry and the TEWL by evaporimetry. The results show that the cutaneous blood flow and the TEWL, parameters of irritation, are increased only by RA, while RAL is well tolerated [11].

*Comparative Study under Normal Conditions*

A randomized open study included 357 female patients [12]. They applied RA, RAL or placebo on the face once a day during 12 months. The frequency of side-effects was evaluated after 1, 6 and 12 months. After 1 month, erythema was observed in 44%, scaling in 35% and burning in 29% of patients treated with RA, compared to 8, 1 and 3%, respectively, of patients treated with RAL. In the group treated with placebo, these side-effects were seen in 3% (erythema) and 1% (scaling, burning). After 12 months of treatment, the frequency of all 3 side-effects was decreased in all 3 groups, but still RAL (erythema 1%, scaling 0%, burning 1%) was much better tolerated than RA (erythema 9%, scaling 9%, burning 6%). The treatment of RA was interrupted during a total of 50 days on an average, the treatment of RAL during 10 and of placebo during 3 days. The overall tolerance of RA was 57%, of RAL 96% and of placebo 97%.

## Topical Tolerance after Long-Term Use

A consequence of the good tolerance of RAL, demonstrated by all clinical studies mentioned above, is the observation that some patients have been using RAL every day

**Table 1.** Topical tolerance of RAL after long-term use

| | |
|---|---|
| Patients, n | 45 |
| Age, years | 22–82 (median 52.4) |
| Male/female | 12/33 |
| Duration of RAL application, months | 12–89 (median 46.6) |
| Patients still using RAL, n | 22 (49%) |
| Follow-up of all patients, months | 15–101 (median 68.3) |
| Follow-up after end of treatment, months | 7–80 (median 48.6) |
| Side-effects | |
| Toxic/allergic reaction | 0 |
| Photosensitivity | 0 |
| (Pre)malignant skin lesions | 0 |

for years. This fact raises the question if RAL in the long-term use causes any topical side-effects. To investigate this question we reviewed the folders of all patients treated with RAL between 1989 and 1997. Of 219 patients, 50 had applied RAL on the face for 1 or more years, the inclusion criterion for our study. Forty-five of these 50 patients could be examined (table 1). Twelve were male and 33 female with a median age of 52.4 years. Reasons for using RAL were photoaging (22 patients), seborrhoeic dermatitis (12 patients), acne (7 patients), rosacea (6 patients), actinic keratosis (3 patients), hyperpigmentation (1 patient) and folliculitis (1 patient). The duration of RAL application on the face and of the follow-up was between 12 and 89 months (median 46.6) and 15 and 101 months (median 68.3), respectively. Twenty-three patients were off treatment at the moment of our evaluation; the time of follow-up after treatment was between 7 and 80 months (median 48.6). In none of the 45 patients did we find any toxic/allergic reaction or any photosensitivity due to RAL. Three patients used RAL to treat actinic keratoses. These premalignant lesions disappeared during 44, 64 and 72 months of RAL application. In 3 patients, 1 respectively 2 actinic keratoses appeared during RAL treatment of 81, 84 and 87 months; regarding the age of these patients (57, 59 and 62 years) and the fact that actinic keratosis can respond to RAL, they do not seem to be due to the RAL treatment. None of the 45 patients presented with any malignant skin lesions like basal or squamous cell carcinoma. This retrospective pilot study demonstrated that RAL in humans does not have local long-term side-effects [13].

## Cosmetovigilance

The cutaneous tolerance of RAL is confirmed by the data of cosmetovigilance. Products with RAL 0.05% have been on the market in France since 1994. Data on files at the manufacturer cosmetovigilance services indicate that for this period of time no significant clinical side-effect has been reported, and complaints of clients have been very rare so far. For cream and emulsion they were below 1 of 10,000 sold products. For gel, probably used especially for 'red faces', they were 2.6 for 10,000 sold products, similar to other cosmetic products for sensitive or inflamed skin. All of these complaints have been investigated and revealed local irritation without any significant consequence. No allergic reaction has been either recorded or published.

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