

INVESTIGATIVE REPORT

Immunofluorescence Localization of Nuclear Retinoid Receptors in Psoriasis Versus Normal Human Skin

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Psoriasis responds favourably to treatment with retinoids but the cellular pathways mediating these effects are poorly understood. Retinoids regulate keratinocyte proliferation and maturation via binding to nuclear retinoic acid receptors (mainly RAR α and RAR γ) which form heterodimers with the 9-cis-RA receptor, RXR α . We have previously shown that mRNA expression of RAR α and RXR α is down-regulated in psoriatic lesions as compared with non-lesional human skin. In the present study, we investigated the protein expression of RAR α , RAR γ and RXR α in normal and psoriatic skin using indirect immunofluorescence analysis. Epidermal keratinocytes of normal and non-lesional psoriatic skin displayed similar nuclear localization of all three receptors; RAR α was detected with decreasing intensity from basal to suprabasal layers, RAR γ showed the opposite trend, whereas RXR α was evenly expressed throughout the epidermis. In lesional psoriatic skin, however, all three receptor proteins showed a much higher staining intensity in the lower half of the epidermis; in particular, RAR α immunoreactivity was low or even absent in the upper layers of epidermis. The results support the idea that psoriasis is associated with abnormal retinoid signalling in lesional epidermis. *Key words: psoriasis; retinoid receptor; retinoic acid; keratinocyte; epidermis.*

(Accepted April 13, 2004.)

Acta Derm Venereol 2004; 84: 363–369.

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Synthetic retinoids and their endogenous counterpart, all-*trans*-retinoic acid (RA), have profound influence on the function of epidermal keratinocytes both *in vitro* and *in vivo*. This characteristic is exploited in the treatment of psoriasis, a genetic disease characterized by epidermal hyperproliferation, disturbed differentiation, and skin inflammation.

Once internalized, retinoids bind to cytoplasmic proteins [cellular retinol-binding protein (CRBP) or cellular retinoic acid-binding protein (CRABPI and II)] and nuclear retinoic acid receptors (RAR α , - β and - γ), which are believed to mediate the genomic effects of

retinoids (1). A second class of retinoid receptors, RXR α , - β and - γ , are activated by 9-cis-RA and phytanic acid, and function as auxiliary heterodimerization partners for RARs, as well as for the vitamin D receptor, liver X receptors, peroxisome proliferator-activated receptors (PPARs) and other members of a superfamily of nuclear receptors (1). The retinoid-activated RAR-RXR heterodimers recognize and bind to RA-response elements (RAREs) in the promoter region of certain genes, thereby recruiting co-activators leading to transcriptional induction of the adjacent gene (1). Retinoids may also interact with other signalling pathways, e.g. activator protein-1 (AP-1), which has been put forward as a key transcriptional regulator in the expression of many marker genes of keratinocyte differentiation. In addition, non-genomic effects have recently been implicated in the mechanism of action of retinoids in keratinocytes (2).

Human epidermal keratinocytes express three major retinoid receptor proteins, in the following order of abundance: RXR α > RAR γ > RAR α (3). According to some investigators, the receptors are mainly expressed in the upper layers of normal epidermis (4, 5) suggesting that they are involved in the process of terminal differentiation. In fact, altered retinoid receptor expression appears to be linked to aberrant growth and differentiation of epidermal keratinocytes; hence reduced retinoid receptor levels were found in mouse skin exposed to tumour promoters and human skin exposed to UV irradiation (6–8) as well as in human squamous cell carcinoma (9–11). Furthermore, a recent study in man showed progressive suppression of RAR and RXR expression going from a normal skin phenotype to premalignant actinic keratosis and invasive squamous cell carcinoma (5).

Valuable insight into the physiological role of retinoid receptors has come from experiments in transgenic mice. For example, developmental defects reminiscent of postnatal vitamin A deficiency were found in RAR α - and RAR γ -null mice (12, 13). A main conclusion from these experiments was that a functional redundancy exists between various RARs, which may allow for one type of receptor to compensate for a missing one. Also, targeted ablation of RAR-mediated signalling in basal keratinocytes leads to abnormal epidermal maturation (14), whereas selective disruption

of RAR α results in abnormal lipid composition of stratum corneum and increased trans-epidermal water loss (15, 16), mimicking the situation in psoriatic lesions (17). Moreover, epidermal RXR α knock-out mice exhibit several skin abnormalities with striking similarities to psoriatic phenotype (e.g. hyperproliferation, thickened stratum corneum and inflammatory cell infiltrates) (18).

In a recent study using a quantitative PCR assay, we have shown that the mRNA expression of RAR α and RXR α is markedly reduced in lesional skin as compared with non-lesional skin in psoriasis patients (19). In the present study, we have investigated RAR α , RAR γ and RXR α protein expression in normal and psoriatic skin biopsies using rabbit polyclonal antibodies and immunofluorescence analysis.

MATERIALS AND METHODS

Biological specimens

Nine patients with chronic and stable plaque psoriasis (eight men, one woman, aged 20–68 years) and nine healthy volunteers (six men, three women, aged 37–83 years) gave written consent to participate in the study approved by the Ethics Committee of Uppsala University. Except for their skin disorder, the patients were healthy and had not received any systemic or topical psoriasis therapy (excluding emollients) for at least 2 weeks prior to sampling. After infiltrating the skin with lidocaine (Xylocaine[®], Astra, Sweden), 3-mm punch biopsies were obtained from normal skin (forearm or buttock in volunteers) and non-lesional (buttock) plus lesional (periphery of nummular lesion of the trunk) skin in psoriasis patients.

Punch biopsies were immediately placed in cold Histocon tissue transport medium (Histolab Products AB, Gothenburg, Sweden), snap-frozen in isopentane and stored at -70°C .

Immunofluorescence procedure

The biopsies were cryo-sectioned (6 μm) and incubated with rabbit polyclonal subtype-specific retinoid receptor antibodies [RAR α (C-20), RAR β (C-19), RAR γ (C-19) or RXR α (D-20); Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA] overnight at 4°C in a humidified chamber.

The specificity of the antibodies had been checked beforehand on Western blot analysis of nuclear extracts prepared from cultured normal human keratinocytes. The antibodies against RAR α , RAR γ and RXR α showed one major band with appropriate molecular weight (data not shown), whereas no band was discerned using the RAR β antibody, thus confirming the absence of RAR β in keratinocytes (4).

The antibodies were used at 1:200, 1:50, 1:400 and 1:800 dilution, respectively. After washing in phosphate-buffered saline, bound immunoglobulins were exposed to biotinylated anti-rabbit IgG secondary antibody for 1 h, and then to Texas Red[®] Avidin D (both from Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min. A goat polyclonal Ku-86 antibody (M-20 at 1:200 dilution; Santa Cruz Biotechnologies) and biotinylated anti-goat IgG secondary antibody was used as positive control of a protein with known nuclear expression. Slides were mounted in Vectashield[®] Mounting Media (Vector Laboratories) containing Hoechst 33258 nuclear stain.

Microscopic analysis

Tissue sections were examined at $\times 20$ magnification using a Leica DMRB microscope equipped with filters for detection of Texas Red (rhodamine) and Hoechst dye fluorescence. Digital images including overlays were captured using Leica QWin version 2.5 software (Leica Imaging Systems Ltd, Cambridge, UK). Texas Red (= RAR and RXR) fluorescence in three epidermal compartments (basal, spinous and granular layer) was scored from 0 to 3 as follows: 0, negative (no receptor-positive nuclei); 1, weak (few nuclei positive); 2, moderate (most nuclei positive); and 3, strong (all nuclei positive). Three microscopists independently evaluated all biopsies blindly.

Statistical analysis

Non-parametric Kruskal-Wallis one-way analysis and Dunn's post-test (analogous to a Bonferroni test) were performed using the GraphPad Prism (version 2.0) software (San Diego, CA, USA).

RESULTS

Expression patterns in normal skin

Immunofluorescence staining of RAR α , RAR γ and RXR α in healthy control epidermis displayed receptor-positive nuclear immunoreactivity with no or weak cytoplasmic localization (Fig. 1a, c, d). As expected, no RAR β fluorescence was observed (Fig. 1b). The nuclear localization of the three former receptors and Ku-86 is further illustrated in Fig. 2 (left panel). When data from all nine controls were compiled, using a scoring system for receptor staining at three different layers of epidermis (Fig. 3a), it emerged that the RAR α expression in normal epidermis was strongest in basal keratinocytes and decreased in suprabasal direction, RAR γ was more intensely stained in suprabasal layers, whereas RXR α was more uniformly distributed across the epidermis with virtually all suprabasal nuclei being positive (Table I; Fig. 4).

Epidermal cells of the hair follicle and sebaceous gland showed strong RAR α and RXR α fluorescence. In particular the peripheral (undifferentiated) sebocytes showed more intense signalling than the central (mature) ones. By contrast, RAR γ and RAR β were weakly expressed by hair follicle keratinocytes and not at all by sebocytes (Fig. 1e–l). This differs from the results of Reichrath et al. (4) who, using a different source of antibody, found a strong RAR γ expression in keratinocytes of the outer root sheath and sebaceous glands.

A strong nuclear RAR α fluorescence was consistently found in dermal fibroblasts (Fig. 1a), endothelial cells and cells of the eccrine sweat glands (Fig. 1m, n). All these cell types were negative for RAR β and RAR γ and only weakly positive for RXR α (results not shown). Negative controls, in which the primary antibody was excluded, showed no staining of the tissue sections (not shown).

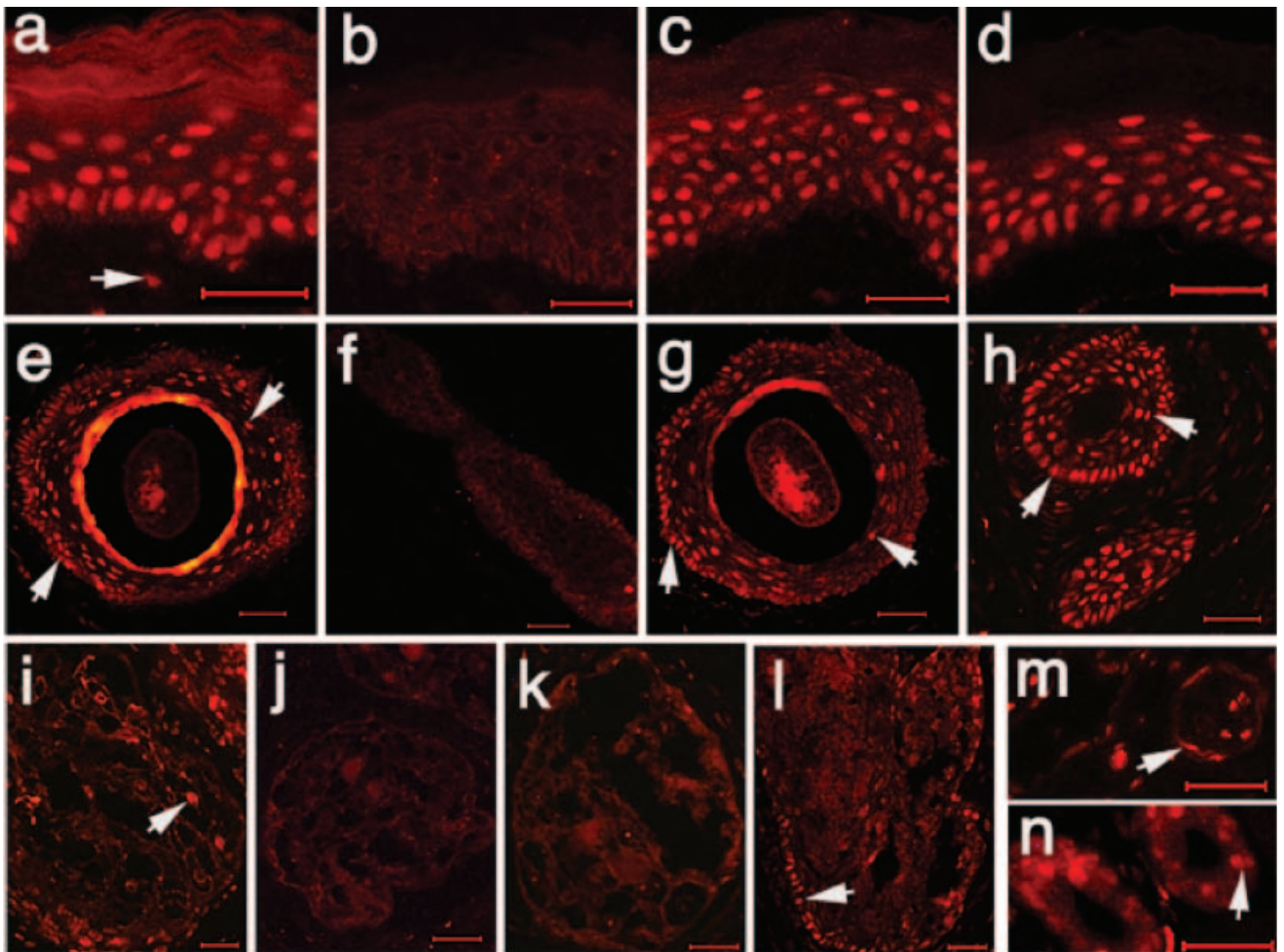


Fig. 1. Immunofluorescence detection of nuclear retinoid receptors RAR α , RAR β , RAR γ and RXR α in normal human epidermis and adjacent structures. Cryostat sections of skin biopsies were incubated with monoclonal antibodies against RAR α (a, e, i, m, n; 1:200 dilution), RAR β (b, f, j; 1:50), RAR γ (c, g, k; 1:400), or RXR α (d, h, l; 1:800) and visualized with Texas Red as described in the Materials and Methods section. Arrowheads indicate nuclear staining in dermal fibroblasts (a), keratinocytes of the inner and outer root sheath (e, g, h), sebocytes (i, l), endothelial cells (m) and cells of the eccrine sweat gland (n). Scale bar: 30 μ m.

Expression patterns in psoriatic skin

Immunofluorescence imaging and scoring of RAR α , RAR γ and RXR α in non-lesional skin displayed no difference compared with healthy control skin (Figs 2 and 4; Table I).

In lesional psoriatic skin, all three receptor proteins were most conspicuous in the lower half of the epidermis (Figs 2, 3b and 4; Table I). This was especially true for RAR γ and RXR α , both of which showed an opposite distribution compared with normal and non-lesional skin, with only weak receptor expression in the superficial layer. Similarly, RAR α staining was sparse or absent at this level of lesional epidermis. In contrast, a normal distribution of RXR α was found in nickel-induced acute eczema, another inflammatory skin disease (data not shown).

The staining pattern of Ku-86, a non-receptor nuclear protein used as positive control, was similar

in lesional compared to non-lesional psoriatic and normal skin (Fig. 2).

DISCUSSION

Our results show for the first time that the various nuclear retinoid receptor proteins exhibit individual expression patterns in normal human epidermis. Thus, whereas RAR γ and RXR α were expressed throughout the entire epidermis, RAR α showed strongest staining in basal nuclei with decreasing intensity in suprabasal direction. The latter finding is in contrast with the weak *in situ* hybridization signal for RAR α previously demonstrated in basal keratinocytes (5), but supports our recent finding that RAR α mRNA and protein expression is higher in undifferentiated versus differentiated keratinocytes in culture (Karlsson et al., unpublished observations).

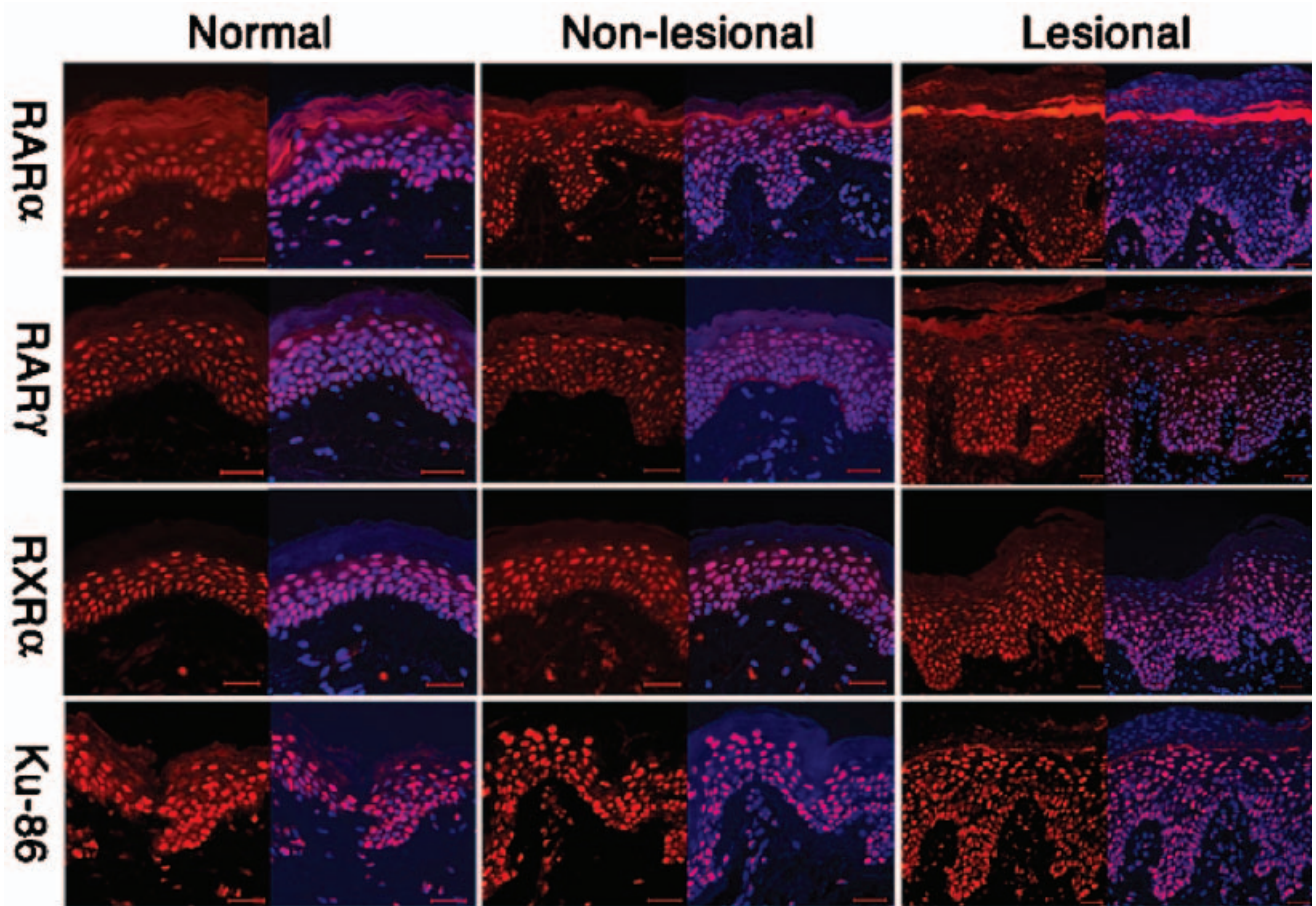


Fig. 2. Nuclear distribution of RAR α , RAR γ and RXR α in normal skin and psoriatic non-lesional and lesional skin. Cryostat sections were incubated with polyclonal antibodies against RAR α , RAR γ , RXR α or Ku-86 (diluted 1:200, 1:400, 1:800 and 1:200, respectively) and studied by *in situ* immunofluorescence as described in Materials and Methods. Images in pairs: left, receptor detection by Texas Red-staining technique; right, overlay image of Texas Red and Hoechst 33258 (nuclei, blue) staining. Co-localized staining appears in pink. The intense red band present in some images is due to non-specific Texas Red staining of stratum corneum (also seen in Fig. 1e and g). Representative results from one healthy donor and one patient. Note the attenuated staining in superficial psoriatic skin. Scale bar: 30 μ m.

In psoriatic skin, although the expression pattern was essentially normal in uninvolved skin, strikingly abnormal distribution of the three prominent receptors was found in lesional skin. In particular, RAR γ and RXR α showed markedly reduced or even absent staining in superficial epidermis, corresponding to the granular layer in normal skin. This coincides with our previous finding of $\geq 50\%$ reduced mRNA expression level for RAR α and RXR α in lesional versus non-lesional psoriatic skin (19). Other investigators have come to somewhat diverging conclusions. No differences in RAR α and RAR γ mRNA between normal and lesional skin were detected by Northern blot analysis (20), and immunohistochemical and immunoblotting studies have either shown normal (21) or only slightly reduced (22) RXR α expression in lesional psoriatic epidermis. The reason for this discrepancy is not known, but could result from using different sources of antibodies and different techniques for quantitation.

The reduced expression of retinoid receptors in superficial psoriatic lesions found in the present study

is most likely a consequence of low mRNA levels. However, increased degradation of the receptor proteins is another option. One of the hallmarks of psoriasis is infiltration of inflammatory cells in epidermis and hence increased levels of pro-inflammatory cytokines such as IFN γ (see ref. 23 and references therein). In adipocytes, IFN γ is known to induce ubiquitin-proteasome-mediated degradation of PPAR γ , another ligand-activated nuclear hormone receptor (24). This pathway has also been implicated as the major degradation route for retinoid receptors in keratinocytes (25). Our preliminary experiments indicate that IFN γ induces degradation of RAR γ and RXR α protein in keratinocytes *in vitro*, and that proteasome inhibitors efficiently block this process (Karlsson et al., unpublished observations). However, a recent immunohistochemical study showed that PPAR α and PPAR γ exhibit a strong nuclear localization in superficial psoriatic epidermis (26), i.e. opposite to the RAR/RXR expression.

Whatever the cause is of the low levels of retinoid

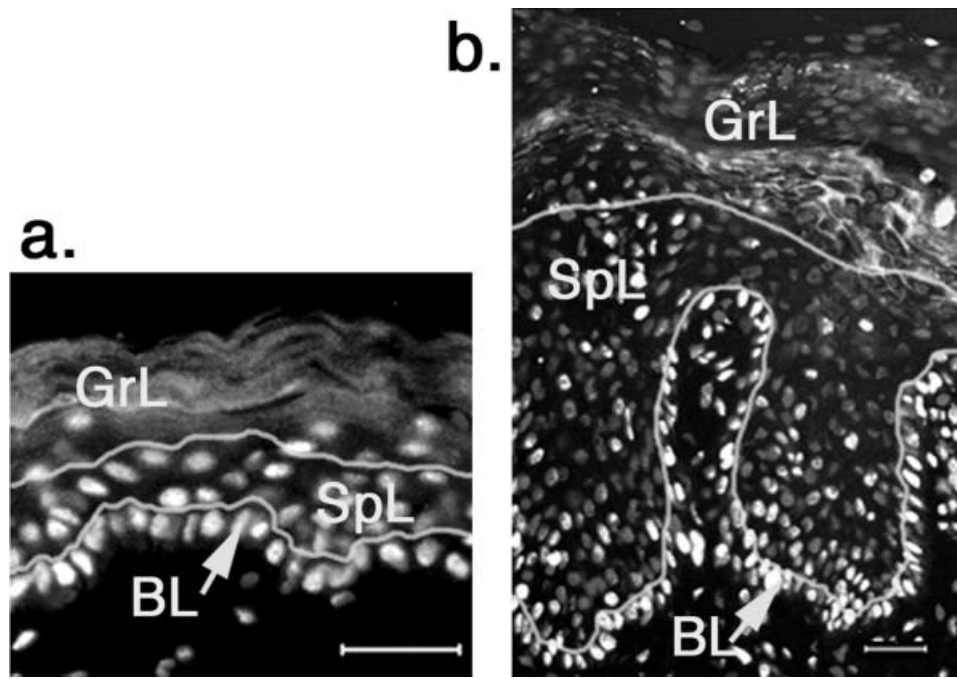


Fig. 3. Schematic illustration of the subdivision of normal or non-lesional skin (a) and lesional psoriatic skin (b) into three epidermal cell layers: BL, basal layer (arrows); SpL, spinous layer; and GrL, granular layer. Although the granular layer *per se* is often absent in psoriatic lesional epidermis, this designation was consistently used to describe the most superficial layer of viable epidermis. Hoechst 33258 staining. Scale bar: 30µm.

receptors in superficial psoriatic epidermis, this abnormality is likely to have biological consequences. One way of incriminating reduced RAR γ and RXR α expression in the pathogenesis relates to the AP-1 signalling pathway. Interference with this transcription factor explains many of the biological effects of retinoids (27), and a correct balance between AP-1 and RAR/RXR signalling is probably crucial for normal keratinocyte differentiation. AP-1 is regarded

as a key transcriptional regulator in the expression of several keratinocyte proteins associated with differentiation (e.g. keratin 1, transglutaminase 1, loricrin and involucrin) and proliferation (e.g. keratins 6 and 16), (see ref. 28 for review). In addition, AP-1 promotes the expression of genes involved in inflammation (23). Many of these genes are up-regulated in psoriatic lesions but can be repressed by anti-psoriatic retinoids such as RA and tazarotene (23). In theory, reduced

Table I. Semi-quantitative assessment of retinoid receptor expression in normal skin, non-lesional and lesional psoriatic skin

Receptor	Layer	Normal skin (n=9) Score ^a				Psoriatic non-lesional skin (n=9) Score ^a				Psoriatic lesional skin (n=9) Score ^a			
		0	1	2	3	0	1	2	3	0	1	2	3
RAR α	BL	0	0	1	8	0	0	2	7	0	0	5	4
	SpL	0	2	4	3	0	2	5	2	1	6	2	0
	GrL	0	2	5	2	0	1	8	0	5	4	0	0
RAR γ	BL	0	0	7	2	0	2	5	2	0	0	3	6
	SpL	0	0	4	5	0	0	4	5	0	0	3	6
	GrL	0	0	3	6	0	0	6	3	1	6	2	0
RXR α	BL	0	0	3	6	0	0	5	4	0	0	8	1
	SpL	0	0	2	7	0	0	1	8	0	1	1	7
	GrL	0	0	2	7	0	1	2	6	1	6	2	0

^aThree microscopists independently and blindly scored the immunofluorescence staining intensity from 0 to 3, and the median value was used to categorize the staining in different layers of each sample. Data represent number of samples falling into each staining category. BL, basal layer; SpL, spinous layer; GrL, granular layer (see Fig. 3 for explanation).

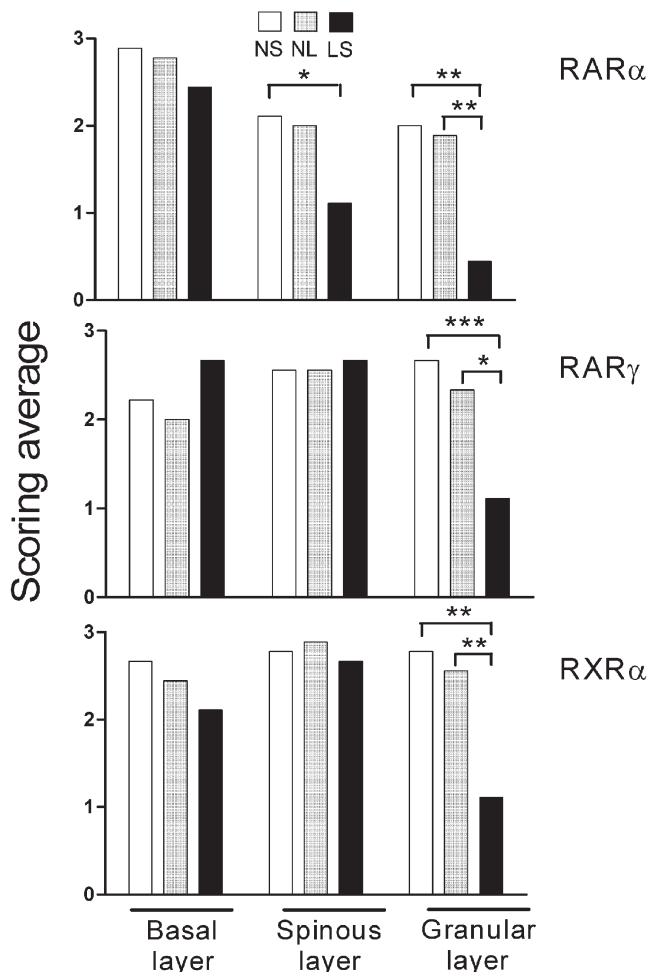


Fig. 4. Compilation of data from Table I showing the average intensity score for receptor expression in different layers of normal epidermis (NS, open bars), non-lesional psoriatic epidermis (NL, grey bars) and lesional psoriatic epidermis (LS, black bars) ($n=9$ for each receptor and type of specimen). Kruskal-Wallis analysis and Dunn's post test were performed where applicable * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

expression of retinoid receptors in psoriatic epidermis might allow for excessive AP-1 signalling and thus disturbed keratinocyte maturation.

Another theory incriminating reduced RAR/RXR levels in the pathogenesis of psoriasis involves the RA-inducible gene for CRABP2, which is strongly up-regulated in psoriatic lesions (29, 30). In contrast to normal skin, which responds to topical RA by increasing the CRABP2 expression, psoriatic epidermis fails to do so (29); it compares in this respect to cultured normal keratinocytes also expressing low amounts of nuclear retinoid receptors (31). In keeping with these findings, it was recently shown that topical RA fails to induce CRABP2 expression in the epidermis of dominant-negative RAR α mutants (15). Under basal conditions, these animals show an unexpected twofold increase in CRABP2 mRNA expression, thus resembling the situation in psoriatic

epidermis. Di et al. (31) have proposed a scenario in which RAR-deficient cells, by some yet unknown mechanism, 'sense' their unresponsiveness to RA and try to compensate for this by up-regulating the CRABP2 expression. CRABP2 has been identified in the nucleus of several cell types (29, 32) and over-expression of the CRABP2 protein enhances RA-mediated transcriptional activation (33, 34). A reduced expression of RAR/RXR in upper epidermis leading to increased CRABP2 expression could thus paradoxically result in increased retinoid signalling in psoriatic keratinocytes with well-known effects of hyperproliferation and de-differentiation.

In conclusion, lesional psoriatic epidermis shows abnormal distribution profiles for RAR α , RAR γ and RXR α characterized by suppressed protein expression in superficial epidermis. Speculatively, this leads to aberrant retinoid signalling and a dysregulation of differentiation in psoriatic epidermis.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Research Council (71X-07133), the Edvard Welander and Finsen foundations, and the Swedish Psoriasis foundation.

We thank I. Pihl-Lundin and E. Hagforsen for their assistance in evaluating the immunofluorescence images.

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