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Decreased mRNA Levels of Retinoic Acid Receptor α, Retinoid X Receptor α and Thyroid Hormone Receptor α in Lesional Psoriatic Skin

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Retinoic acid, vitamin D_3 and triiodothyronine regulate keratinocyte proliferation and differentiation – processes that are disturbed in psoriatic skin – via binding to nuclear receptors for retinoic acid (RAR-α,-γ), vitamin D_3 (VDR), thyroid hormone (TR-α,-β) plus the common heterodimer partners, the 9-cis-retinoic acid receptors (RXR-α,-β). By using a new quantitative real-time polymerase chain reaction assay, the expression of these receptors and three housekeeping genes (cyclophilin, GAPDH and β-actin) was studied in psoriatic skin. The expression of housekeeping genes was consistently 2.7–4.3 times higher in lesional than in non-lesional skin. When the β-actin expression was used to normalize the receptor mRNA values, the RARα, RXRα and TRα transcripts were found to be 58–75% lower in lesional vs. non-lesional skin and the RXRα : RARγ ratio was reduced from 3.2 to 1.5. Topical treatment for 4 days with 0.025% all-*trans*-retinoic acid or calcipotriol under occlusion did not normalize the altered mRNA expression of RARs, RXR and VDR in lesional skin. The results suggest that retinoid and thyroid hormone signalling is abnormal in lesional psoriatic skin, but how this relates to the pathogenesis of the disease is still unclear.

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Hormone-like compounds such as all-*trans*-retinoic acid (RA), 1,25-$(OH)_2$-vitamin D_3 (VD) and triiodothyronine can have a profound influence on keratinocytes both *in vitro* and *in vivo* (1–8). Generally, RA and triiodothyronine inhibit keratinocyte differentiation, whereas VD exerts a pro-differentiating effect. The cellular effects observed probably result from altered transcription of genes regulated by these compounds (9–11).

Psoriasis is characterized by epidermal hyperproliferation, abnormal keratinocyte differentiation and skin inflammation. Although topical RA treatment clearly affects many biochemical and morphological features of differentiating epithelia (12, 13), it is still doubtful whether it can abolish the psoriatic phenotype (14, 15). Indeed, certain synthetic retinoids, e.g. tazarotene (16), seem to do well topically, and calcipotriol (a synthetic VD analogue) has become a standard treatment of plaque psoriasis (17). Whether or not triiodothyronine analogues are also effective in psoriasis is still unclear. A recent clinical trial comparing topical triiodothyroacetic acid and a placebo cream showed no convincing effects after 2 months of treatment (unpublished observation), but synthetic triiodothyronine agonists and antagonists may be worth exploring.

To achieve transcriptional effects, all the aforementioned ligands must bind to their respective receptors, i.e. retinoic acid receptor (RAR) α, β and γ, vitamin D_3 receptor (VDR) and thyroid hormone receptors (TR) α and β. These ligand-receptor complexes recognize specific regulatory sequences in the promoter region of certain genes. A second class of retinoid receptors, RXRs, function as auxiliary heterodimerization partners for RARs, VDR, and TRs. The endogenous ligand for the RXRs has been identified as 9-*cis*-RA (18), but this receptor probably acts as a silent partner in human skin, i.e. without binding to its ligand (19, 20).

Although most of the nuclear receptors known so far have also been recognized in normal human skin (21–23), their putative role in the pathogenesis and treatment of skin disorders remains obscure. In particular, data on the expression level and distribution of the nuclear receptors in diseased skin are still lacking. However, new tools in quantitatively assaying mRNA, such as reverse transcriptase-polymerase chain reaction (RT-PCR) coupled to a probe-based detection of PCR products during their continuous accumulation (i.e. TaqMan assay), have improved sensitivity and specificity of gene expression analysis in small skin biopsies (24).

In the present study, we examined the mRNA levels of RARs, RXRα, VDR and TRs in psoriatic skin using the TaqMan assay. We compared the level of transcripts in non-lesional and lesional skin with that of three different housekeeping genes, and studied the influence of short-term occlusive RA and calcipotriol application on these parameters.

MATERIAL AND METHODS

Biological specimen

Six patients (five men and one woman, age range 34–59 years) with stable chronic plaque psoriasis were included in the study. No systemic or topical psoriasis therapy (excluding emollients) was allowed for at least 3 weeks prior to the study. The patients were healthy except for the skin disease. All patients gave their written consent in a protocol approved by the Ethics Committee of Uppsala University. Non-lesional and lesional skin samples were obtained by shave biopsy from the trunk as previously described (25). Biopsies of non-lesional skin typically consist of epidermis and a minor proportion ($\leq$20%) of papillary dermis (26).

Shave biopsies were also taken from lesional skin exposed to Daivonex™ cream (calcipotriol 50 μg/g, three patients) vs. its vehicle (kindly supplied by LEO Pharmaceutical Ltd, Ballerup, Denmark) and from skin exposed to all-*trans*-RA (0.025% RA in ethanol/propylene glycol solution [70/30 by vol.], six patients) vs. vehicle. The compounds were applied under plastic occlusion for 4 days as described (13). The patients had been previously untreated for 2 weeks and had never received Daivonex™, Tigason/Neotigason™ or any topical retinoids previously.

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RNA preparation and reversed transcription (RT) and RT-PCR

Total RNA was extracted from the skin biopsies and transcribed into cDNA as previously described (22). In short, samples were homogenized in UltraspecII (Biotecx Laboratories Inc., Houston, TX) using a Polytron, and total RNA was extracted. First-strand cDNA was synthesized from 3 μg of total RNA in a 30 μl reaction mixture using oligo-d$(T)_{15}$ as primer and M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD). The cDNA was diluted with 30 μl RNase-free dH_2O to obtain a concentration of 50 ng cDNA/μl. Ordinary RT-PCR of TR isoforms was performed with specific primers (see Table I) as described earlier (22).

Real-time polymerase chain reaction (PCR)

The PCR mixture included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.0 mM $MgCl_2$, 0.2 mM of each dNTP, and 1.25 U AmpliTaq Gold DNA polymerase (Perkin-Elmer, Sundbyberg, Sweden). Oligonucleotide PCR primers and TaqMan™ probes, which contained FAM as 5′-reporter and TAMRA as 3′-quencher, were designed using Primer-Express program (PE-Applied Biosystems, Foster City, CA) and synthesized by PE-Applied Biosystems (Warrington, UK). The sequences are outlined in Table I. Primers and TaqMan™ fluorogenic probes were added to a final concentration of 0.4 μM and 0.2 μM, respectively. Total PCR volume was 25 μl, including 1 μl of the RT reaction, which equals 50 ng total RNA. Duplicate reaction tubes were set up for each sample and transcript under investigation. The tubes were placed in an ABI Prism 7700 System programmed for 40 sequential cycles each comprising heating to 94 °C for 15 s followed by 60 °C for 30 s. A standard curve was generated by amplifying known amounts of a PCR-product (in duplicates) at the same time as the samples. Fig. 1 shows the linearity of the RT-PCR reaction when amplifying nuclear receptor standards using TaqMan assay. The linearity of the amplification of housekeeping gene standards was similar to that for the nuclear receptors (data not shown).

Statistical analysis

One-way ANOVA *t*-test was performed using the JMP software version 3.1 (Cary, NC).

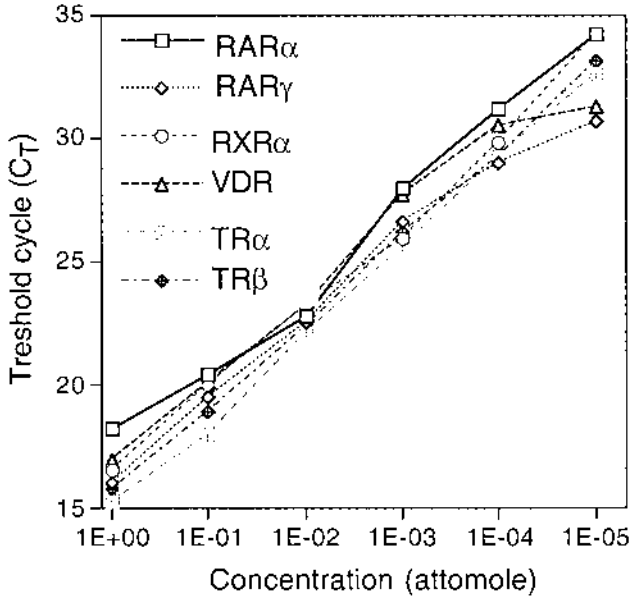

Fig. 1. The amplification of nuclear hormone receptors utilizing TaqMan is linear over a wide range of input molecules. Ten-fold serial dilutions of standards were prepared and amplified in duplicate using a sequence detector (TaqMan apparatus). Mean C_T values (threshold cycle) are plotted as a function of target copy number added to the reaction tube. Thus, the C_T values can be used to quantitate the input target number of unknown cDNA samples. (1E+00 denotes $10^\circ = 1$; 1E−01 denotes 0.1, etc.)

RESULTS

Nuclear receptor mRNA levels in psoriatic skin

Table II gives the number of transcripts encoding the nuclear receptors in non-lesional and lesional psoriatic skin. It is evident from the data that receptor mRNA constitutes only a minute fraction of total mRNA, and that the order of abundance of nuclear receptors in non-lesional skin is RXRα > RARγ > VDR > TRβ ≥ TRα > RARα. The expression level of RARγ, VDR and TRβ was twice as high in lesional as in non-lesional skin, whereas RXRα was similarly

Table I. *Sequence of polymerase chain reaction (PCR) primers and TaqMan™ Fluorogenic DNA probes used. The sequences are given in 5′-3′ orientation*

RT-PCR	*Forward*	*Reverse*	*Product size (bp)*
TRα1	GGTGACTGACCTCCGCATGATCG	GGGAGCTGAATCTATCCAAGGACGAG	259
TRα2	ACCGAAGCGGAATTCTCCATGC	GAGAGGCTGCATTGCCTGCCAGGTC	128
TRβ1	GCCTTACAGCTTGGGACAAACCGAAGC	CATTTCTTCTCCTCCGTTTGGAAGG	252
TRβ2	GCAAGAAATATATGAAGTGCACCC	CAGTGAAATATTCAGGTTGGCTGTA	262

TaqMan	*Forward*	*Reverse*	*TaqMan-probe*
GAPDH	TGGGCTACACTGAGCACCAG	CAGCGTCAAAGGTGGAGGAG	TGGTCTCCTCTGACTTCAACAGCGACAC
Cyclophilin	TGCTGGACCCAACACAAATG	TGCCATCCAACCACTCAGTC	TTCCCAGTTTTTCATCTGCACTGCCA
β-actin	CTGGCTGCTGACCGAGG	GAAGGTCTCAAACATGATCTGGGT	CCTGAACCCCAAGGCCAACCG
RARα	CTCCATGCCGCCTCTCAT	CGGCTGTCCGCTCAGAGT	CAGGCCCTCTGAGTTCTCCAACATTTCCT
RARγ	TGTGCGAAATGACCGGAAC	CTAACTGAGGGCTCAGCTCATAGC	CAGGTGACCCTTCTTCCTTCACCTCTTTCTTC
RXRα	CCCTGTCACCAACATTTGCC	AGAAGTGTGGGATCCGCTTG	AGCAGCCGACAAACAGCTTTTCACCC
VDR	CCTTCACCATGGACGACATG	TCACGTCACTGACGCGGTAC	CCTGGACCTGTGGCAACCAAGACTACA
TRα	GACTGACCTCCGCATGATCG	GAAGAGTTCGGTGGGGCAC	ACGCCAGCCGCTTCCTCCACAT
TRβ	AGAGAGGCGCAGCACGTT	TGAGCTAGTCCAAGTGGTCTGG	AAAAATGAACAGTCGTCGCCACATCTCA
CRABPII	AGCAGAAGCTCCTGAAGGGA	CCCATCGTTGGTCAGTTCTCT	AGGGCCCCAAGACCTCGTGGAC

Table II. *Amount of nuclear receptor mRNA transcripts (relative to total RNA) in non-lesional and lesional psoriatic skin. Values denote mean ± SEM (n = 6)*

Transcript	Non-lesional (10^{-21} mole/100 ng total RNA)	Lesional (10^{-21} mole/100 ng total RNA)
RARα	0.33 ± 0.05	0.37 ± 0.03
RARγ	5.8 ± 1.8	12.6 ± 2.1
RXRα	19.4 ± 1.55	18.7 ± 3.8
VDR	2.80 ± 0.73	5.3 ± 0.76
TRα	1.50 ± 0.26	0.99 ± 0.16
TRβ	1.7 ± 0.16	3.7 ± 0.53

expressed in both types of skin. Consequently, the RXRα: RARγ and RXRα: VDR ratios were much lower in lesional skin (1.5 and 3.5, respectively) compared to non-lesional skin (3.3 and 6.9). Analogously, the TRα: TRβ ratio was 0.3 in lesional skin as opposed to 0.9 in non-lesional skin. Although expressed weakly, all isoforms of TR (α1, α2, β1 and β2) were demonstrable by RT-PCR (Fig. 2).

The above-mentioned figures of receptor transcripts in lesional vs. non-lesional skin have to be regarded with circumspect, because the values are expressed as mole/100 ng total RNA and will thus reciprocally increase when keratins and other major gene products are down-regulated, such as in psoriatic lesions (27). To try to circumvent this pitfall, we also studied in parallel experiments the expression of several housekeeping genes in uninvolved and lesional skin.

Variable expression of housekeeping genes in psoriatic skin

Three housekeeping genes – cyclophilin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin – were studied by quantitative real-time PCR. Fig. 3 shows that the expression of all three genes was higher in lesional as compared to non-lesional skin. The mean lesional/non-lesional ratios for cyclophilin, GAPDH and β-actin were 2.7, 4.4 and 2.9, respectively. In both types of epidermis the expression of GAPDH and β-actin exhibited the smallest individual variations, and were consequently used as internal standards in subsequent analyses.

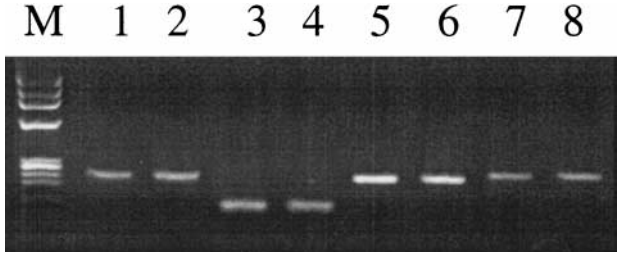

Fig. 2. All isoforms of thyroid hormone receptors (TR) are expressed in psoriatic skin. RT-PCR was used for detection of different isoforms of TRs. cDNA was amplified for 38 cycles with specific primers for the isoforms (see Table II). The expression of TRα1 (lanes 1, 2), TRα2 (lanes 3, 4), TRβ1 (lanes 5, 6) and TRβ2 (lanes 7, 8) in non-lesional (lanes 1, 3, 5, 7) and lesional psoriatic skin (lanes 2, 4, 6, 8) is shown. M denotes molecular weight marker (HaeIII digested ϕX174). Similar results were obtained in five other patients studied (data not shown).

Adjusted expressions of nuclear receptors in lesional and non-lesional psoriatic skin

Fig. 4 shows the expression of RARα, RARγ, RXRα, VDR, TRα and TRβ in psoriatic skin normalized to the expression of β-actin or GAPDH. Very similar results were found when normalizing the data to one or the other of these genes. Contrary to the absolute RARα value (see Table II), the adjusted expression of RARα was significantly ($p < 0.001$) reduced in lesional skin as compared to non-lesional skin. The RXRα and TRα expressions were also significantly lower in lesional skin. In contrast, the adjusted RARγ expression did not differ significantly in the two types of psoriatic skin, thus confirming previous results using other methods (21). Likewise, the adjusted expression of VDR was similar in lesional and non-lesional skin, whereas the TRβ results varied depending on which housekeeping gene was used as internal standard.

Effect of topical retinoic acid and calcipotriol on the expression of RARs, RXRα and VDR lesional psoriatic skin

Fig. 5 shows the changes in the adjusted expressions of RARα, RARγ, RXRα and VDR observed in lesional skin after 4 days of treatment with 0.025% RA or calcipotriol under occlusion. In these experiments, GAPDH alone was used for normalization of data, because calcipotriol markedly reduced the β-actin expression (minus 57 – 90%). It can be seen from the figure that, although there was a tendency to increased values for RARα, RARγ and VDR in RA-treated skin and for RARγ in calcipotriol-treated skin, the differences compared to vehicle-treated skin were not statistically significant.

DISCUSSION

Real-time PCR assay (TaqMan) is a sensitive and relatively new method for quantitative analysis of PCR products. It enables high specificity, since a fluorogenic probe is hybridized to the PCR product after each completed PCR cycle, and does not require any post-PCR manipulations. However, since quantitation of transcripts is based on a fixed amount (100 ng) of cDNA and not on a specified number of cells, it must be understood that all concentration values will be reciprocally influenced by the combined expression of other genes. With this limitation in mind, our TaqMan results showed similar patterns of RARα, RARγ, RXRα, VDR, TRα and TRβ expression in uninvolved psoriatic skin and normal human skin (unpublished observation), but disclosed several clear-cut differences between lesional and non-lesional psoriatic skin (Table II). However, a more refined comparison of the transcript levels in two heterogeneous tissues, such as lesional and non-lesional psoriatic skin, demands that the values are normalized to the expression of a housekeeping gene, i.e. a gene which possesses "stable" transcription. Three candidate genes were tested, i.e. β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclophilin. Surprisingly, all these genes showed a higher expression level in lesional skin as compared to uninvolved skin. This conflicts with a previous report claiming no difference in β-actin mRNA level in non-lesional vs. lesional psoriatic skin when using RT-PCR (28) but fits with a recent in situ hybridization

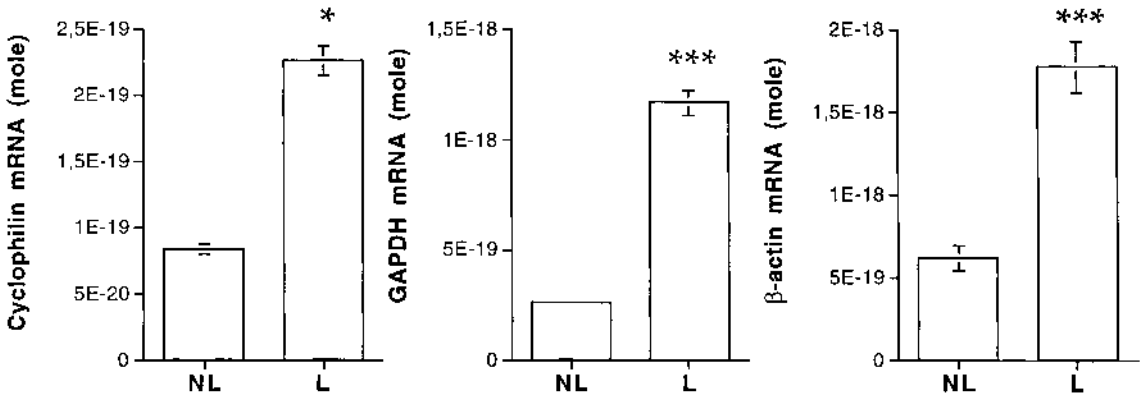

Fig. 3. The mRNA expression of housekeeping genes (cyclophilin, GAPDH and β-actin) was determined in non-lesional (NL) and lesional (L) psoriatic skin by real-time PCR of cDNA derived from total RNA (100 ng). Results shown are means ± SEM ($n = 6$).

study of GAPDH and polyA$^+$ mRNA in psoriatic skin (44). Out of the three genes tested by us, β-actin and GAPDH appeared to be the most useful ones for normalizing receptor expression in lesional skin.

The cause of psoriasis remains unknown but abnormal expression of a ligand-dependent nuclear transcription factor might be a contributing factor. In a report by Noji et al. (29) it was shown that the RARα expression was similar in normal and psoriatic skin using *in situ* hybridization. Yet, others have reported a slightly reduced expression of RXRα in lesional as compared to non-lesional skin using either immunohistochemistry or immunoblotting (30, 31). Our study using real-time PCR and normalization to β-actin expression showed that the RARα and RXRα expressions were decreased in lesional as compared to non-lesional skin. It is doubtful if a reduced expression of RARα substantially affects retinoid signalling in psoriatic skin, since the mRNA level of this receptor is very low in normal human epidermis (32). However, the markedly lower expression of RXRα in psoriatic skin is intriguing, because this receptor type predominates in human keratinocytes (32) and has the ability to heterodimerize with other class II receptors (9). Moreover, the reduced expression of RXRα in lesional skin (implying a 2-fold increase in the RARγ:RXRα ratio) suggests that RARγ-selective agonists may have therapeutic advantages relative to other retinoids used in psoriasis treatment.

All-*trans*-RA is a pan-RAR agonist, which alters keratinocyte proliferation and differentiation. It also reduces the expression of RARγ when applied topically to normal skin (21, 33). The fact that we did not observe any significant

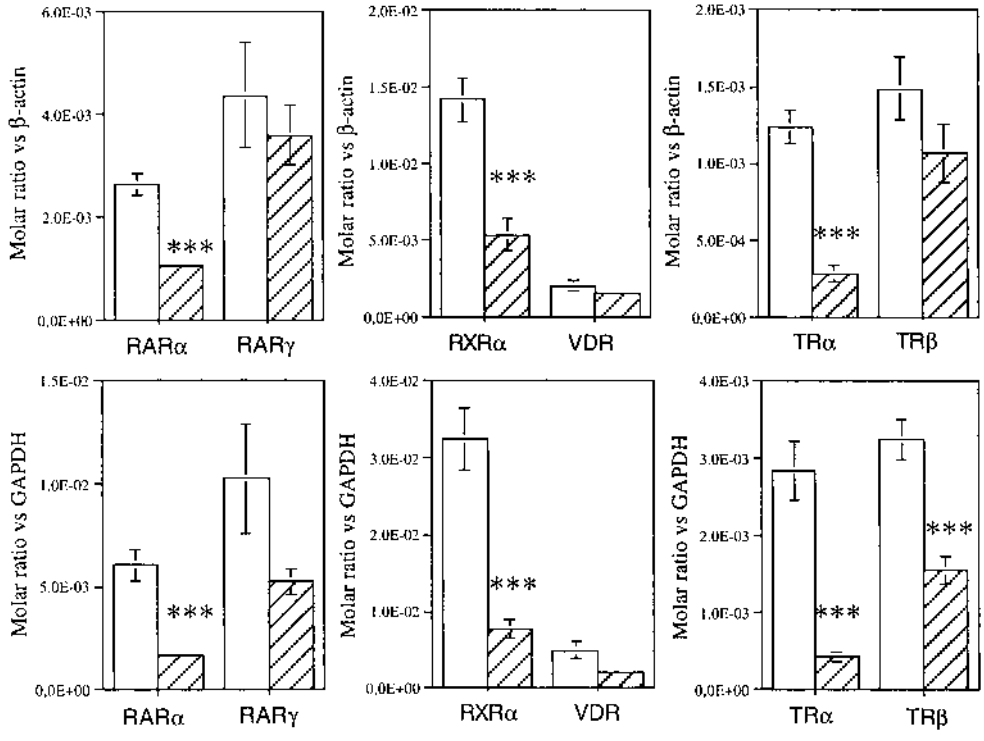

Fig. 4. The β-actin- and GAPDH-adjusted expression of RARα, RARγ, RXRα, VDR, TRα and TRβ in lesional (▨) and non-lesional (□) psoriatic skin shown as the molar ratio to β-actin (upper panel) or GAPDH (lower panel). One-way ANOVA *t*-test was performed; ***$p < 0.001$. Results shown are means ± SEM ($n = 6$).

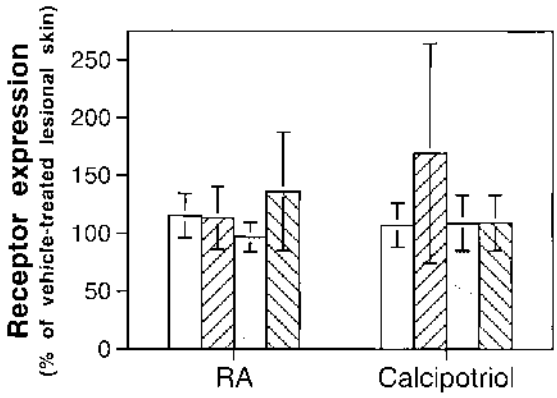

Fig. 5. The expression of RARα (□), RARγ (▨), RXRα (▤) and VDR (▧) in lesional psoriatic skin after 4 days of occlusive treatment with RA ($n=6$) or calcipotriol ($n=3$) (means ± SEM in percent of vehicle-treated skin; all values are related to GAPDH – see text). One-way ANOVA *t*-test showed no significant changes.

changes in the receptor expression in lesional skin treated for 4 days with RA is surprising, and contrasts the *decreased* expression of a retinoid-induced gene, cellular retinoic acid-binding protein II, seen in identically treated samples (unpublished observation).

Vitamin D analogues are commonly used in the treatment of psoriasis, although their mechanism of action is far from being understood (17). In primary hyperparathyroidism, another type of vitamin D_3-responsive disease, a VDR gene polymorphism has recently been found that probably affects the half-life of the VDR transcript (34). However, this type of polymorphism has recently been excluded in psoriasis (35). Previous studies of the VDR expression in psoriatic skin, using RT-PCR, *in situ* hybridization or immunohistochemical methods, have shown partially contradictory results (36, 37). Using quantitative real-time PCR and β-actin normalization, we found the expression of VDR to be virtually identical in non-lesional and lesional psoriatic skin, thus arguing against a primary defect of VD signalling in psoriasis. Notably, whereas vitamin D_3 is known to induce expression of VDR in cultured keratinocytes (38), we did not find any changes in the expression of VDR in psoriatic skin after 4 days of calcipotriol exposure.

Previous information about the TR expression in human skin is sparse (22). However, thyroid hormone is important during epidermal barrier ontogenesis (39) and is known to regulate the expression of several cytokeratins (40). Interestingly, certain subtypes of psoriasis are associated with thyroid dysfunction (41, 42), but whether the endocrine dysfunction affects thyroid hormone signalling in psoriatic skin is not known. In the present study, we found that transcripts for all TR isoforms were present in psoriatic skin, as previously shown in cardiac cells (43), and the TRα expression was specifically down-regulated in lesional skin. How this affects thyroid hormone signalling is difficult to predict, but the concomitant down-regulation of the heterodimer partner RXRα (see above) may exert a synergistic effect. Whether or not thyroid hormone agonists and antagonists specifically binding to TR-α or TR-β can be used in the treatment of psoriasis remains to be investigated.

In summary, our data demonstrate several abnormalities in the mRNA expression of nuclear class II receptors in lesional psoriatic skin. However, these were only evident when data were normalized to the expression of housekeeping genes. Incidentally, the variation in the expression of housekeeping genes in lesional and non-lesional skin is against the dogma that these genes are more or less constantly expressed in all types of tissues. Although, no significant changes in the receptor expression were elucidated by short-term topical RA or calcipotriol treatment of psoriatic lesions, ongoing studies will hopefully clarify whether the altered receptor expression plays a role in the pathophysiology of psoriasis.

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