

The effect of pimecrolimus on expression of genes associated with skin barrier dysfunction in atopic dermatitis skin lesions

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Abstract: The mechanism of action of pimecrolimus (PIM) on atopic lesions is still under consideration. Thus far, we have evidence of its anti-inflammatory and immunomodulatory activity, and recent papers focus on its effect on epidermal barrier function. This study analysed changes in the expression of genes associated with skin barrier dysfunction in atopic dermatitis (AD) skin lesions after 2 weeks of exposure to PIM 1% cream. A real-time quantitative PCR analysis of selected epidermal differentiation complex genes and three alternative pathway keratins was performed in skin biopsies from 11 individuals with AD before and after PIM exposure. The real-time quantitative PCR analysis was compared to non-lesional skin in the same patients. Involucrin, a small proline-rich region (SPRR) 2C gene, and alternative pathway keratin 16 showed significant over-expression in lesional skin followed by significant decrease after PIM therapy. The SPRR1A

gene, S100A9, and keratin 6A were also increased; however, the decrease after PIM treatment was not significant. The changes in S100 A2, A7 and A8 followed a similar course with borderline significance. SPRR4 had a significant decrease in expression in lesional versus non-lesional skin, which persisted after PIM treatment. No significant changes were detected in mRNA expression levels of filaggrin and loricrin. Our results suggest that PIM can be effective in restoring the epidermal barrier in patients with AD at least in part by its impact on expression of genes, which are important for the normal barrier function of skin.

Key words: atopic dermatitis – epidermal differentiation complex – gene expression – pimecrolimus

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Introduction

Pimecrolimus (PIM) is a calcineurin inhibitor and topical agent that has successfully been used in the treatment and prevention of recurrence in patients with atopic dermatitis (AD). If PIM is applied at the first sign of AD exacerbation, it prevents flare progression, modifying the course of AD (1–5). This drug has been widely used clinically for its efficacy but also safety of short-term, long-term and intermittent management of AD. PIM does not induce the skin atrophy and does not increase the incidence of any skin infection in comparison with topical corticosteroids (6–9). Treatment with PIM may be particularly useful for sensitive areas of the skin such as the face, neck and genital region, where the risk for side effects of topical corticosteroids is high (10). PIM cream is indicated in mild to moderate AD, but we know that treatment with PIM may be effective in patients with moderate to severe AD (11). However, the mechanism of action of PIM in AD is still under exploration. Thus far, we have evidence of its anti-inflammatory and immunomodulatory activity. PIM, like tacrolimus and cyclosporin A, impairs T cell signal transduction pathways by blocking calcineurin-induced dephosphorylation and the subsequent nuclear translocation of the nuclear factor of activated T cells (NF-AT), which causes the inhibition of inflammatory cytokine synthesis (12). The increase or only slight decrease in antimicrobial peptides in the skin by PIM has been hypothesized as its beneficial mechanism (12,13), while Jensen et al. (13)

showed recently strong downregulation of antimicrobial peptides after exposure to topical glucocorticoids.

New insights into the pathophysiology of AD indicate that disturbed epidermal barrier can initiate an inflammation modifying innate and adaptive immune response of skin (14). Epidermal differentiation complex (EDC) genes, which are crucial for the proper functioning of the epidermal barrier, play an important role in the development of AD (15). Their expression or variants are known to be altered in AD skin (14–18).

Less is known about the mechanisms of the restoration of the epidermal barrier in lesional atopic skin. The relationship among proteins of the keratin cytoskeleton [i.e. filaggrin (FLG), involucrin (IVL) and loricrin (LOR)], intercellular lipids (i.e. ceramides) and epidermal proteases is essential for the restoration of normal skin barrier function (19). Immunohistochemistry revealed that the disrupted epidermal expression of IVL, LOR, FLG and keratins KRT5, KRT10, KRT14 and KRT16 was nearly normalized after PIM treatment. Normal lipid bilayers, the most important signs of an intact skin barrier, were detected. Epidermal expression of KRT17 remained disrupted (8). Until now, we do not know whether PIM directly affects the epidermal differentiation (at mRNA level) or this is a consequence of PIM anti-inflammatory and immunomodulating properties.

Thus, the aim of our study was to analyse skin gene expression changes after 2 weeks of exposure to PIM 1% cream. The gene

expression profile of the lesional AD skin before and after PIM treatment was compared to the gene expression data collected in non-lesional skin of the same patients with AD. The following groups of genes, selected on the basis of earlier microarray and QPCR studies (16,18), were analysed for their mRNA level changes:

1 Involucrin, LOR, FLG and three small proline-rich region (SPRR1A, SPRR2C and SPRR4) members of the family of short tandem-repeat genes essential to keratinocyte differentiation and cornified envelope (CE) formation;

2 S100 calcium-binding A2, A7, A8 and A9 genes, which are characterized as both EDC genes and innate immunity-controlling genes (especially S100 A7–9);

3 Alternative keratins KRT 6A, 6B and 16, which are characteristic of lesional AD skin and represent the consequence of stress-induced alteration of keratinization.

Methods

Patients

Eleven patients (five women and six men; age 21–48 years, average–33.6 years) with moderate to severe AD were enrolled. The diagnosis of AD was based on the criteria of Hanifin and Rajka (20). The severity of AD was evaluated with a classic SCORing Atopic Dermatitis (SCORAD) according to the European Task Force on AD (21). The SCORAD index included three aspects: extent of disease, disease severity and subjective symptoms. The efficacy of PIM was determined in the representative lesional area of the upper limb by using the partial Eczema Area and Severity Index (pEASI) (8). The change in SCORAD and pEASI was evaluated before and after 2 weeks of PIM treatment.

Therapy with systemic glucocorticoids or UV was stopped for at least 2 months before the study; therapy with topical glucocorticoids and non-steroid immunosuppressants was stopped at 2 weeks. Emollients containing active ingredients, such as urea, were not allowed on study areas. Informed consent was obtained from all patients before enrolment.

Pimecrolimus treatment

Pimecrolimus 1% cream was applied twice daily to all lesional areas including study areas on the upper limb for 2 weeks.

Skin biopsies

Three 4–5 mm skin punch biopsies from representative areas were immediately submerged in of RNA-later solution (Qiagen, Hilden, Germany) and frozen at –80°C for future RNA isolation. Before starting PIM treatment, two samples were taken from one AD lesion and one contralateral non-lesional skin for comparison. The third skin biopsy was taken after 2 weeks of PIM treatment from the same lesion.

RNA isolation, cDNA synthesis and quantitative real-time reverse transcription PCR (QPCR)

As previously described (18), total RNA was extracted using the RNeasy Mini kit (Qiagen), cDNA was synthesized using an Omniscript kit (Qiagen), and the QPCR assay (Roche, Basel, Switzerland) was performed with intron-spanning amplicons using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) in duplicates. Stratagene Human Reference RNA and normal skin RNA were used for standard curves, and the results were normalized against two genes, HADHA and EIF5 (chosen from a larger number by geNorm software-supported analysis).

Statistical analysis

Expression of the investigated genes was first analysed by Friedman's ANOVA test comparison of all three skin biopsies. A *P*-value below 0.1 indicated that the changes were at least borderline significant. *Post hoc* analysis between group comparisons was performed using the paired Wilcoxon test for the comparison of lesional versus non-lesional skin, pretreatment versus post-treatment of lesional skin and post-treatment of lesional versus non-lesional skin.

Results

Two weeks of treatment with PIM led to significant clinical improvement using the SCORAD and pEASI (Wilcoxon test: *P* < 0.05). The pEASI scores of study lesional area decreased by 3–6 points (on a 0–12 scale) (Table S1). PIM was well tolerated, without any significant clinical side effects.

The results of the gene expression study are described below.

Alternative keratin genes

The difference in Friedman's ANOVA was significant for all three of the alternative keratins investigated (Table S2). Two of the three alternative pathway keratinization genes (KRT 6A and 16) demonstrated significantly increased expression in lesional compared to non-lesional skin (Wilcoxon test: *P* < 0.05). The subsequent decrease after 2 weeks of PIM treatment was significant for keratin 16 and 6B, although the individual responses were variable (Fig. 1) and did not correlate well with the clinical response. For KRT16, the difference between treated lesional and non-lesional skin continued to be significant. For KRT 6B, the increase in lesional skin, when compared to non-lesional skin, was not significant using the *post hoc* Wilcoxon test, but a significant decrease (Wilcoxon test: *P* < 0.05) did occur after PIM treatment.

Epidermal differentiation complex genes

Involucrin, the early keratinocyte differentiation marker, demonstrated a significant increase in lesional AD skin compared to non-lesional skin. This increase in gene expression normalized after PIM treatment. The normalization was observed in 6/11 patients, with a >50% decrease in pretreatment values (Fig. 2). However, no correlation with either the degree of clinical response or alternative keratin gene expression was noted. The rise in IVL expression was accompanied by an increase in two of the three investigated SPRR genes. SPRR1A and 2C were significantly increased in lesional skin. The decrease in SPRR2C expression after 2 weeks of PIM treatment was significant, with >50% reductions seen in five patients (Fig. 2). There was a decrease in SPRR1A, but this was not significant. The third gene, SPRR4, had a significant decrease in expression in lesional versus non-lesional AD skin, which persisted after 2 weeks of PIM treatment. Neither FLG nor LOR had a significant change in expression after 2 weeks of PIM treatment (Table S2).

The S100 gene family was represented by four genes: S100A2, A7, A8 and A9. Their expression was increased in lesional skin, with the most distinct increase seen for S100A7–9. The increase in the expression of S100A9 was significant when using the Friedman's ANOVA test. The decrease in expression after PIM, although noticeable (median levels before and after were 83.97 and 11.48, respectively), was not significant in the *post hoc* Wilcoxon test. Nevertheless, the average post-treatment S100A9 level did not differ significantly from the non-lesional AD skin (11.48 vs 6.15, respectively). Changes for other investigated S100 genes

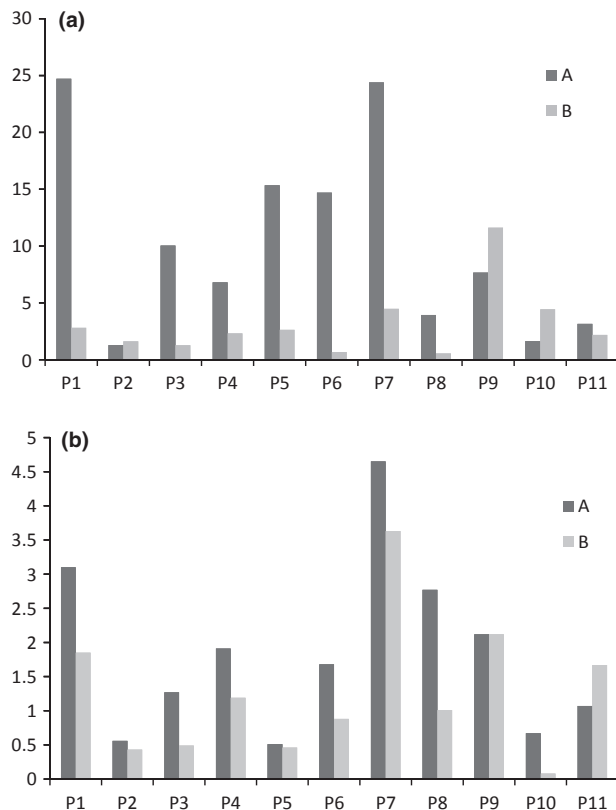


Figure 1. KRT16 (a) and KRT6B (b) gene expression in lesional skin before (A) and after (B) 2 weeks of pimecrolimus treatment from individuals (P1-P11) ordered according to treatment outcome as shown in Table S1. On the Y-axis, relative expression changes are given (in comparison with non-lesional skin).

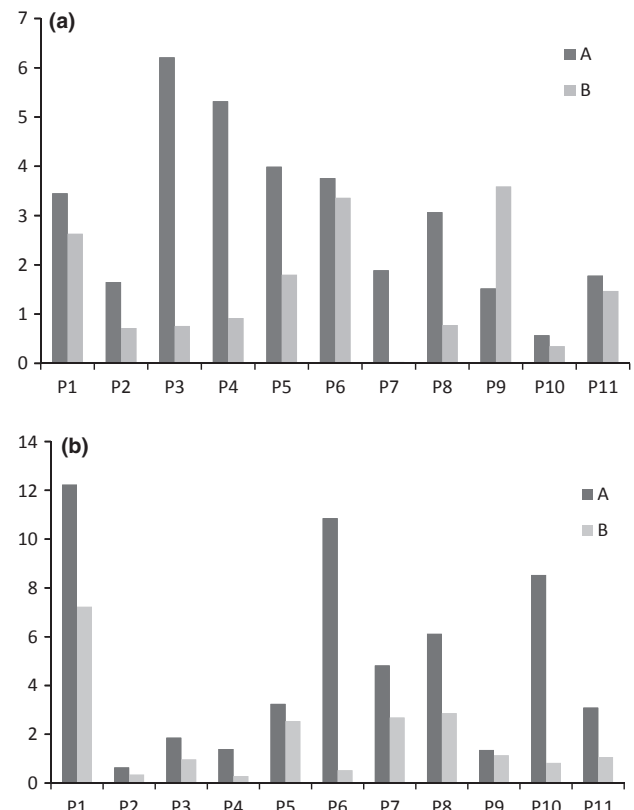


Figure 2. Involucrin (a) and SPRR2C (b) gene expression in lesional skin before (A) and after (B) 2 weeks of pimecrolimus treatment from individuals (P1-P11) ordered according to treatment outcome as shown in Table S1. On the Y-axis, relative expression changes are given (in comparison to non-lesional skin).

were only borderline significant with the Friedman's ANOVA. S100A2 and S100A8 RNA levels normalized after PIM treatment (Table S2).

Discussion

Restoration of the epidermal barrier is an important objective of AD treatment. Topical corticosteroids are effective in AD treatment, but their long-term use is limited by local and systemic adverse events and increasing evidence that they do not address the disturbed epidermal barrier (14). In contrast to glucocorticoids, PIM treatment demonstrated a favourable safety profile even for long-term therapy or the application on sensitive skin (including face and neck) (6,22,23). Long-term, intermittent preventive PIM treatment is safe and superior to conventional use of topical medications in infants, children and adults (2–5). PIM is able to restore disrupted epidermal differentiation in AD skin demonstrated by the clinical observations (1,8).

Our results provide a description of the genes, which are essential for CE formation and for epidermal barrier function, whose expression was changed in lesional skin. PIM regulated expression of these genes to the values observed in non-lesional skin suggesting improved barrier function. These findings have to be confirmed, but these data harmonize with the clinical efficacy and safety of PIM (1,6,8,23). We did not monitor the parameters relevant for the barrier function such as skin hydration and transepidermal water loss. Thus, it is difficult to interpret the lack of

significant correlation between the expression of barrier-associated genes and the clinical outcome measured by SCORAD or pEASI and to conclude on improved barrier function. However, the notion that PIM itself can regulate expression of genes, which are essential for the normal barrier function of skin, also has important implications, suggesting a new mechanism of PIM action (others than NFAT inhibition) and providing a rationale for continuation of such research. This helps to understand the consequences of the transcriptional changes in human skin which accompany chronic AD lesion and to interpret the observed effect of PIM in this context.

Our study shows that the increase in IVL, an early keratinocyte differentiation marker significantly over-expressed in chronic AD lesions, resolves briefly even after a short topical PIM treatment. This finding is contrary to the effect seen on the protein level, as judged by immunohistochemistry after 3 weeks of PIM treatment (8). Although the late keratinocyte differentiation markers, FLG and LOR, did not express significant changes in their mRNA level in our study, they were increased in many patients after PIM treatment, similar to the effects seen in immunohistochemical studies (8). The increase in SPRR 2C expression in lesional AD skin, a transcript related to LOR and cross-linked with LOR on protein level, was reduced after PIM treatment, although no complete normalization of its level was achieved. SPRR1A, another gene in this family known to be up-regulated

in lesional skin (16,18), also decreased after PIM, but not significantly. Notably, median SPRR1A expression in PIM-treated lesional skin was similar to the intact non-lesional AD skin (Table S2). Another gene in this family, SPRR4, was decreased in chronic AD lesions, as shown by our study for the first time. This decrease was not reversed with PIM. From the study of Cabral et al. (24,25), it is known that the SPRR4 protein, inducible mainly by UV irradiation, is practically absent in normal skin. Thus, its PIM-induced decrease may be interpreted as an additional index of PIM-induced normalization of CE gene expression.

In the lesional AD skin, the changes in IVL and SPRR were accompanied by an increase in mRNA level for the S100 genes, although this was only significant for S100A9. The S100A9 expression level was nearly normalized after PIM, but the difference did not reach the level of statistical significance with the Wilcoxon test.

S100 genes constitute a large family, encoding multifunctional molecules containing two EF hand motifs and stimulated by cellular calcium increase. They are injury-induced molecules essential for the formation of the epidermis and for its role in innate immunity, inflammation and even cancer (26,27). S100 genes are also involved in keratinocyte cell cycle progression, cell differentiation and cytoskeletal-membrane interactions. However, the interpretation of their skin expression levels is difficult because they are also abundantly expressed in neutrophils and macrophages (28). For a long period of time, S100A7 (psoriasin) was considered the prototype for psoriasis; however, it is now clear that it is also over-expressed in AD, both on an RNA and protein level (17).

In our study, the most pronounced changes were observed for S100A9. S100A8/A9 increase in expression was observed in hyperproliferative or cytokine-stimulated keratinocytes and, by a positive feedback loop, potentiated their growth (29) and cytokine release (30). Simultaneously, the dimer of S100A8 and A9, as the endogenous ligand of toll-like receptor TLR4, can stimulate innate immunity mechanisms (27,31) and protect skin from a broad spectrum of micro-organisms (28,32). This may be especially important in AD where the induction of human beta defensins is impaired (15). In this context, our finding of quick diminishment of AD-related increases of S100 A8/9 transcripts after PIM treatment may be considered of equivocal significance. On the one hand, S100A8/9 decrease may be interpreted as proof of normalization of EDC gene expression; on the other hand, the innate immunity-related functions of keratinocytes may be impaired before the epidermal barrier is fully restored.

For comparison and timing of changes in expression of EDC genes, we decided to analyse expression gene changes in alternative pathway keratins because the increase in their expression was prominent in the previous microarray-based study (16). For KRT6A and KRT16, a decrease was observed after PIM (significant for KRT 16), but their expression levels were still high in non-lesional skin. In 5/11 patients, the drop exceeded 50% of the lesional skin value, and the median was still >2 times higher than in non-lesional skin. We have to consider that 2 weeks' observation may be too short to fully characterize the effect of PIM on

AD skin gene expression, which is demonstrated by the observation that not all patients experienced a clinically significant improvement (Table S1). In skin biopsies of patients with psoriasis receiving oral PIM, a significant reduction in epidermal thickness, inflammatory infiltrate, parakeratosis and loss of KRT16 staining was noted after 4 weeks of treatment (33). Jensen et al. (8) observed by immunohistochemistry a reduction or absence of KRT16 after 3 weeks of PIM treatment. After PIM, full normalization of gene expression was seen only for KRT6B, which demonstrated the smallest increase in lesional skin. The significant decrease in KRT6B expression after PIM may reflect the beginning of keratin cytoskeleton repair. A similar effect was reported in the healing wound (34). However, keratin 6 is only a broad indicator of perturbed epidermal biology and not hyperproliferation *per se* (34,35). In conclusion, our results suggest that PIM can be effective in restoring the epidermal barrier in patients with AD at least in part by its impact on expression of genes, which are important for the normal barrier function of skin. This effect may explain the clinical observation that long-term, intermittent treatment with topical PIM does not lead to adverse events characteristic of topical glucocorticoids (striae, telangiectasia, atrophy and infection skin).

We show that the profound changes of EDC genes in lesional AD skin, especially IVL, SPRR1A and 2C, partially or completely resolve after topical PIM 1% cream treatment. The reaction of SPRR2C is slower than that of IVL and SPRR1A and more closely parallels the slow diminishment of the alternative keratinization pathway. Also, a similar trend in changes is observed for S100 genes, with the most distinct effect on S100A9 mRNA level. The involvement of SPRR proteins and S100 in innate immunity suggests the need to intensity studies on innate immunity changes after PIM administration. It would be important to determine protein expression levels and protein localization changes (transcript levels do not necessarily translate into changes in protein levels), for example, by performing immunostaining, and correlate these results with parameters relevant for the barrier function.

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Conflict of interest

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical evaluation of the response to pimecrolimus based on SCORAD and pEASI (order of patients according to the efficacy of treatment measured by Δ pEASI).

Table S2. Comparison of gene expression in lesional AD skin investigated before and after PIM treatment compared to the non-lesional skin.

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Table 1 is moved into e-supplement

TABLE 1.

Clinical evaluation of the response to pimecrolimus based on SCORAD and pEASI (order of patients according to the efficacy of treatment measured by Δ pEASI).

Patient	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	
<i>pEASI</i>												
Before treatment	8	8	10	7	9	5	9	10	10	10	6	8,4±1,7
After treatment	2	2	4	2	4	1	5	6	7	7	3	3,9 ±2,1
<i>SCORAD</i>												
Before treatment	46	31	72	48	68	24	53	75	80	72	35	54.9±19.6
After treatment	15	10	64	18	55	11	42	68	70	64	30	40.6±24.5

Table 2 is moved into e-supplement

TABLE 2. Comparison of gene expression in lesional AD skin investigated before and after PIM treatment compared to the non-lesional skin.

* significant difference in comparison of lesional to non-lesional AD skin by paired Wilcoxon test ($p < 0.05$)

** significant difference in comparison of lesional skin treated with pimecrolimus to non-lesional AD skin by paired Wilcoxon test ($p < 0.05$)

ns = nonsignificant

Gene	Non-lesional skin	Lesional skin before treatment	Lesional skin after treatment	Friedman's ANOVA test	Difference in skin expression before and after PIM (Wilcoxon test: $p < 0.05$)
<i>Alternative keratin genes</i>					
KRT6A Median Quartile 1-3	0.79 0.34-3.32	4.61* 1.33-6.23	2.44** 0.21-4.95	0.029	No
KRT6B Median Quartile 1-3	1.81 1.28-2.95	3.01 1.67-4.01	1.6 1.25-2.74	0.035	Yes
KRT16 Median Quartile 1-3	33.43 17.53-95.52	370.06* 246.25-826.64	84.26** 37.35-255.92	0.001	Yes
<i>EDC genes</i>					
IVL Median Quartile 1-3	0.74 0.48-1.26	1.91* 1.44-2.8	0.89 0.49-1.47	0.004	Yes
LOR Median Quartile 1-3	0.63 0.23-1.06	0.42 0.1-0.93	0.66 0.32-1.25	ns	No
FLG Median Quartile 1-3	0.69 0.44-0.94	0.53 0.43-1.19	0.93 0.53-1.94	ns	No
SPRR1A Median Quartile 1-3	3.55 3.25-5.88	11.23* 8.83-16.12	5.29 3.57-10.21	0.003	No
SPRR2C Median Quartile 1-3	17.75 15-57.43	84.44* 52.12-125.23	40.76 11.66-65.59	0.001	Yes
SPRR4 Median Quartile 1-3	1.64 0.4-4.37	0.39* 0.13-1.08	0.19 0.02-2.4	0.029	No
<i>EDC genes known for their involvement in immune response</i>					

S100A2					
Median	5.41	12.72	5.86	0.078	No
Quartile 1-3	3.97-7.35	5.98-25.19	3.38-10.32		
S100A7					
Median	22.54	302.64	86.22	0.078	No
Quartile 1-3	16.58-87.25	129.94-406.16	35.21-234.6		
S100A8					
Median	7.98	122.68	9.21	0.059	No
Quartile 1-3	1.77-24.77	50.44-225.79	2.92-133.49		
S100A9					
Median	6.15	83.97*	11.48	0.012	No
Quartile 1-3	4.29-19.45	23.38-96.87	2.19-60.5		