Overexpression of proapoptotic proteins, Fas, and Bax after topical calcipotriol therapy for psoriasis

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Background
Suppression of the normal apoptotic process is one of the factors implicated in the pathogenesis of psoriasis. One of the actions of calcipotriol in psoriasis might be exerted through induction of apoptosis of keratinocytes through a p53-independent pathway.

Objective
The aim of the present study was to evaluate the expression of both Bax and Fas with its correlation with the apoptotic index and epidermal thickness in psoriatic skin before and at the end of the 8th week of topical calcipotriol therapy.

Methods
Lesional and nonlesional skin biopsy specimens were obtained from 10 patients with generalized plaque psoriasis. Histometric measurements of epidermal thickness were determined and Fas and Bax expressions were also evaluated using the immunoperoxidase technique and apoptotic cells using the terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end labeling method before and at the end of the 8th week of topical calcipotriol therapy.

Results
At the end of the 8th week of topical calcipotriol therapy, the epidermal thickness of psoriatic skin was significantly decreased ($P<0.05$) and a significant increase in both Fas and Bax expression ($P<0.05$) in psoriatic keratinocytes was observed, with no significant increase of either in lymphocytes ($P>0.05$). Meanwhile, the apoptotic index was significantly increased in psoriatic keratinocytes ($P<0.05$), but not in lymphocytes ($P>0.05$). However, there were no statistically significant correlations between proapoptotic markers (Fas and Bax) with either the apoptotic index or the epidermal thickness both before and at the 8th week of therapy.

Conclusion
Our findings suggest that both Bax and Fas overexpression after topical calcipotriol therapy for psoriasis might participate in the induction of apoptosis, especially of keratinocytes.

Keywords:
apoptosis, Bax, calcipotriol, Fas, psoriasis

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Introduction

Psoriasis is a chronic, relapsing, inflammatory, and hyperproliferative skin disease characterized by well circumscribed erythematosquamous lesions [1]. The presence of predominant infiltration of the dermis and epidermis by activated-T cells indicates that the cellular immune system plays a major role in the maintenance of dermal inflammation and epidermal proliferation [2].

Normally, proliferation of keratinocytes is restricted by apoptotic cell death to maintain a constant thickness of the epidermis [3]. However, the epidermal hyperplasia characteristic of psoriasis is suggested to be a result of aberrant epidermal expression of apoptosis-related molecules, leading to suppression of the apoptotic process [4]. Meanwhile, suppression of apoptosis of T lymphocytes leads to their survival, which relates to the chronic and relapsing characteristics of psoriasis [5].

Several proteins of the Bcl-2 gene family are involved in the regulation of programmed cell death either by preventing (Bcl-2, Bcl-xl, Bcl-w, Bcl-B, Bfl-1, and Mcl-1) or by promoting apoptosis (Bax, Bik, Bak, Bad, Bcl-Xs, Bim, Noxa, and Puma). Because many of these proteins are coexpressed in the same cells, the ratio of anti-apoptotic versus proapoptotic proteins determines the inherent susceptibility of a given cell to respond to apoptotic signals. The Bcl-2 gene is located on the chromosome 18 and encodes an inner mitochondrial protein that protects cells from apoptosis by binding to the Bax protein [6]. Bax (Bcl-2-associated X protein), a 21 KDa protein with approximately 21% sequence homology with Bcl-2, has been reported to be present

predominantly in the cytosol, redistributing from its soluble to mitochondrial membrane-bound form in cells undergoing apoptosis [7].

Fas (Apo-1 or CD95) is a 48 KDa membrane protein and a member of the TNF receptor family. Cross-linking of Fas by its ligand, FasL, induces apoptosis of cells expressing Fas on the membrane by triggering a cascade of caspases [8].

Calcipotriol is a vitamin D3 analog used in the treatment of psoriasis through inhibition of cell proliferation, induction of cell differentiation, and modulation of the immune response [9]. El-Domyati *et al.* [10] suggested that one of the actions of calcipotriol in psoriasis might be exerted through induction of apoptosis, especially of keratinocytes, through a p53-independent pathway. Meanwhile, suppression of Bcl-2 expression in lymphocytes may promote apoptosis of dermal lymphocytes, leading to healing of psoriasis. Recently, an in-vitro study of Tiberio *et al.* [11] confirmed that calcipotriol induces apoptosis of psoriatic keratinocytes.

The aim of the present study was to evaluate the expression of both Bax and Fas with its correlation with the apoptotic index and epidermal thickness in the psoriatic skin before and at the end of the 8th week of topical calcipotriol therapy.

Patients and methods

This retrospective study was conducted on 10 patients with generalized plaque psoriasis and was approved by the Committee for Postgraduate Studies and Research of Al-Minya University. The patients had not received topical, systemic antipsoriatic treatment, or phototherapy for at least 3 months before starting the study. After an informed written consent, all patients were treated with a topical calcipotriol ointment 50 µg/g Daivonex (LEO Pharmaceutical Products, Sarath Ltd, Athens, Greece) twice daily for 8 weeks. Patients were instructed to apply a thin film of the ointment to the lesions and to the surrounding nonlesional skin up to 15 cm all around the lesions, up to a maximum of 90 g/week (three tubes), according to the area affected. The severity of psoriasis was evaluated using the PASI score (Psoriasis Area and Severity Index) according to Fredriksson and Pettersson [12] before treatment and weekly after starting treatment up to 8 weeks. The patients' initial serum calcium level was measured and every month after starting the treatment.

From each patient, double 4-mm punch skin biopsy specimens were taken before and at the end of the 8th week of calcipotriol treatment; one was from a psoriatic plaque (lesional biopsy) and the other was from the normal-appearing perilesional skin (nonlesional biopsy, 15 cm away from the lesion). These sections were subjected to histopathological (hematoxylin and eosin) and immunohistochemical examination. A light microscope [Accu-Scope #3025 five headed (A3025-5)-OLYMPUS] with a built-in camera (digital camera E-330 SLR; Olympus, Japan) was used to examine and photograph the sections.

Histometric evaluation of epidermal thickness

A computer-assisted program (analySIS Five Olympus Soft Imaging Solutions GmbH, Munster, Germany) was used to measure epidermal thickness in hematoxylin and eosin-stained sections of biopsy specimens before and at the end of the 8th week of calcipotriol treatment. The mean epidermal thickness was determined by measuring the distance between the outermost surface of the epidermis excluding the stratum corneum and the dermo–epidermal junction at five points through the entire length of three examined sections.

Immunohistochemical examination

To evaluate the Bax and Fas expression in the keratinocytes and lymphocytes, the sections were stained by the primary antibodies [(Anti-Bax; code no.: E3381, ready-to-use 1ry antibody; SPRING BIOSCIENCE, Fermont, California, USA), (Anti-Fas; code no.: M3554; DAKO, Carpinteria, California, USA; at a dilution of 1 : 30 with labeled streptavidin biotin + horseradish peroxidase method)] that were applied to cover each section, and then slides were placed in a humidity chamber and incubated for 2 h (for both Bax and Fas antibodies). The ready-to-use detection system (code no.: K0673, DAKO LSAB2 system, peroxidaseDAB; DAKO) was used to demonstrate antibodies' expression according to the manufacturer's instructions. All tissue sections were stained under similar conditions to ensure equal staining quality.

The level of apoptosis markers' expression in keratinocytes was evaluated, by two blinded histopathologists, in accordance with the scoring system devised by Liang *et al.* [13]. This system results in a score ranging from 0 to 3 for both the degree of positivity (percentage of positively stained epidermal cells: 0; $<1\%$, 1; 1–10%, 2; 10–50%, 3; $>50\%$) and the degree of intensity of staining [from faint-brown (score 1) to deep-brown (score 3)]. The sum of the two scores was taken as the level of expression.

The expression of either Fas or Bax was evaluated in dermal lymphocytes according to Yildiz *et al.* [5] in a score ranging from 0 to 4 staining as follows: 0, none; 1, $<25\%$; 2, 26–50%; 3, 51–75%; and 4, $>75\%$ staining of the lymphocytes.

For the detection of apoptotic cells, sections from lesional and nonlesional biopsy specimens of the 10 patients before and at the 8th week of calcipotriol therapy were stained by the terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method using an in-situ apoptosis detection kit (TA4625, TACS TDT kit; R&D Systems Inc., Minnesota, USA). The apoptotic index was evaluated according to Kikuchi and Nishikawa [14] as follows:

$$\text{Apoptotic index (AI)}\,(\%) = (\text{TUNEL} + \text{ve cells} / \text{total cells counted}) \times 100.$$

Statistical analysis

Data were statistically analyzed using SPSS for Windows, Version 16.0.1, Chicago, Illinois, USA). Statistical analysis included descriptive analysis as mean ± SD, paired-sample and independent-sample *t*-test, and correlation coefficient (*r*) for the results. Significance was expressed in terms of a *P* value, which was considered significant when it was 0.05 or less.

Results

The study included 10 patients with generalized plaque psoriasis (eight men and two women). Their age ranged from 20 to 73 years (mean, 47.3 ± 16.4 years). The duration of psoriasis ranged from 3 months to 16 years (mean, 8.1 ± 5.1 years). Before starting treatment, the PASI score ranged from 5.7 to 20.3 (mean, 14.8 ± 4.5). At the 8th week of treatment, the PASI score was significantly reduced, ranging from 1.3 to 8.2 (mean, 5 ± 2.1; $P<0.05$).

Before treatment, the serum calcium level ranged from 8.8 to 10.9 g/dl (mean, 9.4 ± 0.7). At the 8th week of treatment, the level did not change significantly and ranged from 8.2 to 10.9 (mean, 9.2 ± 0.7; $P>0.05$). No local irritation or dermatitis was recorded in the treatment period.

Histopathological examination

Before treatment, lesional skin biopsy specimens revealed the characteristic features of psoriasis. At the 8th week of treatment, these features were markedly reduced. It was observed that the epidermal changes were much more reduced than the dermal changes.

Nonlesional skin biopsy specimens revealed an apparently normal epidermis and dermis. Before treatment, dilated blood vessels were detected in four biopsy specimens and a mild lymphocytic infiltrate was detected in one biopsy specimen. At the 8th week of treatment, dilated blood vessels were detected in two biopsy specimens and a mild infiltrate was also detected in only one biopsy specimen.

Histometric evaluation of epidermal thickness

The epidermal thickness of lesional biopsy specimens of patients with psoriasis ranged from 122.8 to 198.6 μm (mean, 156.47 μm ± 24.09), which was statistically significantly higher compared with nonlesional skin ($P<0.05$). At the 8th week of treatment, the epidermal thickness was significantly decreased (mean, 77.82 μm ± 15.96; $P<0.05$) (Fig. 1).

However, the epidermal thickness of nonlesional biopsies ranged from 57.4 to 75 μm (mean, 68 μm ± 6.31). At the 8th week of treatment, the nonlesional epidermal thickness showed no statistically significant difference (mean, 67.40 μm ± 6.65; $P>0.05$).

Fas and Bax expression

It was noteworthy that Bax-positive keratinocytes demonstrated a cytoplasmic staining pattern in all positive biopsy specimens. Before treatment, the mean Bax expression was 0.72 ± 0.56 in the keratinocytes and 0.06 ± 0.13 in lymphocytes in psoriatic lesions. At the 8th week of calcipotriol therapy, Bax expression in keratinocytes was significantly increased (mean, 2.16 ± 0.88; $P<0.05$), with no significant increase in its expression in lymphocytes (mean, 0.14 ± 0.29; $P>0.05$) (Table 1). In nonlesional biopsy specimens, the mean Bax expression was 0.61 ± 0.40 in the keratinocytes, with no statistically significant difference between nonlesional and lesional biopsy specimens ($P>0.05$). At the 8th week of calcipotriol therapy, Bax expression of keratinocytes in nonlesional skin was not significantly increased (mean, 0.60 ± 0.46; $P>0.05$) (Table 2).

As regards Fas expression, membranous staining was observed in all Fas-positive specimens. Before treatment, the mean Fas expression was 0.28 ± 0.19 in keratinocytes and 0.11 ± 0.21 in lymphocytes in psoriatic lesions. At the 8th week of treatment, Fas expression in keratinocytes was significantly increased (mean, 2.36 ± 1.10; $P<0.05$), whereas it was not significantly increased in lymphocytes (mean, 0.15 ± 0.29; $P>0.05$) (Table 1). In nonlesional biopsy specimens, the mean Fas expression was 0.22 ± 0.16 in keratinocytes in the psoriatic lesions, with no statistically significant difference between nonlesional and lesional biopsy specimens ($P>0.05$). Meanwhile, at the 8th week of therapy, Fas expression of keratinocytes in non-lesional skin was not significantly increased (mean, 0.19 ± 0.17; $P>0.05$). The lymphocytes of nonlesional skin were negative for both Bax and Fas before and at the 8th week of treatment (Table 2).

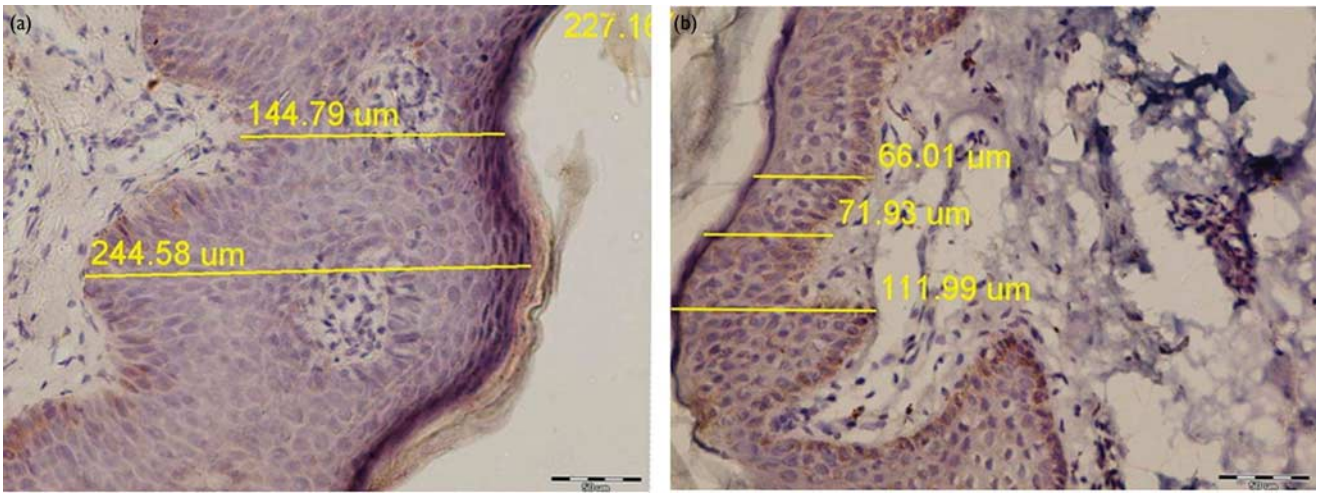
In both pretreated lesional and nonlesional biopsy specimens stained by either Bax or Fas, the stain was mainly observed in the basal cell layer, whereas few positive keratinocytes were seen in squamous cell layers. At the 8th week of calcipotriol treatment, the stain was observed markedly in both basal and squamous cell layers (Figs 2 and 3).

Detection of apoptotic cells (TUNEL)

The TUNEL method was used to detect apoptotic cells in epidermal keratinocytes and lymphocytes. Few apoptotic keratinocytes were demonstrated in both basal and squamous cell layers of three pretreated lesional biopsy specimens and the mean apoptotic index was 0.03 ± 0.05. At the 8th week of treatment, the apoptotic index was significantly increased (mean, 0.81 ± 0.54, $P<0.05$) as apoptotic keratinocytes were demonstrated in eight posttreated lesional biopsy specimens (Fig. 4). No apoptotic lymphocytes were demonstrated in pretreated lesional biopsy specimens whereas two posttreated biopsy specimens showed a few apoptotic lymphocytes (mean, 0.03 ± 0.07; $P>0.05$) (Table 1).

As regards nonlesional biopsy specimens, two nonlesional pretreated biopsy specimens showed a few apoptotic keratinocytes and the mean apoptotic index was 0.02 ± 0.04. At the 8th week of treatment, the apoptotic index was not significantly increased as a few apoptotic keratinocytes were detected in only three biopsy specimens

Figure 1.



(a) Histometry of psoriatic lesion before treatment showing increased epidermal thickness. (b) At the 8th week of calcipotriol therapy, the epidermal thickness was significantly decreased (hematoxylin and eosin; × 200).

(mean, 0.03 ± 0.05; $P > 0.05$). No apoptotic lymphocytes were demonstrated in all biopsy specimens (Table 2).

Meanwhile, there was no statistically significant correlation between proapoptotic markers (Fas and Bax) with either the apoptotic index or the epidermal thickness both before and at the 8th week of therapy ($P > 0.05$). Moreover, there was no correlation between Fas and Bax expression both before and at the 8th week of calcipotriol therapy ($P > 0.05$).

Discussion

Psoriasis is one of the most common skin diseases, characterized by excessive growth and aberrant differentiation of keratinocytes [15]. Although the pathogenesis of psoriasis is not completely understood, it is widely accepted that T cells play an important role [16].

Calcipotriol is a vitamin D3 analog that is considered by some dermatologists as a first-line treatment for chronic plaque psoriasis [17]. In-vitro studies have shown that vitamin D3 analogs decrease proliferation, induce differentiation of keratinocytes, and have a strong immunomodulating effect [9].

In the present study, the PASI score was significantly reduced at the 8th week of treatment. There were no reported side effects throughout the treatment period.

A biopsy of lesional skin of psoriasis has many characteristic findings including increased epidermal thickness, parakeratosis, lymphocyte-rich inflammatory infiltrate, and multiple dilated blood vessels [18]. At the 8th week of treatment of psoriatic plaques, there is a progressive decrease in the inflammatory infiltrate, reduction in the epidermal hyperplasia, and restoration of the granular layer [19]. In the present study, at the 8th week of treatment, the histological abnormalities of psoriasis were diminished, with more improvement in the epidermal changes than the dermal changes.

Before treatment, the epidermal thickness of psoriatic skin was significantly increased compared with nonlesional skin. At the 8th week of calcipotriol therapy, the epidermal thickness of lesional biopsy specimens was significantly decreased; however, it was not significantly changed in nonlesional skin. To the best of our knowledge, there are no reports in the literature on the effect of calcipotriol on the epidermal thickness, measured by the histometry technique, in both lesional and nonlesional skin of patients with psoriasis.

The characteristic epidermal hyperplasia of psoriasis is suggested to be a result of aberrant epidermal expression of apoptosis-related molecules, leading to suppression of the apoptotic process [4].

Bax is a member of the Bcl-2 family and one of the main inducers of apoptosis. Bax immunoreactivity was present in normal epidermis and its appendages, with the suprabasal compartments being stained more strongly than basal keratinocytes [20]. The results of the present study showed no statistically significant increase in Bax expression in pretreated psoriatic lesions compared with nonlesional biopsy specimens. This was in agreement with the result of Tomkova *et al.* [20], who examined the expression of Bax in five normal biopsy specimens, five psoriatic lesions, and other malignant lesions and reported similar Bax expression in both normal skin and pretreated psoriatic lesion. However, Kocak *et al.* [21] and Batinac *et al.* [22] reported that Bax protein was strongly expressed in almost all the psoriasis specimens and there was a statistically significant increase in Bax expression in psoriatic epidermis compared with the normal epidermis.

Table 1. Mean Bax and Fas expression and the apoptotic index in psoriatic plaques before and at the 8th week of calcipotriol therapy

	Bax				Fas				Apoptotic index			
	Keratinocytes		Lymphocytes		Keratinocytes		Lymphocytes		Keratinocytes		Lymphocytes	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Range	0–1.7	0.9–3.6	0–0.4	0–0.9	0.1–0.5	0.6–4.2	0–0.6	0–0.8	0–0.1	0–1.7	0	0–0.2
Mean	0.72 ± 0.56	2.16 ± 0.88	0.06 ± 0.13	0.14 ± 0.29	0.28 ± 0.19	2.36 ± 1.10	0.11 ± 0.21	0.15 ± 0.29	0.03 ± 0.05	0.81 ± 0.54	0	0.03 ± 0.07
P	$P < 0.05$		$P > 0.05$		$P < 0.05$		$P > 0.05$		$P < 0.05$		$P > 0.05$	

As regards Fas expression in keratinocytes, the present study showed no statistically significant difference between lesional and nonlesional biopsy specimens. These results were not in agreement with those of Gilhar *et al.* [23] and Yang *et al.* [24], who found increased expression of Fas in keratinocytes in psoriatic epidermis.

It was found in the current study that both Bax and Fas were expressed in lymphocytes of three lesional biopsy specimens. However, Tomkova *et al.* [20] observed numerous Bax-positive, infiltrating lymphocytes in all the five specimens of psoriasis examined.

Before treatment, there were very few apoptotic keratinocytes in three pretreated lesional biopsy specimens stained by the TUNEL technique, with no statistically significant increase in the apoptotic index of pre-treated lesional biopsy specimens compared with nonlesional biopsy specimens. No apoptotic lymphocytes were demonstrated in pretreated lesional biopsy specimens. This means that few keratinocytes in psoriatic lesions undergo apoptosis like nonlesional skin, which is in agreement with the finding of Weil *et al.* [25], who reported that some epidermal keratinocytes in normal skin undergo apoptosis to balance the proliferation of cells in the basal cell layer. Also, Iizuka *et al.* [26] reported that this dynamic equilibrium of proliferation and apoptosis is maintained in psoriasis but in an accelerated manner. However, Yang *et al.* [24] observed more noticeable apoptosis in keratinocytes than in lymphocytes in normal skin and nonlesional psoriatic skin, with the appearance of few apoptotic keratinocytes or lymphocytes in lesional psoriatic skin. Wrone-Smith *et al.* [27] and Laporte *et al.* [28] suggested that keratinocytes in the psoriatic lesions have a phenotype that resists apoptosis. The results of the present study agree with those of Kocak *et al.* [21], who showed that the psoriatic epidermal hyperplasia may result from excessive mitogenic stimuli that promote an increase in the proliferative cell compartment, rather than being a consequence of the complete loss of the antiproliferative control.

At the 8th week of calcipotriol therapy, both Bax and Fas expression were significantly increased in keratinocytes, but not in lymphocytes in psoriatic lesions.

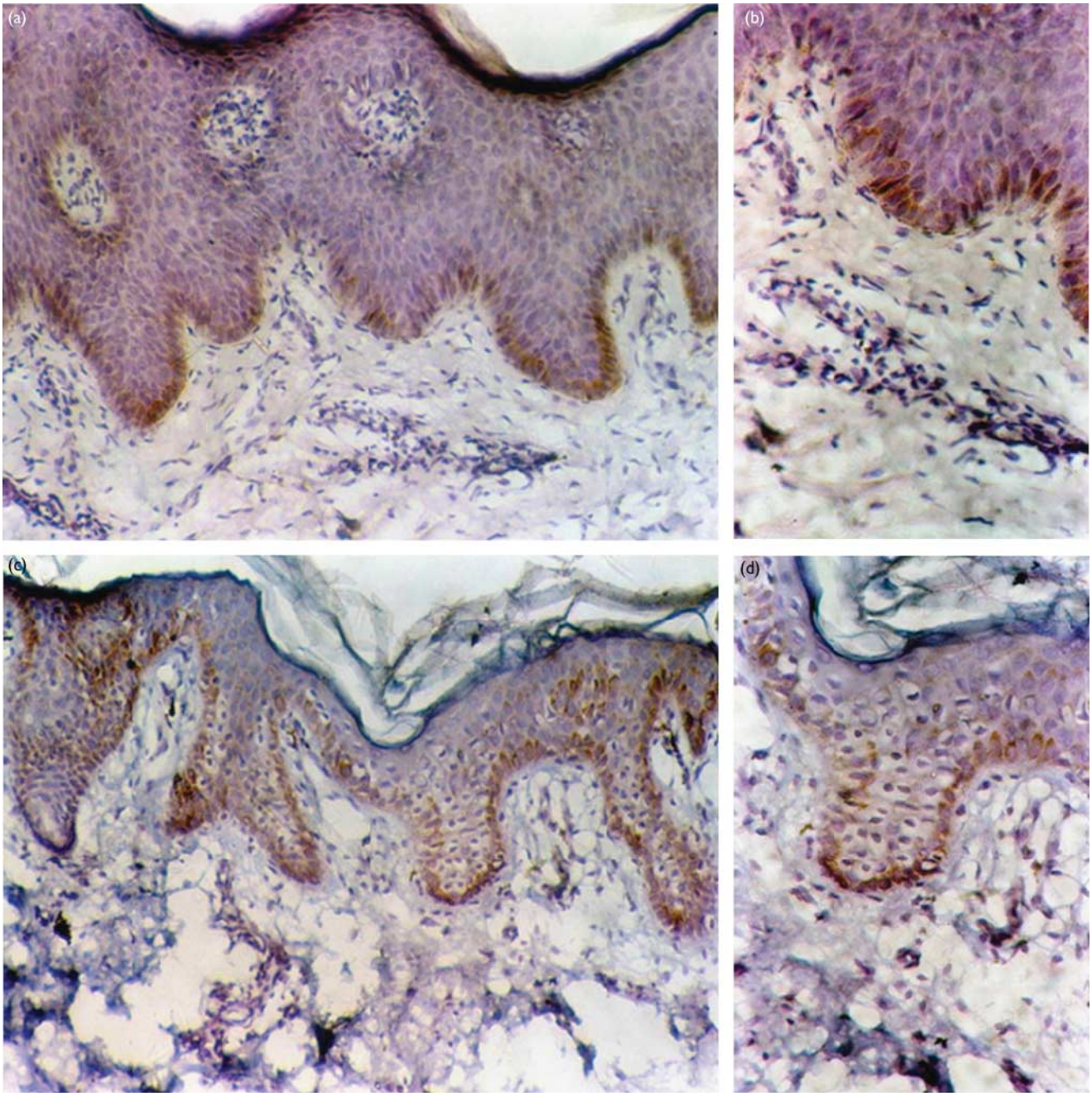
Although there is little information about the effect of calcipotriol on apoptosis, it was reported that vitamin D analogs can induce apoptotic death of tumor cells such as retinoblastoma and colorectal cancer cells through a p53 pathway in certain cancer cells or through p53-independent pathways in others [29,30]. In psoriatic lesions, Adişen *et al.* [31] compared the effects of calcipotriol and methylprednisolone aseponate treatments on Bcl-2, p53, and ki-67 expressions and found that both calcipotriol and methylprednisolone aseponate decrease the p53 and ki-67 expression and increase Bcl-2 expression. However, it should further be elucidated whether these changes represented the usual response of psoriatic keratinocytes to any antipsoriatic medication.

El-Domyati *et al.* [10] confirmed that calcipotriol induced apoptosis especially of keratinocytes through a

Table 2. Mean Bax and Fas expression and apoptotic index in nonlesional skin before and at the 8th week of calcipotriol therapy

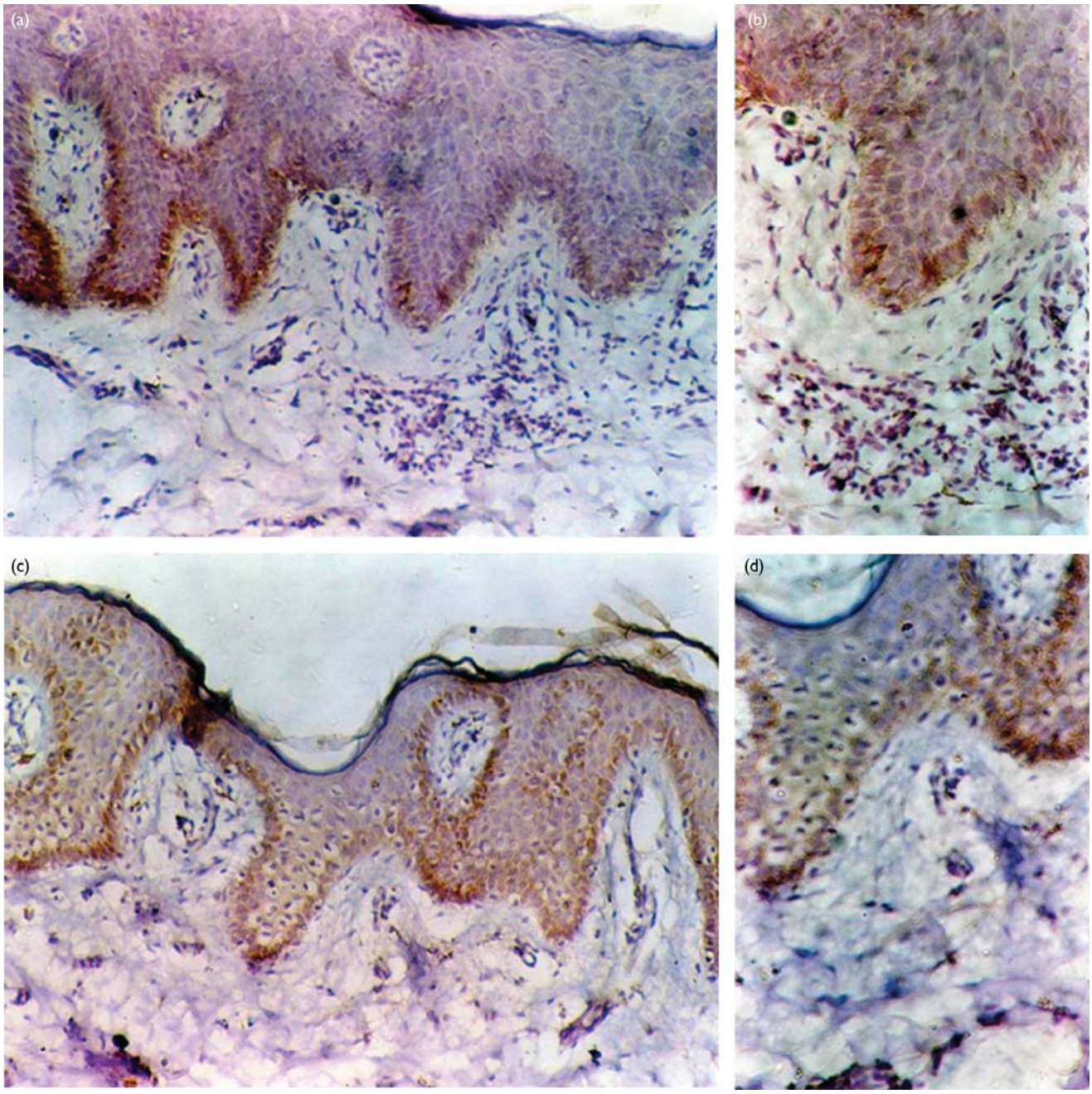
	Bax				Fas				Apoptotic index			
	Keratinocytes		Lymphocytes		Keratinocytes		Lymphocytes		Keratinocytes		Lymphocytes	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Range	0–1.5	0–1.4	0	0	0–0.4	0–0.5	0	0	0–0.1	0–0.1	0	0
Mean	0.61 ± 0.40	0.60 ± 0.46	0	0	0.22 ± 0.16	0.19 ± 0.17	0	0	0.02 ± 0.04	0.03 ± 0.05	0	0
P	*P* > 0.05		*P* > 0.05		*P* > 0.05		*P* > 0.05		*P* > 0.05		*P* > 0.05	

Figure 2.



Immunohistochemical staining with Bax. (a, b) Psoriatic lesion before treatment showing cytoplasmic staining of Bax expression in the basal layer and little in squamous cell layers. (c, d) Cytoplasmic staining of Bax expression is significantly increased in the basal and squamous cell layers at the 8th week of calcipotriol treatment [Immunoperoxidase; (a, c); × 100, (b, d); × 200].

Figure 3.



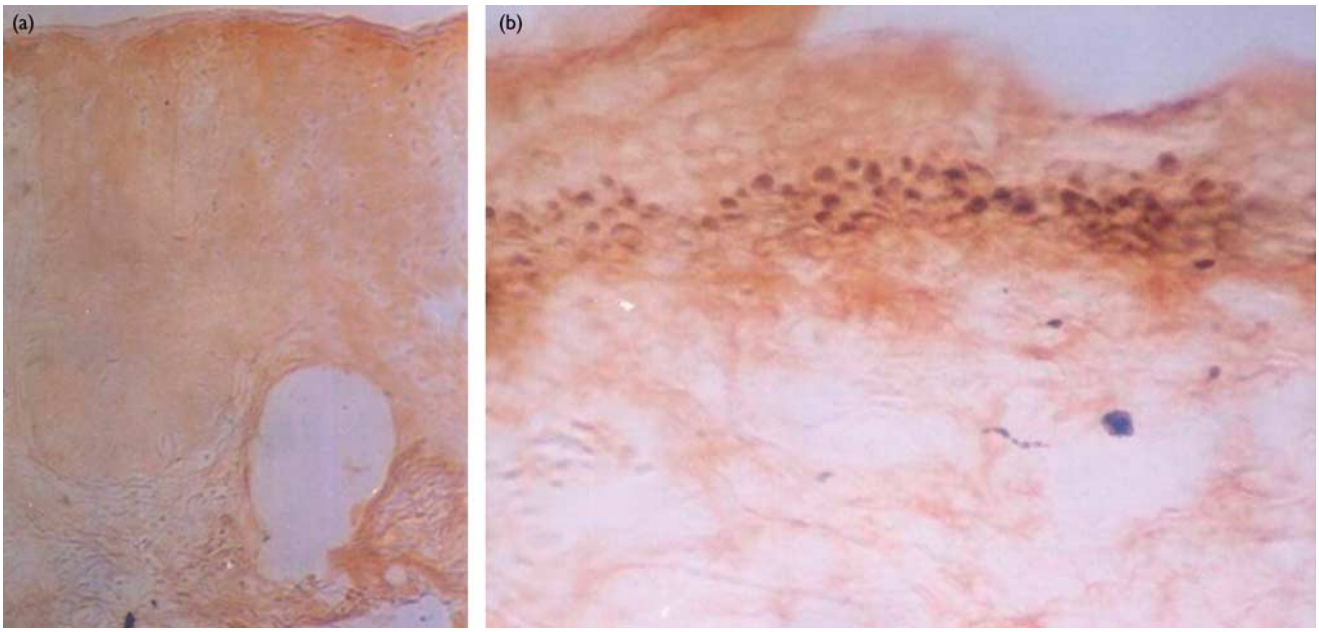
Immunohistochemical staining with Fas. (a, b) Psoriatic lesion before calcipotriol treatment showing membranous staining of Fas expression in the basal layer and little in squamous cell layers. (c, d) Membranous staining of Fas expression is significantly increased in the basal and squamous cell layers at the 8th week of calcipotriol treatment (Immunoperoxidase; a, c; × 100, b and d; × 200).

p53-independent pathway. Recently, Tiberio *et al.* [11] reported that the number of cultured apoptotic keratinocytes, detected by confocal microscopy, after incubation with calcipotriol was significantly higher in lesional than in perilesional keratinocytes or nontreated psoriatic keratinocytes.

In the present study, at the 8th week of calcipotriol treatment, the apoptotic index in posttreated lesional keratinocytes was significantly increased, with no statistically significant increase in the apoptotic index of posttreated lesional lymphocytes. This indicates an increase in the apoptotic process that is consistent with an improvement in both histological architecture and clinical status. This result is in agreement with that of Laporate *et al.* [28], who reported that healed psoriatic plaques are associated with apoptosis. However, the present study observed that calcipotriol mainly affects keratinocytes rather than lymphocytes.

It is worth noting that no previous reports have studied the effect of calcipotriol on both Fas and Bax expressions and their effect on apoptosis in both keratinocytes and lymphocytes of psoriatic skin.

Figure 4.



(a) Psoriatic lesion before treatment showing no apoptotic cells. (b) Apoptotic keratinocytes in the basal and squamous cell layers at the 8th week of calcipotriol therapy (terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end labeling; × 200).

Unfortunately, no significant correlations were detected between proapoptotic markers (Fas & Bax) for either epidermal thickness or the apoptotic index both before and at the 8th week of therapy. This discrepancy in the correlation results may be attributed to the small sample group of patients in the present study. Therefore, future large-scale studies are recommended.

In conclusion, Fas and Bax keratinocyte overexpression after topical calcipotriol therapy for psoriasis might participate in the induction of apoptosis.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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