INVESTIGATIVE REPORT

Novel Quantitative Immunofluorescent Technique Reveals Improvements in Epidermal Cell Populations after Mild Treatment of Psoriasis

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A novel antibody labelling technique, the Zenon technique, was used in fluorescent immunohistochemistry for a better characterization of epidermal cell populations in a quantitative approach. With this technique, differences in proliferation and differentiation characteristics were shown between psoriatic and normal epidermis. The sensitivity of the method was investigated by assessing the effect of a mild topical treatment versus an emollient.

Frozen sections of non-treated psoriatic epidermis and psoriatic epidermis treated once daily with either an emollient or betamethasone-17-valerate for only 2 weeks were compared immunohistochemically. Antibodies against keratin 6, 10 and 15 were labelled with the Zenon technique, whereas antibodies against the Ki-67 antigen and β-1 integrin were covalently FITC-labelled. Using image analysis, these markers were measured in the epidermis in a standardized manner.

Treatment of psoriasis with short-term topical steroid resulted towards normalization of Ki-67 antigen, β-1 integrin, keratin 10 and keratin 6 expression, which are parameters for proliferation and differentiation. Although treatment with an emollient showed hardly any clinical response, changes towards a more normal phenotype could already be detected in several epidermal markers using this method. *Key words: Zenon; immunohistochemistry; psoriasis; epidermis.*

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The epidermis is a constantly renewing epithelium. The capacity for self-renewal of the epidermis is presumed to be preserved by stem cells, which are situated in the basal layer. These stem cells develop transient amplifying cells (TACS), which have a limited capacity to proliferate before differentiation and formation of a corneocyte (1–3). Psoriasis is characterized by epidermal hyperproliferation, disturbed differentiation and inflammation. It has been shown that the process of keratinocyte differentiation is altered in psoriasis.

According to our current understanding psoriasis should be considered a T-cell-mediated disease. Therefore, T cells are supposed to have a regulatory role in the altered keratinocyte differentiation. Indeed, specific anti-T-cell treatments, such as alefacept and efalizumab, have a substantial therapeutic efficacy in psoriasis (4). Established anti-psoriatic treatments have more broad effects on various aspects in the pathogenesis of psoriasis. Topical vitamin D analogues and corticosteroids inhibit the proliferation of epidermal keratinocytes and the former drug also enhances the cornified envelope formation, which indicates an effect on keratinocyte differentiation (5). Furthermore, application of an emollient also seems effective to some extent, resulting in a slight modification of proliferation and differentiation (6–8).

Increased recruitment of cycling epidermal cells has been shown to be the most important mechanism of epidermal proliferation in psoriasis. This phenomenon can be quantified by calculating the number of Ki-67-positive nuclei, which is impressively increased in the psoriatic epidermis (9–13). The altered differentiation in the psoriatic epidermis is shown by the replacement of keratin 10, representing differentiating cells, by keratin 6 (14–19). β-1 Integrin expression in the basal layer is important for studying stem cells, which reside in this compartment (2, 20–23). Keratin 15 may be a marker for stem cells in the hair follicle and is also expressed in the basal layer of the interfollicular epidermis. However, expression of keratin 15 disappears in a hyperproliferative state like psoriasis (24, 25).

Major differences in expression of the above-mentioned epidermal cell markers between normal and psoriatic epidermis were shown with fluorescent immunohistochemistry using the Zenon technique, a novel labelling technique (26). This technique is a simple and rapid technique. It conjugates primary antibodies quantitatively and the labelled antibodies can be used in the same way as covalently labelled primary antibodies (27). The combination of this technique with image analysis offers a possibility to describe morphology and show co-expression of proteins. Co-expression is demonstrated by labelling two antibodies with different fluorochromes. Using a combination of a proliferation and a

differentiation marker is useful for the demonstration of TACs, which migrate from the basal layer into the upper layers of the epidermis. After being released from the basal layer, expression of β-1 integrin on their cell surface is still present but diminished. After proliferation, these cells become committed to terminal differentiation and express both Ki-67 and keratin 10. The effect of treatment on the differentiation process is reflected by co-expression of keratin 10 and keratin 6, which can be quantified with this technique (26).

The aim of the present study was to investigate the sensitivity of the combination of the Zenon technique with standardized image analysis, i.e. to see whether or not minor differences in expression patterns of epidermal markers for proliferation and differentiation could be detected by this method in patients with mild psoriasis.

MATERIALS AND METHODS

Patients and biopsies

After obtaining informed consent, skin punch biopsy specimens were collected from five patients (four women and one man, aged 57.6 years $\pm$ 8.8) with mild-to-moderate plaque psoriasis. They did not receive any topical treatment for at least 2 weeks or systemic therapy for at least 4 weeks before specimens were obtained. A 4-mm punch biopsy was taken from two representative lesions after local anaesthesia with xylocaine 1%/adrenaline. One lesion was then treated with betamethasone-17-valerate 0.1% cream once daily, while the other lesion was treated with an emollient (50% cremor lanette I (15 g lanette wax SX, 20 g cetiol V, 4 g sorbitol solution, 0.15 g sorbinic acid, aqua ad 100 g) in vaselinum album; vaseline-lanette). After 2 weeks of treatment, a second 4-mm punch biopsy was taken. All specimens were embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen and stored at $-80°C$. Frozen sections were chosen because of their advantages regarding the use of surface antigens and fluorescent techniques.

Erythema, infiltration and scaling of the lesions were recorded using a 5-point scale from 0='none' to 4='extremely severe' before and after treatment. The SUM score, which is the total score for erythema, infiltration and scaling, was calculated.

Fluorescent immunohistochemistry and Zenon labelling technique

This method has been described previously (26). In brief, the Zenon labelling technique makes it possible to label a primary antibody rapidly and quantitatively, which can be used in the same way as covalently labelled primary antibodies. With this technique, the primary antibodies are specifically coupled with fluorescent-labelled Fab fragments of secondary antibodies directed against their Fc regions. The Zenon technique was used according to the protocol of the manufacturer (Molecular Probes, Eugene, USA) with minor modifications. Monoclonal antibodies were labelled and/or diluted and incubated on 6-μm frozen sections of the biopsies. Monoclonal antibodies against keratin 10 (Monosan, Uden, The Netherlands; RKSE60) and keratin 6 (Novocastra Laboratories, Newcastle upon Tyne, UK; LHK6B) were labelled using the Zenon labelling technique and were used for examination of epidermal differentiation.

For assessment of epidermal proliferation, Ki-67 antigen (DakoCytomation, Copenhagen, Denmark; MIB-1 FITC), covalently FITC-labelled, was used. Monoclonal antibodies against β-1 integrin (DakoCytomation, Copenhagen, Denmark; CD29 FITC, clone K20) and keratin 15 (Neomarkers, Fremont, USA; LHK15) were used for analysis of the basal layer of the epidermis. β-1 Integrin was covalently labelled with FITC, whereas keratin 15 was labelled using the Zenon labelling technique.

For the double-staining procedure, equal volumes of two antibody dilutions were mixed and incubated on the slides. Keratin 10 was combined with Ki-67, with β-1 integrin and with keratin 6, while β-1 integrin was also mixed with keratin 15. The initial manufacturers' concentrations of the antibodies were diluted in 1% bovine serum albumin (BSA: Sigma, St Louis, USA) in phosphate-buffered saline and used in the following concentrations: anti-keratin 10 and anti-keratin 6, 1:30; anti-β-1 integrin, 1:100; and anti-Ki-67 antigen and anti-keratin 15, 1:50.

The sections and monoclonal antibodies were examined using an immunofluorescence microscope (Axioskop 2 MOT, Zeiss) and images were photographed using a digital camera (Sony, DXC-390P 3 CCD) and 'Image J' software (Scanalytics, Fairfax, USA). Photography was standardized by setting the shutter off and by securing the light intensity. Total exposure to light was less than 5 seconds for each frame. For each antibody, 10 different parts of the epidermis were photographed. These images were analysed with IP-lab software (Scanalytics) in a standard manner. The intensity and percentage of positive signal in the epidermis was measured with standardized settings.

For double-staining and co-expression, photographs of the same part of the epidermis were taken for each antibody by switching to the correct filters and light intensity and these pictures were merged using IP-lab.

Statistical analysis

Statistical analysis was performed using a t-test: two-sample assuming unequal variances and standard regression software and a t-test: paired two samples for means. *p* values of <0.05 were considered statistically significant. Mean $\pm$ SEM are used.

RESULTS

The clinical SUM scores revealed a reduction in erythema, induration and scaling for both vaseline-lanette and betamethasone-17-valerate treatment (from 5.6 ± 0.7 to 4.6 ± 0.5 and from 6.0 ± 0.7 to 2.8 ± 0.2 (mean $\pm$ SEM), respectively). However, only the latter response was statistically significant ($p < 0.05$).

Ki-67 expression revealed a significant reduction from 112 ± 8.1 positive cell nuclei per mm basal membrane before treatment to 48 ± 4.1 positive cell nuclei per mm basal membrane after treatment with betamethasone-17-valerate ($p < 0.05$). Treatment with vaseline-lanette also showed a significant decrease from 98 ± 7.6 to 65 ± 5.8 positive cell nuclei per mm basal membrane after treatment ($p < 0.05$). The reduction after betamethasone-17-valerate treatment was significantly higher ($p < 0.05$) as compared with the improvement following vaseline-lanette treatment of these plaques (Fig. 1A). However, no statistically significant differences were found in the vaseline-lanette- and betamethasone-17-valerate-treated psoriatic lesions
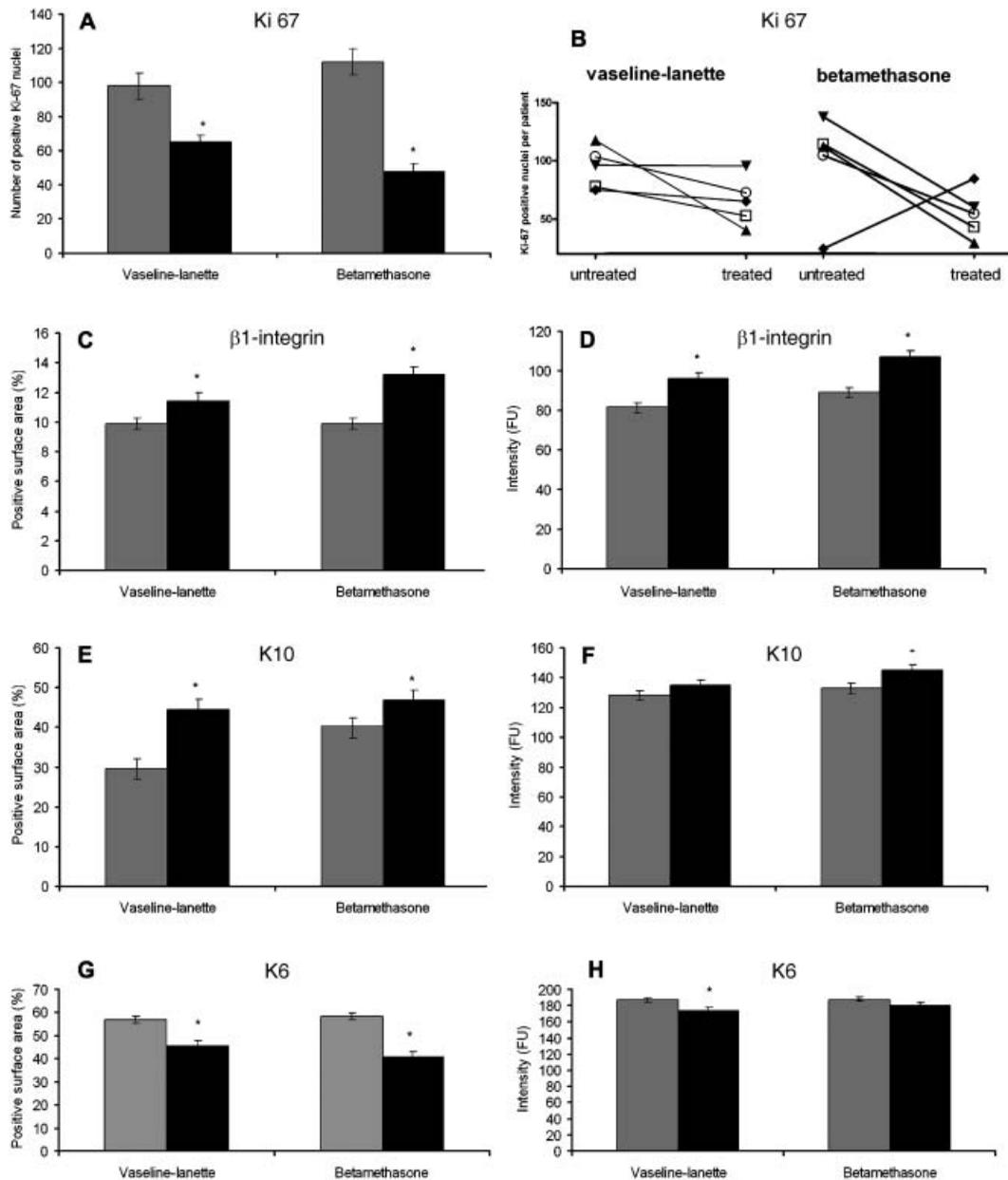
Fig. 1. (A) Number of Ki-67-positive nuclei /mm length of basement membrane before (grey bar) and after (black bar) treatment (*$p < 0.05$). (B) Number of Ki-67-positive nuclei before and after treatment with vaseline-lanette and betamethasone-17-valerate per patient (the symbols in the vaseline-lanette- and betamethasone-treated group refer to the same patient). (C) β-1 Integrin-positive surface area as a percentage of epidermis before (grey bar) and after (black bar) treatment. (D) Intensity of β-1 integrin expression assessed as units of fluorescence before (grey bar) and after (black bar) treatment. (E) Keratin 10-positive surface area as a percentage of epidermis before (grey bar) and after (black bar) treatment. (F) Intensity of keratin 10 expression assessed as units of fluorescence before (grey bar) and after (black bar) treatment. (G) Keratin 6-positive surface area as a percentage of epidermis before (grey bar) and after (black bar) treatment. (H) Intensity of keratin 6 expression assessed as units of fluorescence before (grey bar) and after (black bar) treatment.

when comparing the results paired per patient ($p=0.10$ and $p=0.17$, respectively). A tendency towards normalization is shown (Fig. 1B).

β-1 Integrin is expressed in the basal and some suprabasal layers in the untreated psoriatic epidermis. After treatment, the expression in the suprabasal layers was diminished and more confined to the basal layer (Fig. 3). The increase of positive surface area for β-1 integrin reached statistical significance for both

vaseline-lanette and betamethasone-17-valerate (from $9.9 \pm 0.4\%$ to $11.4 \pm 0.6\%$ and from $9.9 \pm 0.5\%$ to $13.2 \pm 0.7\%$, respectively ($p < 0.05$) with a statistically higher increase for betamethasone-17-valerate compared with vaseline-lanette ($p < 0.05$) (Fig. 1C). Also, for β-1 integrin intensity a significantly higher increase was found for both vaseline-lanette and betamethasone-17-valerate treatment (from 81.6 ± 2.3 FU to 96.3 ± 2.9 FU and from 89.3 ± 3.1 FU to 107.2 ± 2.9 FU,

respectively; $p < 0.05$) (Fig. 1D). Due to statistically unequal intensities for β-1 integrin between both sets of untreated plaques, the difference in increase for vaseline-lanette and betamethasone-17-valerate could not be compared.

Keratin 10 has a suprabasal expression pattern, which is heterogeneous in the untreated psoriatic lesion and becomes more homogenous after treatment (Figs 2 and 3). Keratin 10 expression showed a significant increase in positive surface area following both vaseline-lanette and betamethasone-17-valerate treatment (from $29.7 \pm 2.3\%$ to $44.6 \pm 2.5\%$ and from $40.2 \pm 2.9\%$ to $46.9 \pm 1.8\%$, respectively; $p < 0.05$). These increases cannot be compared with each other because of significantly different positive surface areas for keratin 10 between both untreated plaques. For the intensity of keratin 10 a significant increase was seen after betamethasone-17-valerate treatment (from 132.9 ± 3.2 FU to 145.3 ± 1.9 FU; $p < 0.05$), while the increase with vaseline-lanette treatment from 128.2 ± 3.3 FU to 135.2 ± 3.1 FU did not reach statistical significance ($p = 0.12$). While keratin 10 intensity was equal in both sets of untreated plaques, betamethasone-17-valerate treatment revealed a significantly higher increase of
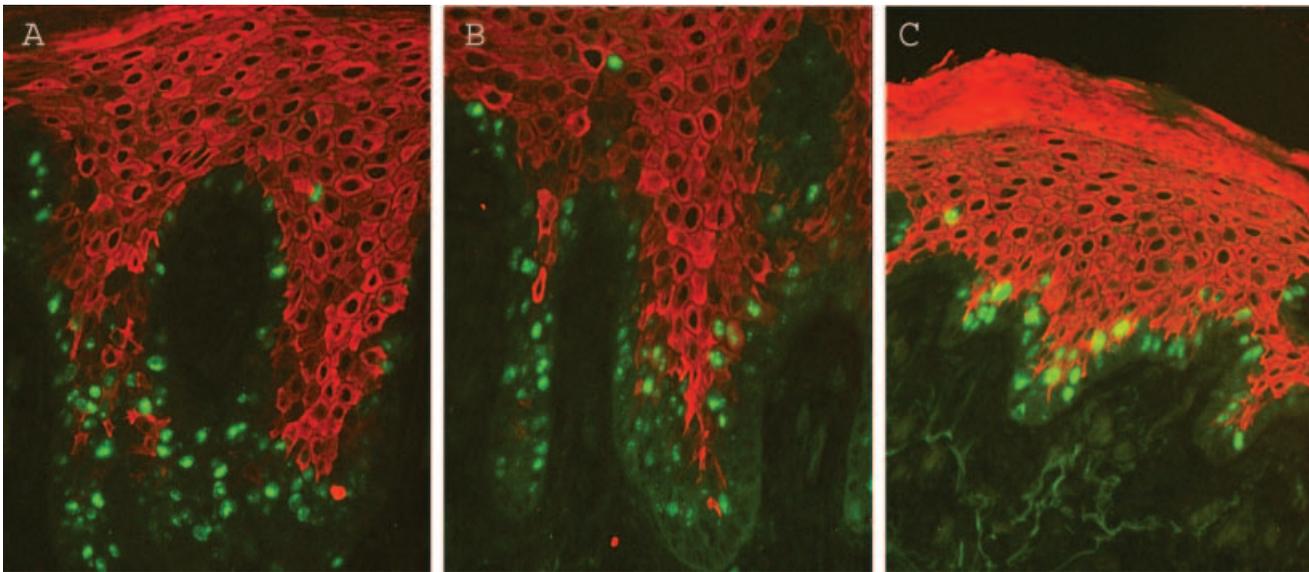

Fig. 2. Double-staining of Ki-67 (green) with keratin 10 (red) before treatment (A), after treatment with vaseline-lanette (B) and after treatment with betamethasone-17-valerate (C).

Fig. 3. Double-staining of β-1 integrin (green) with keratin 10 (red) before treatment (A), after treatment with vaseline-lanette (B) and after treatment with betamethasone-17-valerate (C).
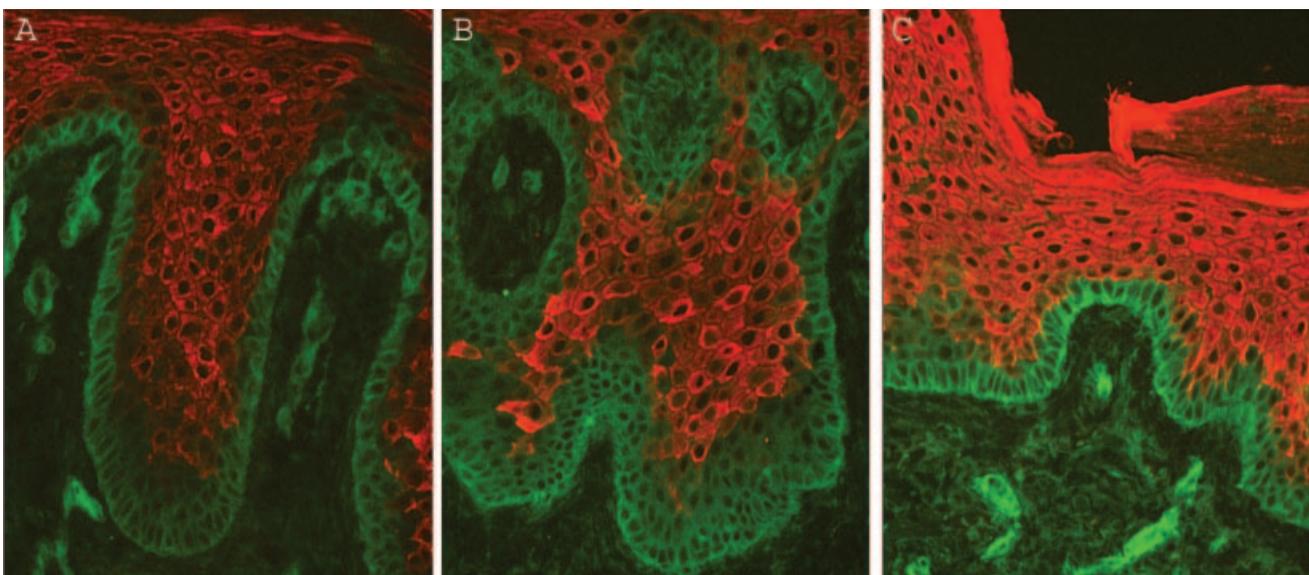
intensity in comparison with vaseline-lanette treatment (Fig. 1E, F).

Keratin 6 is also expressed in the suprabasal layers of the epidermis in psoriatic lesions. In the untreated lesions a more homogenous pattern of expression was seen, while after 2 weeks of treatment the pattern was more heterogeneous (data not shown). A significant decrease within the same range was found for positive surface area after both vaseline-lanette and betamethasone-17-valerate treatment (from $56.9 \pm 1.5\%$ to $45.5 \pm 2.3\%$ and from $58.2 \pm 1.6\%$ to $40.9 \pm 3.2\%$, respectively; $p < 0.05$) with no statistical differences between both untreated plaques ($p = 0.58$) (Fig. 1G). For keratin 6 intensity, vaseline-lanette treatment showed a significant decrease (from 187.2 ± 2.7 FU to 173.9 ± 3.8 FU; $p < 0.05$). Betamethasone-17-valerate also revealed a decrease in intensity (from 187.8 ± 2.8 FU to 180.1 ± 4.3 FU), which did not reach statistical significance ($p = 0.14$) (Fig. 1H).

Untreated psoriatic lesions did not express keratin 15; neither treatment with betamethasone-17-valerate nor with vaseline-lanette resulted in its expression.

Double staining of Ki-67 with keratin 10 visualizes an overlap in expression in both vaseline-lanette- and betamethasone-17-valerate-treated epidermis. Some Ki-67-positive nuclei reside in keratin 10-positive cells. However, Ki-67-positive nuclei are more confined to the basal layer after betamethasone-17-valerate treatment, whereas in untreated and with vaseline-lanette-treated psoriatic epidermis, Ki-67-positive nuclei are also present in suprabasal layers (Fig. 2).

In the double-staining of β-1 integrin with keratin 10, β-1 integrin-positive cells are found in the basal layer after treatment with betamethasone-17-valerate. Also, in this treatment group, a few cells, which express both beta-1 integrin and keratin 10, are seen. In the untreated epidermis and the epidermis treated with vaseline-lanette, no co-expression was shown (Fig. 3).

Double-staining of keratin 6 with keratin 10 in the untreated epidermis shows keratinocytes expressing both keratin 6 and keratin 10 (yellow/orange cells) and keratinocytes only expressing keratin 6 (green cells). These double-positive cells are localized throughout the epidermis. No cells that were only positive for keratin 10 were seen in the untreated epidermis. However, following treatment with betamethasone-17-valerate, the epidermis revealed cells that only expressed keratin 10 (Fig. 4).

DISCUSSION

In order to demonstrate the sensitivity of the combination of the Zenon labelling technique and microscopic image analysis, the effect of a mild treatment and an emollient on the psoriatic epidermis was investigated. Betamethasone-17-valerate in a cream base was used once daily for 2 weeks and this can be regarded as a mild treatment (5). The effect of such a mild corticosteroid treatment is markedly different from a potent or ultra-potent corticosteroid. For example, a potent corticosteroid diminishes proliferation, resulting in a reduction of the number of Ki-67-positive nuclei below the normal range after treatment (28).

Although emollients have a slight clinical effect on the psoriatic skin, it has been shown in previous studies that a
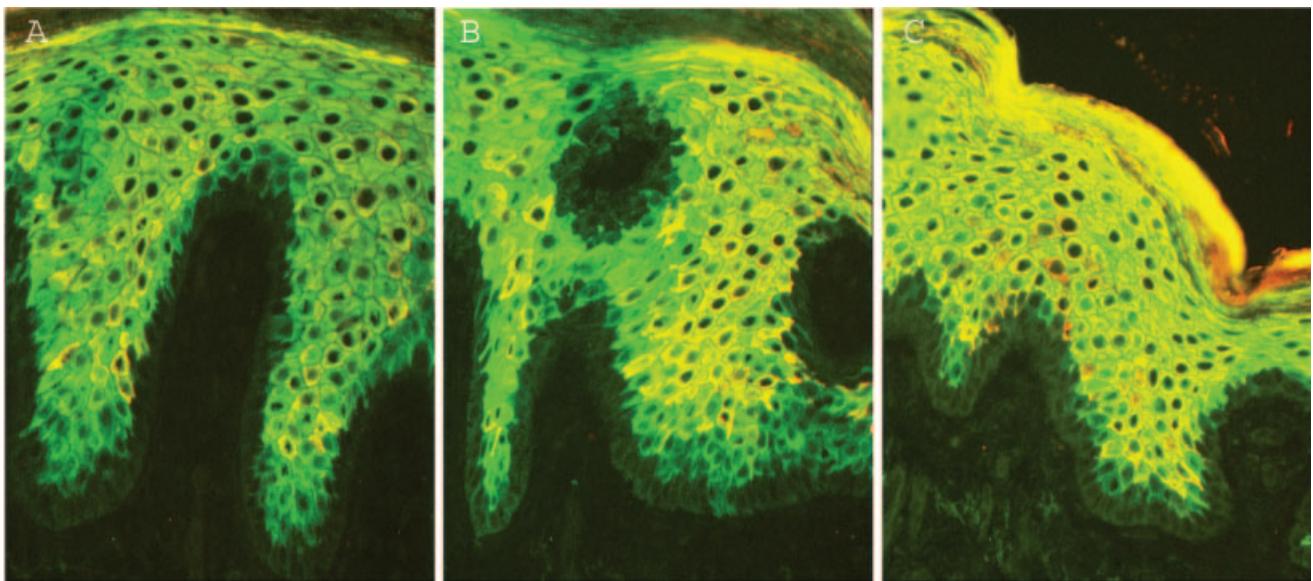

Fig. 4. Double-staining of keratin 6 with keratin 10 before treatment (A), after treatment with vaseline-lanette (B) and after treatment with betamethasone-17-valerate (C).

vehicle alone also has a small therapeutic effect in the psoriatic epidermis (29, 30). Therefore, treatment with an emollient was chosen to investigate the sensitivity of this novel method to quantify proliferation and differentiation aspects in the epidermis. A decrease of proliferation, which is shown by a diminished number of Ki-67-positive nuclei and an increase of β-1 integrin surface area and intensity was seen following treatment with the corticosteroid. The β-1 integrin-positive surface area increases during treatment, while the epidermis clearly loses its suprabasal β-1 integrin expression. The β-1 integrin-positive surface area is measured relative to the thickness of the epidermis (total surface area). Because of decreasing number of cell layers in the epidermis during treatment and therefore the total surface area, the β-1 integrin-positive surface area can increase relatively. The improvement of the differentiation markers, keratin 6 and keratin 10, was evident but less prominent. For keratin 10, this had been observed in earlier studies (31). Although treatment with vaseline-lanette only tends to provide a slight improvement clinically, differences were already evident for all epidermal markers using this novel method. The present methodology permits demonstration of the anti-psoriatic effect of a mild treatment and even an emollient on the epidermis, which only causes a subtle clinical improvement.

An anti-proliferative effect of emollients has been shown in the literature, which is confirmed by these data (7, 29). The double-staining, however, still reveals the presence of Ki-67-positive nuclei in the keratin 10-positive compartment and co-expression of β-1 integrin and keratin 10 is not present, suggesting that emollients do not influence the suprabasal compartment, which contains late TACs. Although double-staining of keratin 6 and keratin 10 shows more co-expression of both proteins after treatment with an emollient, cells expressing only keratin 10 are not present. However, a small effect of an emollient on keratinocyte differentiation is shown.

The effect of a mild corticosteroid on the suprabasal compartment is more evident. After treatment with a corticosteroid, the Ki-67-positive nuclei are more confined to the basal layer, the suprabasal expression of β-1 integrin disappears and keratin 10 expression occurs just above the basal layer. An effect on differentiation is indicated in the double-staining of keratin 6 and keratin 10, which reveals a few cells expressing only keratin 10. Therefore, double-staining clearly differentiated the effect of a topical corticosteroid from an emollient, focusing on the TACs as target cells for pharmacological intervention.

Probably owing to the relatively small number of participating patients in this study, the differences mentioned above were not found to be statistically significant by analysing the data paired per patient. However, this study was not designed to investigate the size of the therapeutic effect, but to show the sensitivity of this method. Therefore only mild therapies were used.

In our opinion, comparison of the data using a t-test in a non-paired manner was permitted.

In conclusion, the methodology presented in this communication permits highly sensitive discrimination of anti-psoriatic effect of an active treatment versus an emollient. Corticosteroids affect the suprabasal compartment, probably by affecting the TACs. Further studies on the role of TACs in mediating therapeutic effects of anti-psoriatic treatments are worthwhile.

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