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Epidermal cell DNA content and intermediate filaments keratin 10 and vimentin after treatment of psoriasis with calcipotriol cream once daily, twice daily and in combination with clobetasone 17-butyrate cream or betamethasone 17-valerate cream: a comparative flow cytometric study

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Summary

Calcipotriol and corticosteroids, two therapy modalities frequently prescribed in the treatment of psoriasis, are often used in combination. The aim of the present study was to determine whether the cell biological response pattern of concurrent use of calcipotriol and corticosteroids is different from calcipotriol monotherapy. Forty patients with chronic plaque psoriasis were divided at random in four parallel groups and treated for 8 weeks with: (1) calcipotriol cream (50 μg/g once daily); (2) calcipotriol cream twice daily; (3) calcipotriol and clobetasone 17-butyrate (0·5 mg/g) creams; and (4) calcipotriol and betamethasone 17-valerate (1 mg/g) creams. Before and after treatment keratotome biopsies were taken and single cell suspensions prepared for flow cytometric analysis. Flow cytometric multiparameter quantification of markers for proliferation (TO-PRO-3), differentiation (antikeratin 10) and inflammation (antivimentin) was used to evaluate all four therapy modalities.

A statistically significant decrease of the percentage of basal cells in S- and G_2M-phase (proliferation) was obtained with all therapy modalities, except for calcipotriol monotherapy applied once daily. A significant reduction of the number of vimentin-positive cells (non-keratinocytes) was observed following combined treatment with calcipotriol and clobetasone butyrate. In contrast, monotherapy with calcipotriol had virtually no effect on the number of vimentin-positive cells.

It can be concluded that: (i) calcipotriol monotherapy, applied once daily was less antiproliferative compared with twice daily applications of calcipotriol or the combined treatment with corticosteroids and that (ii) the combination of calcipotriol and corticosteroids proved to have a marked effect on the percentage of non-keratinocytes, in contrast to the modest effect of calcipotriol.

Vitamin D_3 analogues interfere with various characteristics of the psoriatic plaque: epidermal hyperproliferation, impaired differentiation and cutaneous inflammation. *In vitro* studies demonstrated that calcipotriol inhibits proliferation and stimulates differentiation in cultured human keratinocytes. Several inflammatory processes are modulated by calcipotriol.[1–5] *In vivo*, topical application of calcipotriol to psoriatic plaques resulted in decreased epidermal DNA synthesis and a shift of the expression pattern of epidermal cytokeratins towards normalization.[6–9] Furthermore, calcipotriol has been reported to influence different cytokines[10] and to change the numbers of several immunocytes (polymorphonuclear leucocytes, CD1a positive cells, T lymphocytes, monocytes) in psoriatic plaques.[8,9,11,12] Interference with epidermal proliferation has been reported to be the most conspicuous effect of calcipotriol treatment, whereas interference with inflammation is less marked.[9] Using absolute counts, however, a significant reduction of infiltrate cells in the epidermis has been claimed.[8] In this respect it is of relevance that the combination of topical treatment with calcipotriol and low-dose systemic cyclosporin (2 mg/kg per day) proved to be a highly effective combination, indicating that the immunomodulating effect of cyclosporin might compensate for the relatively low immunosuppressive capacity of calcipotriol *in vivo*.[13]

Double-blind, placebo-controlled studies showed that

effective and safe antipsoriatic therapy can be obtained with calcipotriol in a concentration of 50 μg/g.[14,15] In a right–left comparison calcipotriol induced a significantly larger reduction in PASI score compared with 0·1% betamethasone 17-valerate ointment.[16] In a parallel group study no significant difference in the reduction of PASI score was demonstrated between calcipotriol and betamethasone, but patients' efficacy assessment was significantly in favour of calcipotriol.[17] Calcipotriol ointment was also more effective and better accepted than short-contact dithranol therapy.[18] However, an important limitation of calcipotriol monotherapy twice daily is the occurrence of irritative dermatitis in 10–20% of patients.[19] Once daily application of calcipotriol might decrease side-effects while maintaining clinical efficacy. Also concurrent use of topical calcipotriol and topical corticosteroids might reduce the frequency of irritative dermatitis while maintaining or even improving the clinical result. Recently, a large multicentre study was initiated to establish the clinical efficacy and tolerability of a once-daily schedule of calcipotriol treatment and a combination of calcipotriol with a low and a medium strength corticosteroid. The results of this study (Leo Pharmaceutical Products, Denmark, data on file) will be published as a full report.

The aim of the present study was to analyse the response pattern of psoriatic skin with respect to epidermal proliferation (percentage basal cells in S- and G_2M-phase), epidermal differentiation (percentage cells expressing the differentiation marker keratin 10) and cutaneous inflammation (relative number of vimentin positive cells) to various treatment schedules: (1) calcipotriol cream once daily; (2) calcipotriol cream twice daily; (3) calcipotriol cream in combination with clobetasone 17-butyrate (0·5 mg/g); or (4) calcipotriol cream in combination with betamethasone 17-valerate cream (1 mg/g). These response patterns were evaluated using a recently developed flow cytometric multiparameter technique with simultaneous quantification of DNA content and two intermediate filament proteins in epidermal single cell suspensions.[20,21] Single cell suspensions were prepared from skin samples of 40 patients who participated in the above-mentioned comparative double-blind parallel group multicentre study at the Department of Dermatology, Nijmegen.

Materials and methods

Patients and biopsies

Forty patients with stable chronic plaque psoriasis participated in the study after informed consent. No systemic treatment had been used for at least 6 weeks. Excluded were patients who were pregnant, breastfeeding or expected to become pregnant. After a wash-out period of 2 weeks, in which only an emollient (Danatekt$^{(R)}$) was permitted, patients were at random assigned to one of four therapy groups. These consisted of 8 weeks treatment with a morning application of calcipotriol cream 50 μg/g and an evening application of either (1) vehicle of calcipotriol cream, (2) calcipotriol cream 50 μg/g, (3) clobetasone butyrate cream 0·5 mg/g, or (4) betamethasone valerate cream 1 mg/g on psoriatic lesions of extremities and trunk. Assessment of clinical scores for erythema, induration and desquamation (five-point scale) and of the area of the psoriatic lesions was performed every 2 weeks. Patients were withdrawn from the study before 8 weeks treatment if all lesions had cleared. Before and after treatment in total two keratotome biopsies (thickness 0·4 mm, 2 cm^2) were taken from the same test lesion for flow cytometric analysis.

Cell isolation procedure

Epidermal single cell suspensions were prepared as described before.[21] Briefly, the keratotome biopsies were incubated in phosphate-buffered saline (PBS) containing 0·25 mg/ml trypsin (Sigma, St Louis, MO, U.S.A.) and 3·0 mg/ml dithioerythritol (Sigma) for 30 min at 37°C. Then, in PBS containing 10% heat-inactivated newborn calf serum (HINCS, Life Technologies Ltd, Paisley, U.K.) the dermis was separated from the epidermis with a fine forceps. The remaining epidermis was gently mixed on a vortex to loosen the keratinocytes, resulting in a single cell suspension. After discarding the horny layer, the suspension was centrifuged, the supernatant removed and the cells fixed in 70% ice-cold ethanol. The cell suspension was stored at −20°C until staining and flow cytometric analysis.

Staining procedure

Triple labelling was performed, using a DNA fluorochrome combined with two antibodies against intermediate filaments. The procedure has been described by us in detail before.[21] To assess proliferation, the DNA fluorochrome TO-PRO-3 iodide (TP3, Molecular Probes, Eugene, OR, U.S.A.) was used.[20] TP3 intercalates with double-stranded DNA and permits measurement of the proliferative activity of cells by quantification of the percentage of cells in S- and G_2M-phase. As TP3 also

binds to RNA to some extent, it was used in combination with RNase. To study inflammation Vim3B4 (Novocastra Laboratories Ltd, Newcastle upon Tyne, U.K.) was used. This IgG2a-type mouse monoclonal antibody stains vimentin, an intermediate filament-type protein which occurs in mesenchymal cells.[22] In the present study, PMNs, lymphocytes, monocytes, macrophages, melanocytes and Langerhans cells were stained by Vim3B4. The IgG1-type mouse monoclonal antibody RKSE60 (Department of Molecular Biology, University of Maastricht, the Netherlands) was used as differentiation marker. RKSE60 is directed against keratin 10, an intermediate filament-type protein that is expressed in differentiating keratinocytes.[23,24] Three-colour fluorescence was obtained with the fluorochromes fluorescein-isothiocyanate (FITC) and phycoerythrin (PE), which were conjugated to monoclonal goat antibodies against mouse IgG2a and mouse IgG1, respectively (Southern Biotechnology Associates, Birmingham, AL, U.S.A.), in combination with TP3.

Approximately $1-2 \times 10^5$ cells of the cell suspensions were washed in PBS, filtered to remove clumps and horny material, and resuspended in 500 μl of a solution with Vim3B4 diluted 1:50 and RKSE60 diluted 1:15 in PBS. After incubation for 30 min at room temperature in the dark, the cells were washed in PBS containing 1% HINCS, resuspended and incubated for 15 min at 5°C in a solution of 500 μl PBS, containing 2 μl goat–antimouse–PE, 10 μl goat–antimouse–FITC, 10 μl normal goat serum and 5 μl HINCS. After a third washing step DNA staining was performed by addition of 300 μl TP3 (1 μmol/l in PBS) and 50 μl RNase (1 mg/ml in PBS) (Sigma, St Louis, MO, U.S.A.).

Flow cytometric analysis

The flow cytometric measurements and analysis were performed before the treatment codes were revealed. From each sample 5000–10,000 gated cells were measured and analysed using an EPICS® Elite flow cytometer (Coulter, Luton, U.K.) equipped with a dual laser system. PE and FITC were excited with an air-cooled argon ion laser (15 mW, 488 nm). TP3 was excited with a HeNe laser (10 mW, 633 nm). Fluorescence was measured using bandpass filters of 520–530 nm (green, FITC), 555–595 nm (orange, PE) and 670–680 nm (red, TP3). The area/peak ratio of the red signal (DNA) was used to discriminate between doublets of diploid cells (clumps) and real single tetraploid cells.[25] After setting appropriate gates with the EPICS® Elite software percentages of vimentin- and keratin 10-positive cells were calculated. Using Multicycle™ software (Phoenix Flow Systems, San Diego, CA, U.S.A.) the percentages of basal keratinocytes in S- and G_2M-phase of the cell cycle (proliferation) were calculated from DNA histograms.

Statistical analysis

Changes in the relative numbers of vimentin positive cells, keratin 10 positive keratinocytes and basal cells in S- and G_2M-phase before and after treatment were analysed using the paired *t*-test for means (two-tail). Differences between therapies were assessed using the two-sample *t*-test assuming equal variances (two-tail). Analysis of the correlation between clinical and flow cytometric scores was performed by calculation of the Pearson correlation coefficient.

Results

Clinical response

Of 40 included patients 39 completed the present study. One subject was withdrawn at week 4 because of severe generalized itching and worsening of psoriasis. This patient had used calcipotriol cream twice daily for 3 weeks. All four therapy regimens resulted in a statistically significant reduction of PASI scores. Percentages were 50% (calcipotriol once daily, D), 64% (calcipotriol twice daily, DD), 58% (calcipotriol in combination with clobetasone, DC) and 55% (calcipotriol in combination with betamethasone, DB). Between the four therapies no statistically significant difference in the decrease of the PASI score could be demonstrated. Clearance of the test lesions was reached in 10 subjects: values for each therapy group were 2 (D), 1 (DD), 3 (DC) and 4 (DB). In five subjects even clearance of all lesions was reached. These patients all had used calcipotriol in combination with a corticosteroid (DC: 3, DB: 2).

Flow cytometric analysis

From one patient the initial keratotome biopsy proved to contain exclusively the most superficial layers of the epidermis. Therefore, flow cytometric assessment was performed on 76 epidermal cell suspensions obtained from test lesions of 38 patients. The percentage of intact cells per sample (corrected for debris and clumps) (mean ± standard error of mean) was 78·1 ± 1·2 (range 48·7–93·5). The clinical scores and the flow cytometric

analyses of all biopsies before and after treatment are shown in Figure 1. It can be seen that the percentage of vimentin-positive cells correlates with the clinical expression of inflammation, i.e. erythema ($r = 0{\cdot}26$, $P < 0{\cdot}05$), that the relative number of keratin 10 positive keratinocytes (differentiation) correlates inversely with the score for desquamation ($r = -0{\cdot}40$, $P < 0{\cdot}01$), and that the percentage of basal keratinocytes in S- and G_2M-phase (proliferation) correlates with the induration score of the psoriatic test lesions ($r = 0{\cdot}53$, $P < 0{\cdot}0001$).

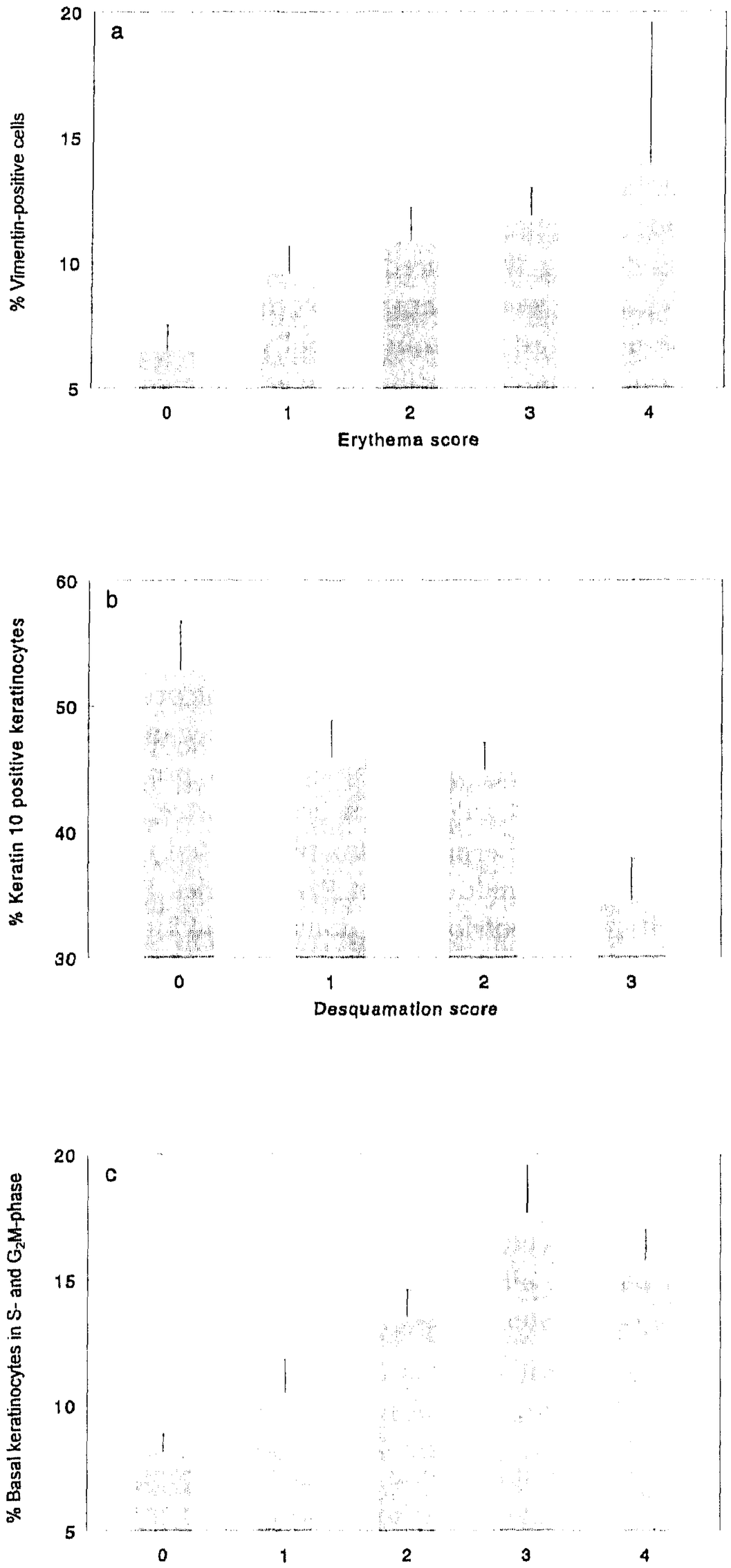

Figure 1. Correlation of clinical scores and flow cytometric analyses (means ± SEM) of all test lesions ($n = 76$). (a) Percentages of vimentin positive cells vs. erythema score. (b) Percentages of keratin 10 positive keratinocytes vs. desquamation score. (c) Percentages of basal keratinocytes in S- and G_2M-phase vs. induration score.

Table 1 summarizes the expression of the markers for proliferation, differentiation and inflammation before and after treatment. All therapy regimens except the once daily application of calcipotriol resulted in a statistically significant decrease of the percentage of basal keratinocytes in S- and G_2M-phase. Following calcipotriol once daily and twice daily the reductions of this parameter were 24% ($P = 0{\cdot}24$) and 34% ($P < 0{\cdot}05$), respectively, whereas the reductions following treatment with calcipotriol/clobetasone 17-butyrate and calcipotriol/betamethasone 17-valerate were 44% ($P < 0{\cdot}01$) and 47% ($P < 0{\cdot}01$), respectively. With respect to the number of vimentin-positive cells a statistically significant decrease was reached in the calcipotriol/clobetasone butyrate-treated group (47%, $P < 0{\cdot}05$). Values for the other therapy groups were 29% ($P = 0{\cdot}19$) for calcipotriol/betamethasone, 21% ($P = 0{\cdot}34$) for calcipotriol once daily and 23% ($P = 0{\cdot}42$) for calcipotriol twice daily. The percentage of differentiated keratinocytes (keratin 10 positive) increased in all therapy groups. Statistical significance was only reached with calcipotriol/clobetasone butyrate (35%, $P < 0{\cdot}05$).

Discussion

From the present flow cytometric evaluation the following conclusions were drawn: (i) twice daily treatment with calcipotriol cream results in a substantial decrease of the percentage of cells in S- and G_2M-phase in the basal cell layer, without a significant effect on the percentage of vimentin-positive cells; (ii) once daily treatment with calcipotriol cream does not result in a significant reduction of the percentage of cells in S- and G_2M-phase in the basal cell layer; (iii) combination of calcipotriol and topical steroids results in a more marked decrease of the epidermal proliferative activity compared with calcipotriol treatment; (iv) a marked reduction of the percentage of vimentin positive cells is observed following the concurrent use of calcipotriol and topical steroids whereas calcipotriol monotherapy has virtually no effect on this marker; and (v) an increase of the relative number of differentiated keratinocytes is only reached after concurrent use of calcipotriol and corticosteroids.

Table 1. Percentages of vimentin-positive cells, keratin 10 positive keratinocytes and basal keratinocytes in S- and G_2M-phase in epidermal cell suspensions prepared from keratotome biopsies taken from test lesions of psoriasis patients during treatment (means ± SEM)

		Vimentin		Keratin 10		S and G_2M	
		Baseline	After treatment	Baseline	After treatment	Baseline	After treatment
Calcipotriol once daily	$n = 9$	10·8 ± 2·0	8·5 ± 1·0	41·0 ± 5·3	50·2 ± 3·9	14·1 ± 2·3	10·8 ± 2·2
Calcipotriol twice daily	$n = 9$	10·9 ± 2·2	8·4 ± 1·1	44·3 ± 4·4	54·3 ± 4·1	15·2 ± 1·2	10·1 ± 1·9*
Calcipotriol/clobetasone 17-butyrate	$n = 9$	14·4 ± 1·8	7·8 ± 0·8*	35·7 ± 3·3	48·2 ± 5·3*	16·1 ± 2·5	8·9 ± 1·0**
Calcipotriol/betamethasone 17-valerate	$n = 11$	15·5 ± 2·8	10·7 ± 1·5	37·9 ± 4·2	40·5 ± 3·1	16·5 ± 2·3	8·7 ± 1·3**

*/** Significant differences (compared with baseline) at the $P < 0{\cdot}05$ and $P < 0{\cdot}01$ level, respectively

In the present study the effect of the different treatment schedules was assessed using flow cytometric quantification of epidermal growth, and of markers for differentiation and inflammation. These markers were chosen in analogy to the clinical features of the psoriatic lesion, i.e. erythema, induration and desquamation that are assessed in the PASI score. In previous studies the methodology was evaluated in epidermal hyperproliferation induced by sellotape stripping,[26] after application of leukotriene B_4 to normal skin[27] and in psoriatic patients before and following treatment.[21] In Figure 1 the correlation between the percentage of basal cells in S- and G_2M-phase and induration, between the percentage of vimentin-positive cells and erythema, and between the percentage of keratin 10 positive keratinocytes and scaling further substantiates the relationship between clinical features of the psoriatic lesion and the cell biological equivalent.

In a previous immunohistochemical study it was shown that calcipotriol ointment twice daily had a minor effect on cutaneous inflammation.[9] In a recent study using the same flow cytometric analytical method it was shown that Tacalcitol® (1α,24 dihydroxyvitamin D_3, 4 μg/g) ointment, applied once daily, had a substantial effect on epidermal proliferation (34% reduction of the percentage basal keratinocytes in S- and G_2M-phase) without a significant effect on the percentage of vimentin-positive cells.[21] The present study on calcipotriol demonstrates a similar preponderance of the antiproliferative effect. As once daily treatment with calcipotriol cream had a minor effect on epidermal growth of the psoriatic lesion, twice daily application seems to be a more optimal approach.

Insight into the *in vivo* action of antipsoriatic treatments helps to define promising combination schedules. The present study indicates that concurrent use of calcipotriol and a topical corticosteroid has a more pronounced effect on epidermal hyperproliferation and inflammation, compared with monotherapy with calcipotriol. In this respect it is of interest that after clobetasol 17-propionate monotherapy, the reduction of the percentage of basal cells in S- and G_2M-phase was 72% and the reduction of the percentage of vimentin positive was 62% (unpublished data). Surprisingly, a marked difference between both combination therapies was observed with respect to the expression of the differentiation-related keratin 10: the increase after treatment with calcipotriol and clobetasone 17-butyrate was 35% and following treatment with the combination of calcipotriol and betamethasone 17-valerate 7%.

In conclusion, the present flow cytometric study lends support for the hypothesis that the effect of calcipotriol once daily is inferior to calcipotriol twice daily and that the combination of calcipotriol and a corticosteroid has a better antipsoriatic efficacy compared with calcipotriol monotherapy.

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References

1 van de Kerkhof PCM. Biological activity of vitamin D analogues in the skin, with special reference to antipsoriatic mechanisms. *Br J Dermatol* 1995; 132: 675–82.

2 Binderup L, Bramm E. Effects of a novel vitamin D_3 analogue MC 903 on cell proliferation and differentiation *in vitro* and calcium metabolism *in vivo*. *Biochem Pharmacol* 1988; 37: 889–95.

3 Kragballe K, Wildfang IL. Calcipotriol MC903, a novel vitamin D_3 analogue, stimulates terminal differentiation and inhibits proliferation of cultured human keratinocytes. *Arch Dermatol Res* 1990; 282: 164–7.

4 Bagot M, Charue D, Leses M-C *et al.* Immunosuppressive effects of 1,25-dihydroxyvitamin D_3 and its analogue calcipotriol on epidermal cells. *Br J Dermatol* 1993; 130: 424–31.

5 Müller K, Svenson M, Bendtzen K. 1,25-dihydroxyvitamin D_3 and a novel vitamin D analogue MC903 are potent inhibitors of human interleukin 1 *in vitro*. *Immunol Lett* 1988; **17**: 361–6.
6 de Mare S, de Jong EMGJ, van de Kerkhof PCM. DNA content and K_s8.12 binding of the psoriatic plaque lesion during treatment with the vitamin D_3 analogue MC903 and betamethasone. *Br J Dermatol* 1990; **123**: 291–5.
7 Holland DB, Roberts SG, Russell A *et al.* Changes in epidermal keratin levels during treatment of psoriasis with the topical vitamin D_3 analogue MC903. *Br J Dermatol* 1990; **122**: 284 (Abstr.)
8 Berth-Jones J, Fletcher A, Hutchinson PE. Epidermal cytokeratin and immunocyte responses during treatment of psoriasis with calcipotriol and betamethasone valerate. *Br J Dermatol* 1992; **126**: 356–61.
9 de Jong EMGJ, van de Kerkhof PCM. Simultaneous assessment of inflammation and epidermal proliferation in psoriatic plaques during long-term treatment with the vitamin D_3 analogue MC903: modulations and interrelations. *Br J Dermatol* 1991; **124**: 221–9.
10 Oxholm A, Oxholm P, Staberg B, Bendtzen K. Expression of interleukin-6-like molecules and tumour necrosis factor after topical treatment of psoriasis with a new vitamin D analogue (MC903). *Acta Derm Venereol (Stockh)* 1989; **69**: 385–90.
11 Verburgh CA, Nieboer C. Local application of vitamin D_3 derivative MC903 in psoriasis: influence on cellular infiltrate, Langerhans cells and keratinocyte (KC) markers. *J Invest Dermatol* 1989; **93**: 310 (Abstr.)
12 Mallett RB, Coulson IH, Purkis PE *et al.* An immunohistochemical analysis of the changes in the immune infiltrate and keratin expression in psoriasis treated with calcipotriol compared with betamethasone ointment. *Br J Dermatol* 1990; **123**: 837 (Abstr.)
13 Grossman RM, Thivolet J, Claudy A *et al.* A novel therapeutic approach to psoriasis with combination calcipotriol ointment and very low-dose cyclosporine: Results of a multicenter placebo-controlled study. *J Am Acad Dermatol* 1994; **31**: 68–74.
14 Kragballe K. Treatment of psoriasis by the topical application of the novel cholecalciferol analogue calcipotriol (MC 903). *Arch Dermatol* 1989; **125**: 1647–52.
15 Dubertret L, Wallach D, Souteyrand P *et al.* Efficacy and safety of calcipotriol (MC903) ointment in psoriasis vulgaris. *J Am Acad Dermatol* 1995; **27**: 983–8.
16 Kragballe K, Gjertsen BT, de Hoop D *et al.* Double-blind, right/left comparison of calcipotriol and betamethasone valerate in treatment of psoriasis vulgaris. *Lancet* 1991; **337**: 193–6.
17 Cunliffe WJ, Berth-Jones J, Claudy A *et al.* Comparative study of calcipotriol (MC 903) ointment and betamethasone 17-valerate ointment in patients with psoriasis vulgaris. *J Am Acad Dermatol* 1992; **26**: 736–43.
18 Berth-Jones J, Chu AC, Dodd WAH *et al.* A multicentre, parallel-group comparison of calcipotriol ointment and short-contact dithranol therapy in chronic plaque psoriasis. *Br J Dermatol* 1992; **127**: 266–71.
19 van de Kerkhof PCM. Vitamin D_3 und seine Analoge in der Dermatologie. *Z Hautkr* 1994; **69**: 219–26.
20 van Hooijdonk CAEM, Glade CP, van Erp PEJ. TO-PRO-3 iodide, a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry. *Cytometry* 1994; **17**: 185–9.
21 Glade CP, van Erp PEJ, van Hooijdonk CAEM *et al.* Topical treatment of psoriatic plaques with 1α,24 dihydroxyvitamin D_3: a multiparameter flow cytometrical analysis of epidermal growth, differentiation and inflammation. *Acta Derm Venereol (Stockh)* 1995; **75**: 381–5.
22 Bauer FW, Boezeman JBM, van Engelen L *et al.* Monoclonal antibodies for epidermal population analysis. *J Invest Dermatol* 1986; **87**: 72–5.
23 Ramaekers FCM, Puts JJ, Moesker O *et al.* Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours: an overview. *Histochem J* 1983; **15**: 691–713.
24 Leigh IM, Purkis PE, Whitehead P, Lane EB. Monospecific monoclonal antibodies to keratin 1 carboxy terminal (synthetic peptide) and to keratin 10 as markers of epidermal differentiation. *Br J Dermatol* 1993; **129**: 110–19.
25 Bauer FW, Boezeman JBM. Flow cytometric methods in human skin with respect to cell cycle kinetics. In: *Psoriasis: Cell Proliferation* (Wright NB, Camplejohn RS, eds). Edinburgh: Churchill Livingstone, 1983; 104–16.
26 Glade CP, Seegers BAMPA, Meulen EFJ *et al.* Multiparameter flow cytometric characterization of epidermal cell suspensions prepared from normal and hyperproliferative skin using an optimized thermolysin–trypsin protocol. *Arch Dermatol Res* 1996; **288**: 203–10.
27 Glade CP, Botermans RJG, van Erp PEJ, van de Kerkhof PCM. The dynamics of the response of normal skin to single and multiple epicutaneous leukotriene B_4 applications analysed by three-colour flow cytometry. *Acta Derm Venereol (Stockh)* 1995; **25**: 437–40.