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## INVESTIGATIVE REPORT

# A Left/Right Comparison of Twice-Daily Calcipotriol Ointment and Calcitriol Ointment in Patients with Psoriasis: The Effect on Keratinocyte Subpopulations

Mannon E.J. FRANSSEN, Gys J. DE JONGH, Piet E.J. VAN ERP and Peter C.M. VAN DE KERKHOF

*Department of Dermatology, University Medical Centre Nijmegen, The Netherlands*

Vitamin D3 analogues are a first-line treatment of chronic plaque psoriasis, but so far, comparative clinical studies on calcipotriol and calcitriol ointment are sparse, and in particular no comparative studies are available on cell biological effects of these compounds *in vivo*. Using flow cytometric assessment, we investigated whether these compounds had different effects on the composition and DNA synthesis of epidermal cell populations responsible for the psoriatic phenotype. For 8 weeks, 20 patients with psoriasis vulgaris were treated twice daily with calcipotriol and calcitriol ointment in a left/right comparative study. Before and after treatment, clinical assessment of target lesions was performed, together with flow cytometric analysis of epidermal subpopulations with respect to keratin (K) 10, K6, vimentin and DNA distribution. Treatment with each compound resulted in a substantial clinical improvement, a reduction of the K10–K6– population and an increase of the K10+K6– population. A correlation was found between the clinical response of calcipotriol and the K10+K6– population, and the clinical response of calcitriol and the K10+K6– population, as well as the percentage of cells in the S, G<sub>2</sub> and M phase of the cell cycle within the K10–K6– population, suggesting that the analogues have a different preference for affecting the K10+K6– pool (committed differentiated cells) or affecting the K10–K6– pool (basal cells). **Key words:** calcitriol; calcipotriol; psoriasis; keratinization; proliferation; flow cytometry.

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M.E.J. Franssen, Department of Dermatology, University Medical Centre Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands. E-mail: m.franssen@derma.umcn.nl

For several years, vitamin D3 derivatives have been used in the topical treatment of psoriasis. These derivatives are thought to inhibit proliferation and promote differentiation of keratinocytes of lesional psoriatic skin. As disturbed keratinization and increased proliferation, together with inflammation, are key marks of psoriasis, a number of analogues were investigated for their anti-psoriatic potential.

Calcipotriol (Daivonex<sup>®</sup>, 50 µg/g ointment, Leo Pharmaceutical Products, Denmark) has been investigated intensively during the last decade, and has proven to be a valuable tool in the management of chronic plaque psoriasis. A review by Ashcroft et al. (1), based on a large number of randomized controlled trials, showed that calcipotriol was at least as effective as potent topical corticosteroids, 1 $\alpha$ ,25-dihydroxycholecalciferol (calcitriol), short-contact dithranol, tacalcitol and coal tar. Recently, Scott et al. (2) presented an overview of studies on the use of calcipotriol ointment in the management of psoriasis. They reconfirmed the superior efficacy of a twice-daily calcipotriol ointment regimen to the treatments as mentioned above, and concluded that calcipotriol ointment is valuable as a first- or second-line therapy option for the management of mild to moderate psoriasis.

In the last decade, calcitriol ointment (Silkis<sup>®</sup>, 3 µg/g ointment, Galderma Laboratories, France) has been added to the armamentarium available for the topical treatment of psoriasis vulgaris. A few clinical studies have been performed to establish the efficacy of calcitriol ointment as an anti-psoriatic drug. A review by Kowalzick (3) concluded that calcitriol applied twice daily was as effective as short-contact dithranol treatment (4). Furthermore, combining calcitriol with other anti-psoriatic therapies such as ultraviolet B phototherapy or topical corticosteroids was as effective as each therapy alone and thereby proved to have a radiation- and corticosteroid-sparing effect (3, 5, 6). To date, however, conclusive comparative clinical studies between calcipotriol and calcitriol are not available.

Active vitamin D3 inhibits cell proliferation, induces cell differentiation and modulates inflammation. Treatment with calcipotriol ointment applied twice daily for 8 weeks has been reported to normalize nearly completely the morphological appearance of the psoriatic lesion, except for persistence of capillary enlargement (2). Findings from our group included a significant reduction of the number of neutrophils, a reduction of epidermal cell recruitment and expression of keratin (K) 16, as a marker of abnormal keratinization (7–9). Immunohistochemical data of a calcitriol ointment regimen, showed a significant decrease in the number of Ki-67-positive cells, and a significant increase in filaggrin staining after 4 weeks of twice-daily treatment,

together with modulation of the dermal infiltrate with a reduction of neutrophils and T cells (10). These data suggested that a reduction of epidermal proliferation, normalization of keratinization and modulation of dermal inflammatory cells are important *in vivo* effects of calcipotriol and calcitriol.

As the receptor binding of calcitriol and calcipotriol are comparable, the biological effects of both vitamin D3 analogues are likely to be similar. However, the pharmacokinetics of these compounds are markedly different. Vitamin D serum protein binding of calcipotriol is less and the inactivation of calcipotriol is much faster compared with calcitriol (11, 12). The concentrations of calcipotriol (50 µg/g) and calcitriol ointment (3 µg/g) are also highly different. Therefore a comparative analysis regarding the cell biological action spectrum is indicated.

The aim of the present study was to compare a regimen of calcipotriol and calcitriol ointment applied twice daily in patients with chronic plaque psoriasis to elucidate the behaviour of the suprabasal and basal compartment, and to establish the biological effect of each therapy on different epidermal cell populations responsible for the psoriatic phenotype in a within-subject comparative study. The relationship between these effects and clinical responses was investigated.

## MATERIALS AND METHODS

### *Subjects and study design*

The present study was performed as an open left/right comparative study between calcipotriol ointment and calcitriol ointment in patients with psoriasis vulgaris. After giving informed consent, 20 patients from our outpatient department (12 men and 8 women, mean age 54 years, range 29–71) with psoriasis vulgaris participated. Patients were diagnosed with stable and mild to moderate chronic plaque psoriasis. Patients had symmetrical psoriatic lesions on the trunk and/or on extremities. Within at least one body region the score for extent, as part of the Psoriasis Area and Severity Index (PASI) score was at least 2. Patients who were pregnant, lactating or expected to become pregnant were excluded. Patients must not have received any systemic anti-psoriatic treatment or (chemo-) phototherapy during the 6 weeks prior to study enrolment. All topical treatments except emollients were discontinued for 2 weeks prior to enrolment. Patients with a known or suspected abnormality of calcium metabolism or hypercalcaemia were excluded. Exposure to sun, UVB or UVA light was not allowed during the entire study period.

Patients were instructed to treat all psoriatic lesions twice daily with calcitriol ointment on the left side, and calcipotriol ointment on the right side of the body. Psoriatic lesions in the flexures or on the face were included. In case of scalp psoriasis, patients were allowed to use a moderate corticosteroid formulation three times a week on the scalp. Patients were instructed to wash their hands between the application of each ointment and after the applications.

Efficacy of both treatments was evaluated by clinical scoring before treatment and after 8 weeks of treatment. The PASI was used as an overall assessment (13). The target lesions were at least 15 cm<sup>2</sup> and located symmetrically on the

trunk or extremities. Using a scale ranging from 0 (no) to 8 (severe), the degree of erythema, induration and desquamation was estimated for each target lesion, resulting in a sum of scores. At each visit, the target lesions were outlined using transparent paper, and the area of each target lesion was calculated. Using the same method, a global sum score and global area was calculated for each side of the body.

### *Biopsy procedure*

Before and after 8 weeks of treatment, 3-mm punch biopsies (Stieffel, Offenbach am Main, Germany) were taken from the target lesions on the trunk and extremities. Biopsies were obtained after local anaesthesia with xylocaine/adrenaline 1:100 000 (Astra Pharmaceutica BV, Zoetermeer, The Netherlands) and kept in phosphate-buffered saline (PBS, NPBI, Emmer-Compascuum, The Netherlands) at 4°C.

### *Cell isolation procedure*

Epidermal single cell suspensions were prepared as described previously (14). In brief, biopsies were placed in an overnight solution of 2.5 ml PBS with Ca<sup>2+</sup>/Mg<sup>2+</sup>, containing 0.5 mg/ml thermolysin (Protease type X, Sigma, St Louis, MO, USA) and kept at 4°C. After incubation, the epidermis and dermis were gently separated using two fine forceps, and the epidermis was incubated at 37°C in 2.5 ml PBS, containing 0.025 mg/ml trypsin (Sigma) and 0.3 mg/ml dithioerythritol (DTE, Sigma). After 30 min, the trypsinization was stopped by adding 10% newborn bovine serum (NBS), and the cell suspension was gently mixed on a vortex, allowing keratinocytes to detach from the remaining stratum corneum. After centrifuging and decanting the supernatant, the remaining cell pellet was fixed in 70% ethanol and kept at -20°C until further processing.

### *Staining procedure*

The staining procedure and the flow cytometric assessment have been described previously (15). We adjusted the protocol by adding a marker for inflammation (16). In brief: a four-parameter protocol allowed simultaneous assessment of epidermal proliferation, hyperproliferation-associated differentiation, keratinization and inflammation. An IgG<sub>1</sub>-isotype mouse monoclonal antibody directed against cytokeratin 10, RKSE60 (clone RKSE60, Sanbio, Uden, The Netherlands), was used to assess epidermal differentiation. To assess hyperproliferation-associated differentiation, LHK6B (NCL-CK6, Novocastra Laboratories, New Castle upon Tyne, UK), an IgG<sub>2a</sub>-isotype mouse monoclonal antibody directed against cytokeratin 6 was used. Mesenchymal cells were measured by using V9 (clone V9, Sanbio, Uden, The Netherlands), an IgG<sub>1</sub>-isotype mouse monoclonal antibody directed against vimentin.

A sample of approximately 2 × 10<sup>5</sup> cells from each cell suspension was washed, and incubated with a solution containing RKSE60, LHK6B and V9 at a dilution of 1:20 in the dark for 30 min at room temperature. After a washing step, a solution containing goat anti-mouse (GAM) IgG<sub>1</sub> conjugated with phycoerythrin (GAM-PE, Southern Biotechnology Associates, Birmingham, AL, USA) at a dilution of 1:100, and goat anti-mouse IgG<sub>2a</sub> conjugated with fluorescein isothiocyanate (GAM-FITC, Southern Biotechnology Associates) at a dilution of 1:50 was added and incubated on ice for 15 min. As a DNA stain 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes, Eugene, OR, USA) was diluted 1000 times in PBS containing

0.1% Triton X-100 and added to each sample, with a final concentration of 1  $\mu$ M.

#### Flow cytometric analysis

After the staining procedure, all samples were measured and analysed with an EPICS Elite flow cytometer (Coulter, Luton, UK) with a dual laser system. An air-cooled argon ion laser (20 mW, 488 nm) excited PE and FITC, while a water-cooled argon ion laser tuned at 365 nm (80 mW) excited DAPI. By separating the two laser beams spatially, the fluorescent signals were detected 40  $\mu$ s apart. Finally, bandpass filters at 450 nm (DAPI), 525 nm (PE) and 575 nm (FITC) conducted the signals to photomultipliers and a gated amplifier.

The area/peak ratio of the DAPI signal (DNA) combined with scatter parameters was used to exclude debris, and to discriminate between doublets and real tetraploid cells, as described previously (17). Using WinList software (WinList 4.0, Verity Software House, Topham, ME, USA), regions were set for K10-positive cells, K6-positive cells, K6/K10 co-expressing cells and vimentin-positive cells. A count stop was set at 10 000 single cells. Using ModFit software (ModFit LT 2.0, Verity Software House), the proportion of cells in different phases of the cell cycle was calculated, as described previously (18).

#### Statistical analysis

For estimating the changes in the clinical scores (PASI, sum of scores, area) during both treatments, we performed a repeated two-way analysis of variance (ANOVA). The changes in the epidermal subpopulations during treatment with calcipotriol and calcitriol ointment were also analysed by a repeated two-way ANOVA. To establish the relationship between changes in clinical scores and changes in the epidermal subpopulations, a stepwise multiple regression

analysis was performed and a correlation coefficient was determined.

## RESULTS

#### Clinical response

The PASI score before treatment was  $7.89 \pm 0.58$  ( $n=20$ ) and the percentage of body surface involved was  $3.09 \pm 0.36$  for the calcipotriol-treated side and  $3.23 \pm 0.37$  for the calcitriol-treated side. These values indicate that patients suffered from mild to moderate disease. Finally, after 8 weeks of treatment, the PASI score showed an overall decrease of 54% from  $7.89 \pm 0.58$  to  $3.61 \pm 0.42$  ( $P < 0.001$ ).

The sum of scores for the target lesion showed a remarkable improvement during 8 weeks of treatment for both therapies, as depicted in Fig. 1a. Treatment with calcipotriol resulted in a decrease of 74% from  $12.6 \pm 0.93$  to  $3.25 \pm 0.51$  after 8 weeks ( $P < 0.001$ ). Treatment with calcitriol gave an improvement of the sum of scores of 65% from  $12.85 \pm 0.92$  to  $4.55 \pm 0.52$  in 8 weeks ( $P < 0.001$ ).

Treatment with both vitamin D analogues resulted in a significant decrease in the area of the target lesion after 8 weeks, as shown in Fig. 1b. Treatment with calcipotriol resulted in a decrease of 43% to  $21.85 \pm 3.63$  after 8 weeks of treatment ( $P < 0.05$ ). During calcitriol treatment, the affected area improved by 48% after 8 weeks to  $19.85 \pm 2.87$  ( $P < 0.05$ ). The global sum of scores improved significantly during 8 weeks of treatment for both calcipotriol and calcitriol ointment,

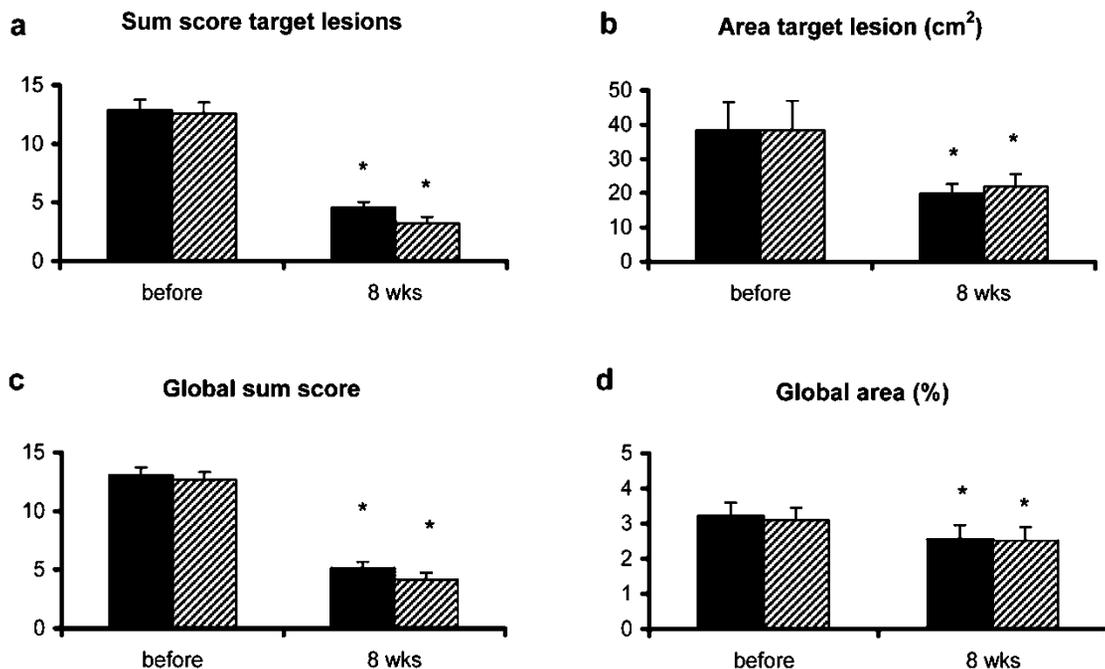


Fig. 1. Mean sum of scores  $\pm$  SEM (a) and mean area (cm<sup>2</sup>)  $\pm$  SEM (b) for the target lesions before and after treatment with calcitriol (black bars) and calcipotriol (striped bars). Global sum of scores  $\pm$  SEM for all treated lesions (c) and global area (%)  $\pm$  SEM for all treated lesions (d) before and after treatment with calcitriol (black bars) and calcipotriol (striped bars).

which is illustrated in Fig. 1c. The global area score showed a significant decrease after 8 weeks of vitamin D treatment (Fig. 1d).

#### Changes in epidermal subpopulations

No significant changes in the percentage of vimentin-positive cells before and after each treatment were detected. Regarding the subpopulations of K10- and/or K6-expressing cells (depicted in Fig. 2a and b), the subpopulation of K10+K6- cells showed a significant increase from  $23.05\% \pm 4.13$  to  $39.25\% \pm 4.50$  during calcipotriol treatment ( $P < 0.05$ ). During calcitriol treatment, the number of K10+K6- cells increased from  $23.52\% \pm 4.07$  to  $32.56\% \pm 4.34$ ; however, this increase was not significant. No significant differences were found between both therapies. The K10-K6- expressing cells showed a significant decrease from  $48.92\% \pm 2.85$  to  $40.10\% \pm 2.62$  during calcipotriol treatment ( $P < 0.05$ ). During calcitriol treatment, the number of K10-K6- cells decreased from  $50.80\% \pm 2.2$  to  $43.98\% \pm 2.58$ , but again this was not significant. No significant differences with respect to the changes in the K10-K6- population were found between both therapies. The subpopulation of K10+K6+ cells and K10-K6+ cells showed no significant changes during both calcipotriol and calcitriol treatment.

With regard to the overall K10 or K6 expression, overall K10 expression showed a significant increase during calcipotriol treatment from  $42.52\% \pm 3.73$  to  $54.82\% \pm 3.11$  ( $P < 0.05$ ). Treatment with calcitriol resulted in an increase of K10 expression from  $41.10\% \pm 3.07$  to  $49.32\% \pm 3.34$ , but this was not significant. No significant differences with respect to these changes were found between the two therapies. The overall K6 expression did not show significant changes during either calcipotriol or calcitriol treatment. Assessment of cells in the S, G<sub>2</sub> and M phase of the cell cycle within the K10-K6- compartment showed no significant changes during either treatment.

#### Comparing clinical results with changes in epidermal subpopulations

A stepwise multiple regression analysis was performed, correlating the changes in clinical scores to changes in

the different epidermal subpopulations. As clinical scores, we assessed the changes in the sum score and the area of the target lesion, together with changes in the sum score and the area of all treated lesions during both calcipotriol treatment and calcitriol treatment. Using flow cytometric analysis, changes in epidermal subpopulations with respect to K10- or K6-expressing cells, K10/K6 co-expressing cells, vimentin-positive cells, and the percentage of cells in SG<sub>2</sub>M phase of the cell cycle in the population of K10-K6- cells were assessed. During anti-psoriatic treatment, changes in the composition of epidermal populations resulted in the improvement of the clinical scores of a psoriatic lesion. Therefore, a stepwise multiple regression analysis was performed, linking the effect of each vitamin D3 treatment on a clinical score to a specific epidermal subpopulation which covariates with the changes in the clinical score. An example is shown in Fig. 3; it illustrates the effect of calcipotriol treatment on the global sum of scores, which is predicted from the changes in the K10+K6- subpopulation. In Table I, the results of all clinical scores are depicted, together with the correlation coefficients (R) for each corresponding epidermal subpopulation. As can be seen, clinical improvement during calcipotriol treatment, which resulted in a decrease of both the sum score of the target lesion and of all treated lesions, is highly correlated with the changes in the K10+K6- subpopulation. Clinical improvement during calcitriol treatment resulting in a decrease of the global sum score, correlated with changes in the K10+K6- subpopulation, and with changes in the percentage of cells in the SG<sub>2</sub>M phase of the cell cycle in the K10-K6- compartment. The correlating epidermal subpopulations are depicted, which correlate with the decrease in the global sum score during calcitriol treatment. As can be seen, the  $\beta$  coefficient of the percentage of cells in the SG<sub>2</sub>M phase of the cell cycle is five times higher than the  $\beta$  coefficient for the K10+K6- subpopulation, suggesting a preferential effect of calcitriol on this subpopulation.

#### DISCUSSION

In the present study, a relevant clinical improvement was found after treatment with both calcipotriol and

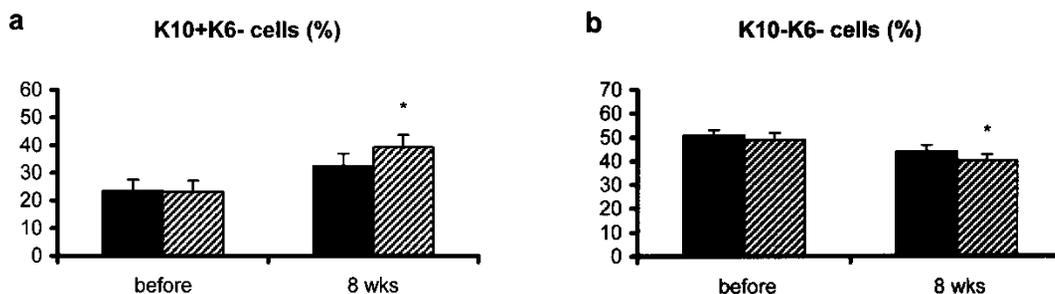


Fig. 2. Mean percentage of K10+K6- cells ( $\pm$ SEM) (a) and mean percentage of K10-K6- cells ( $\pm$ SEM) (b) before and after 8 weeks of treatment with calcitriol (black bars) and calcipotriol (striped bars).

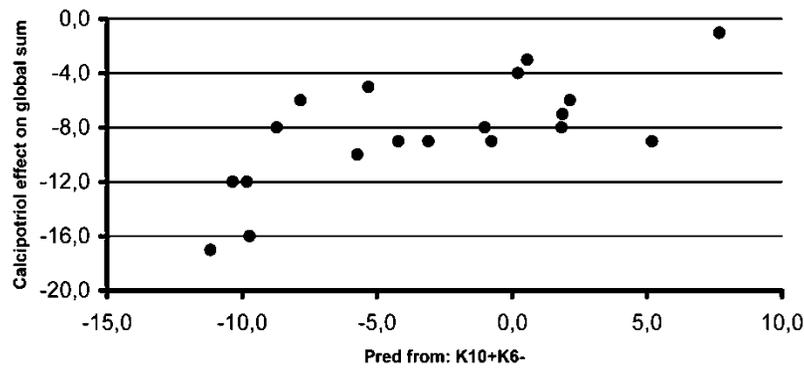


Fig. 3. An example of a stepwise multiple regression test, correlating changes in clinical scores to changes in epidermal subpopulations during treatment. The correlation of the calcipotriol effect on the global sum score of all treated lesions and the K10+K6- population is depicted.

Table I. Results of a stepwise multiple regression test, correlating changes in clinical scores to changes in epidermal subpopulations during treatment with calcitriol and calcipotriol

Ointment	Clinical score	R (corr. coeff.)	Corresponding epidermal subpopulation	P value
Calcipotriol	Sum target	0.716	K10+K6-	0.000258
Calcipotriol	Area target	0.675	K10-K6+	0.058853
Calcipotriol	Sum global	0.713	K10+K6-	0.000287
Calcipotriol	Area global	Not available		
Calcitriol	Sum target	0.640	%SG <sub>2</sub> M K10-K6-	0.008417
Calcitriol	Area target	Not available		
Calcitriol	Sum global	0.709	K10+K6-*	0.044621
Calcitriol	Sum global	0.709	%SG <sub>2</sub> M K10-K6-**	0.008141
Calcitriol	Area global	0.543	K10+K6-	0.011031

\* $\beta$  coefficient  $-0.404$ , \*\* $\beta$  coefficient  $2.085$ . Notice a  $\beta$  coefficient 5 times higher for the percentage of cells in SG<sub>2</sub>M phase of the cell cycle in the K10-K6- population as compared with the K10+K6- population.

calcitriol ointment twice daily. The overall PASI score, comprising both body sides, improved by 54%, which is in line with percentages found in other studies (4, 10, 19, 20). Both treatments resulted in a clinically relevant improvement as judged by the investigator and experienced by the patient.

Regarding the effect of both calcipotriol and calcitriol ointment on the epidermal cell populations responsible for the psoriatic phenotype, a flow cytometric assessment of epidermal keratinization, proliferation and inflammation showed only mild changes in these subpopulations. We did find a significant increase in the K10+K6- subpopulation and a significant decrease in the K10-K6- subpopulation during calcipotriol treatment. During calcitriol treatment a similar tendency was found, without reaching the level of statistical significance. These data suggest a normalization of keratinization, together with a decrease in the number of basal cells. However, we could only observe a tendency towards normalization in the number of hyperproliferative K10-K6+ cells; again without reaching the level of significance. Furthermore, we did not observe a statistically significant change in the number of cells in the S, G<sub>2</sub> or M phase of the cell cycle. Gerritsen et al. (10) reported a significant

decrease in the number of Ki-67-positive cells after 4 weeks of calcitriol treatment. With respect to the *in vivo* effects of calcitriol, no analyses of the percentage of cells in the S, G<sub>2</sub> or M phase of the cell cycle using flow cytometry have been reported. Treatment with calcipotriol also showed a significant decrease in the Ki-67-positive cells, according to van der Vleuten et al. (8). Furthermore, de Mare et al. (9) reported a significant decrease of cells in the SG<sub>2</sub>M phase of the cell cycle during calcipotriol treatment. However, the previous flow cytometric analysis has been carried out by single parameter flow cytometry, and it is highly likely that differences in epidermal subpopulations have confused these previous results, suggesting differences in the percentage of cells in the SG<sub>2</sub>M phase of the cell cycle.

Taking these findings together, vitamin D3 analogues are effective in the treatment of psoriasis, but the mode of action has still to be elucidated. Because of the *in vitro* effects of vitamin D3 and its derivatives, one may speculate whether the hormone acts on keratinocytes, antigen-presenting cells, T cells or a combination of these. However, *in vivo* studies as described above indicate that the effect of vitamin D3 analogues in psoriasis is primarily due to an effect on keratinocyte proliferation.

We performed a correlation test linking the clinical results to the flow cytometric data. As depicted in Table I, clinical improvement during calcipotriol treatment correlated with changes in the subpopulation of K10+K6- cells. This suggests a preferential effect of calcipotriol on epidermal differentiation, resulting in normalization of keratinization. During calcitriol treatment, a similar correlation was found between the clinical changes and the K10+K6- subpopulation. Nevertheless, a fivefold stronger correlation was found with the number of cells in the SG<sub>2</sub>M phase of the cell cycle within the K10-K6- subpopulation. This suggested not only an effect of calcitriol on suprabasal keratinocytes resulting in normalization of differentiation, but even more distinctly treatment with calcitriol seemed to have a more preferential effect on the basal compartment in the epidermis.

Antiproliferative and keratinization-enhancing effects of vitamin D3 analogues are mediated by activation of the vitamin D3 receptor. *In vitro* studies have not revealed any difference in this respect between calcipotriol and calcitriol. In the *in vivo* situation, however, the differences between both treatments with respect to pharmacokinetics could well have an impact on the skin compartment on which each of them might have a preferential effect. Indeed, the present study suggests that the K10+K6- population is more relevant to the anti-psoriatic efficacy of calcipotriol as compared to the effects on the K10- population, whereas both the K10+ (suprabasal compartment comprising committed differentiated cells) and K10- populations (basal compartment comprising stem cells and transit amplifying cells) are relevant to the effects of calcitriol.

Further studies reconciling the keratinocyte subpopulations (stem cells, transit amplifying cells and committed keratinizing cells) may help to define more precisely the *in vivo* mode of action of treatments that interfere with epidermal proliferation and differentiation.

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