

Topical Treatment of Psoriatic Plaques with $1\alpha,24$ Dihydroxyvitamin D_3 : A Multiparameter Flow Cytometrical Analysis of Epidermal Growth, Differentiation and Inflammation

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The clinical efficacy and tolerability of the vitamin D_3 analogues calcitriol, calcipotriol and $1\alpha,24$ dihydroxyvitamin D_3 in the treatment of psoriasis have been assessed in various clinical studies. *In vitro* and *in vivo* investigations have shown interference of these compounds with epidermal growth, keratinisation and inflammation. In this study we quantified the *in vivo* cell biological effects during treatment of psoriatic plaques with $1\alpha,24$ dihydroxyvitamin D_3 . By using a flow cytometric triple labelling procedure, we could discriminate different epidermal subpopulations, permitting precise assessment of epidermal cell cycle kinetics. Twenty patients with plaque-type psoriasis were treated in a double-blind placebo-controlled left-right comparative study with $1\alpha,24$ dihydroxyvitamin D_3 ointment (4 $\mu\text{g/g}$ applied once daily) for 8 weeks. Epidermal cell suspensions prepared from keratome biopsies taken before and after treatment were stained with TO-PRO-3 iodide (a new DNA fluorochrome) and monoclonal antibodies against keratin 10 (as a marker for differentiation) and vimentin (as a marker for inflammation), simultaneously. The flow cytometric analyses showed a significant decrease of proliferating basal keratinocytes in verum-treated lesions, whereas such a decrease was not observed in placebo-treated lesions. The amount of keratin 10-positive keratinocytes increased and the presence of vimentin-positive cells decreased in cell suspensions derived from both verum- and placebo-treated lesions, but these effects were not significant. We conclude that multiparameter flow cytometry promises to be an adequate approach to assess the interference of antipsoriatic treatments with cutaneous inflammation, epidermal proliferation and keratinisation. Topical $1\alpha,24$ dihydroxyvitamin D_3 seems to exert its *in vivo* antipsoriatic effect mainly through an inhibition of epidermal growth. **Key words:** skin; therapy; epidermal proliferation; cytometry.

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In 1985 Morimoto & Kumahara described a patient with senile osteoporosis whose psoriasis cured after oral administration of 1α hydroxyvitamin D_3 , a prodrug of calcitriol (1). Their observation caused a renewed interest in the class of vitamin D_3 analogues as an antipsoriatic principle (2, 3). During the last decade the clinical efficacy of $1\alpha,25$ dihydroxyvitamin D_3 (calcitriol) (4–8) and several of its derivatives, including 1α monohydroxyvitamin D_3 (4), calcipotriol (9–12) and $1\alpha,24$ dihydroxyvitamin D_3 ($1\alpha,24$ (OH) $_2$ D_3) (Tacalcitol) (13–15) has been documented. Calcipotriol (50 $\mu\text{g/g}$), $1\alpha,24$ (OH) $_2$ D_3 (2–4 $\mu\text{g/g}$) and calcitriol (3–15 $\mu\text{g/g}$) can be applied topically. Calcipotriol

and $1\alpha,24$ (OH) $_2$ D_3 exert a decreased calcitropic effect compared to calcitriol (15, 16). Furthermore, neither hypercalcemia nor hypercalciurea were observed in patients who received a daily total dose of 80 μg $1\alpha,24$ (OH) $_2$ D_3 for 7 days (17). Calcitriol induced irritation of the skin at concentrations of 15 $\mu\text{g/g}$ in 6.3% of the patients, whereas concentrations of 3 $\mu\text{g/g}$ caused irritation in 3.4% of patients (7, 8). Irritation of the skin, especially of the face, was seen in 4.3–19.5% of patients treated with calcipotriol (11, 18). Remarkably, with respect to $1\alpha,24$ (OH) $_2$ D_3 , so far, only a slight irritation has been reported in less than 1% of patients (15). Therefore, $1\alpha,24$ (OH) $_2$ D_3 has expanded the horizon of vitamin D_3 treatment of psoriasis, as facial and flexural lesions can be treated with this analogue without significant irritation.

In vitro data on mouse and human keratinocytes and *in vivo* data on guinea pigs, but also studies in psoriatic patients, have shown the capability of $1\alpha,24$ (OH) $_2$ D_3 to suppress DNA synthesis and to induce epidermal differentiation (16, 19–22). These findings have been confirmed in psoriasis *in vivo* by a previous immunohistochemical study from our department, showing a substantial inhibitory effect of $1\alpha,24$ (OH) $_2$ D_3 with respect to epidermal proliferation and a significant modulation of cutaneous inflammation and keratinisation (23). Histological scoring of stained sections, however, is semi-quantitative. A more objective and quantitative method is required for an accurate and reliable assessment of the effect of antipsoriatic therapy.

Multiparameter flow cytometry allows a simultaneous, quantitative, statistically accurate analysis of different cell parameters and is a useful tool for studies on skin pathology (24, 25). Triple labelling combining double immunophenotyping and assessment of DNA content is impeded by a considerable spectral overlap (26–28). Only to a limited extent could these difficulties be overcome (29, 30). Recently, by using the new DNA stain TO-PRO-3 iodide (TP3), we were able to design a procedure in which DNA content and expression of two intermediate filament proteins were measured simultaneously in single cell suspensions from epidermis (31). Simultaneous discrimination and quantification of three different epidermal subpopulations, i.e. non-keratinocytes, differentiated and basal keratinocytes, became possible, allowing a precise quantification of the proliferative activity of the basal compartment.

The aim of the present study was to quantify flow cytometrically in terms of epidermal proliferation, differentiation and inflammation, the response of the psoriatic lesion to a therapeutical concentration of $1\alpha,24$ (OH) $_2$ D_3 (4 $\mu\text{g/g}$ Tacalcitol ointment applied once daily).

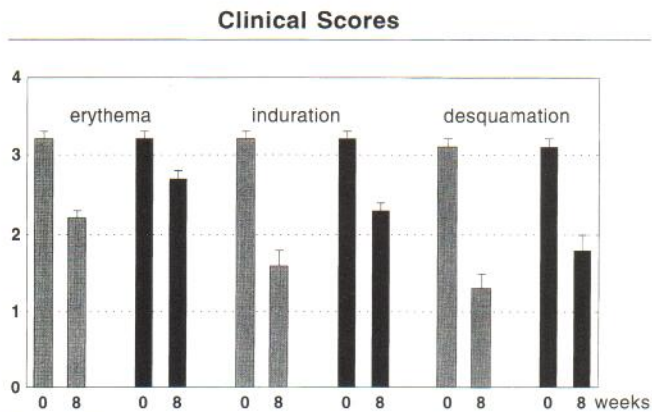


Fig. 1. Clinical scores before and after 8 weeks' treatment with $1\alpha,24$ dihydroxyvitamin D_3 ($4 \mu\text{g/g}$) ointment (shaded bars) and ointment base only (black bars).

MATERIALS AND METHODS

Patients and skin samples

Twenty patients (11 females and 9 males; age range 22–66 years, mean age 43.5 years) with symmetrically distributed chronic plaque psoriasis participated in the investigation, after informed consent had been given. The mean duration of psoriasis was 20.2 years. The study design was a placebo-controlled double-blind left-right comparison. $1\alpha,24$ ($\text{OH})_2 D_3$ (Talcitol, Hermal AG, Hamburg, Germany) was manufactured in an ointment base consisting of paraffinum subliquidum, diisopropyladipat and vaseline album. The verum comprised $4 \mu\text{g/g}$ $1\alpha,24$ ($\text{OH})_2 D_3$ ointment. The placebo contained the ointment base only. In the previous 2 months, the patients had not received systemic therapy, and in the previous 4 weeks no topical treatment had been applied. After an initial wash-out phase of 2 weeks, the patients were treated for 8 weeks with $1\alpha,24$ ($\text{OH})_2 D_3$ on one body half and placebo on the other body half. The maximum dose of $1\alpha,24$ ($\text{OH})_2 D_3$ was $40 \mu\text{g}$ per day. Before and during treatment clinical improvement was assessed using the Psoriasis Area and Severity Index, including scoring for erythema, induration and scaling using a five-point scale. Blood investigations were carried out every 2 weeks.

In each patient two symmetrically localised lesions were selected for the cell biological assessment. Before treatment keratome biopsies (0.4 mm thick and $\pm 1 \text{ cm}^2$) using a small dermatome (Coriotome 6B333, Aesculap AG, Tuttlingen, Germany) were taken from these lesions. After 8 weeks of treatment biopsies were taken from the same areas. In this way, from each patient four biopsies (two $1\alpha,24$ ($\text{OH})_2 D_3$ -treated and two placebo-treated) were obtained. Before initiation of the study Medical Ethical Committee approval had been obtained.

Preparation of cell suspension

Epidermal cell suspensions were prepared using a modification of a trypsinisation procedure previously described by Gommans et al. (32). In brief, the biopsies were washed in phosphate buffered saline (PBS) and floated with dermal side downwards on a solution containing 0.025% w/v trypsin (Sigma, St. Louis, USA) and 0.3% w/v dithioerythritol (Sigma, St. Louis, USA) in PBS for 30 min at 37°C . Then, in a solution containing 10% v/v heat-inactivated newborn calf serum (HINCS, Life Technologies Ltd., Paisley, UK) in PBS, the dermis was peeled off with a fine forceps. After the basal cells had been removed by waving in the solution, the dermis was discarded. After gentle ragitation ("Vortex" mixer) of the remaining epidermis for 1 min, the transparent horny layer was removed. The epidermal cells obtained in this way were centrifuged, resuspended in 2–3 ml ice-cold ethanol (70% v/v) and stored at -20°C until use.

Staining procedure

A triple labelling was performed. DNA content was measured using the new DNA fluorochrome TO-PRO-3 iodide (TP3, Molecular Probes,

Eugene, USA). To assess epidermal keratinisation, the IgG₁-type monoclonal antibody RKSE60 (gift from Prof. F. C. Ramaekers, Department of Molecular Biology, University of Maastricht, the Netherlands) was used. RKSE60 is directed against keratin 10, an intermediate filament-type protein of 56.5 kd that is exclusively expressed in suprabasal, i.e. differentiating keratinocytes in normal and psoriatic skin (33). To assess epidermal inflammation, the IgG_{2a}-type monoclonal antibody Vim 3B4 (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) was used. Vim 3B4 is directed against vimentin, the intermediate filament-type protein that is expressed in cells of mesenchymal origin (34, 35). In the epidermis Vim 3B4 stains non-keratinocytes, i.e. dendritic cells and (especially in psoriatic skin) inflammatory infiltrate cells. The second step of the indirect immunofluorescent staining was performed with monoclonal goat antibodies against mouse IgG₁ and IgG_{2a}, conjugated to phycoerythrin (PE) and fluorescein-isothiocyanate (FITC), respectively (Southern Biotechnology Associates, Birmingham, USA).

Aliquots ($750 \mu\text{l}$) of the cell suspensions, which contained $1-2 \times 10^5$ cells, were washed in PBS, filtered to remove clumps and horny material, and resuspended in $500 \mu\text{l}$ of a solution with Vim 3B4 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 $500 \mu\text{l}$ PBS, containing $2 \mu\text{l}$ goat-anti-mouse-PE, $10 \mu\text{l}$ goat-anti-mouse-FITC, $10 \mu\text{l}$ normal goat serum and $5 \mu\text{l}$ HINCS. After a third washing step DNA staining was performed by addition of $300 \mu\text{l}$ TP3 ($1 \mu\text{M}$ in PBS) and $50 \mu\text{l}$ RNase (1 mg/ml in PBS) (Sigma, St. Louis, USA).

Flow cytometric analysis

From each sample 5,000 gated cells were measured and analysed using an EPICS[®] Elite flow cytometer (Coulter, Luton, UK), equipped with a dual laser system. Both FITC and PE were excited with an air-cooled argon ion laser (15 mW , 488 nm). TP3 was excited with a HeNe laser (10 mW , 633 nm). After determination of the electronic compensation for spectral overlap in earlier experiments, which was minimal in the case of TP3, fluorescence was measured using bandpass filters at 525 nm (green, FITC), 575 nm (orange, PE) and 675 nm (red, TP3). The ratio area/peak of the red signal (DNA) was used to discriminate between doublets of diploid cells and real tetraploid cells (25). After setting appropriate gates with the Elite software, percentages of vimentin- and keratin 10-positive cells were calculated. With the aid of Multicycle[™] software (Phoenix, Flow Systems, San Diego, USA) the percentage of basal keratinocytes in SG₂M phase of the cell cycle was calculated from DNA histograms.

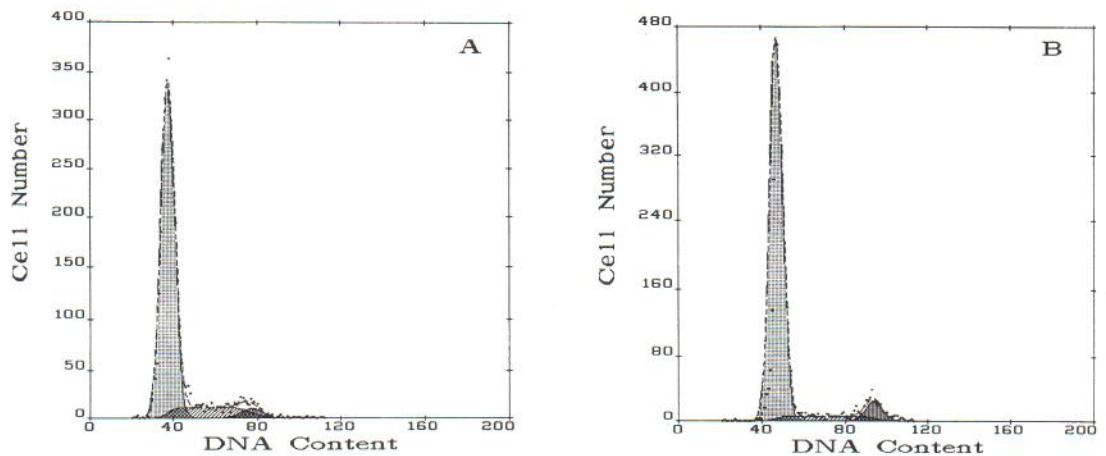
RESULTS

Clinical response

Thirteen patients showed a moderate to marked improvement of their psoriatic lesions. One patient, who also showed a marked improvement, was withdrawn from the study after 6 weeks because of an exacerbation of psoriatic arthritis, requiring additional medication. The mean PASI score showed a significant reduction after 8 weeks of treatment for both $1\alpha,24$ ($\text{OH})_2 D_3$ - and placebo-treated lesions. $1\alpha,24$ ($\text{OH})_2 D_3$ -treated lesions showed a mean reduction of the PASI score of 48%. The pretreatment PASI score was 5.3 ± 0.4 (mean \pm SEM) and the posttreatment PASI score was 2.8 ± 0.4 ($p \leq 0.0001$, Wilcoxon test for matched pairs). The mean PASI score in placebo-treated lesions decreased 28% (pretreatment 5.4 ± 0.4 and posttreatment 3.9 ± 0.4 , $p \leq 0.0001$). Comparison of posttreatment PASI scores between the $1\alpha,24$ ($\text{OH})_2 D_3$ - and placebo-treated lesions showed a significant difference in favour of the $1\alpha,24$ ($\text{OH})_2 D_3$ -treated lesions ($p \leq 0.01$).

As shown in Fig. 1 the clinical severity scores for erythema, induration and desquamation of the biopsied lesions all showed

Fig. 2. DNA histograms of epidermal cell suspensions prepared from keratome biopsies taken from psoriatic plaques (a) before and (b) after 8 weeks' treatment with $1\alpha,24$ dihydroxy-vitamin D_3 ($4 \mu\text{g/g}$) ointment.



a significant reduction, which was more substantial for $1\alpha,24$ $(\text{OH})_2 D_3$ -treated lesions. The mean score for erythema in $1\alpha,24$ $(\text{OH})_2 D_3$ -treated lesions decreased from 3.2 ± 0.1 to 2.2 ± 0.1 ($p \leq 0.0004$), and in placebo-treated lesions from 3.2 ± 0.1 to 2.7 ± 0.1 ($p \leq 0.008$). The mean score for induration showed a reduction from 3.2 ± 0.1 to 1.6 ± 0.2 ($p \leq 0.0003$) in $1\alpha,24$ $(\text{OH})_2 D_3$ -treated, and a reduction from 3.2 ± 0.1 to 2.3 ± 0.1 ($p \leq 0.0007$) in placebo-treated lesions. With respect to desquamation the mean score in $1\alpha,24$ $(\text{OH})_2 D_3$ treated lesions decreased from 3.1 ± 0.1 to 1.3 ± 0.2 ($p \leq 0.0003$), and in placebo-treated lesions from 3.1 ± 0.1 to 1.8 ± 0.2 ($p \leq 0.0007$). Comparison of posttreatment clinical scores revealed a significant difference. The reductions of erythema, induration and scaling were more substantial at the $1\alpha,24$ $(\text{OH})_2 D_3$ -treated sites compared to placebo-treated sites at the levels $p \leq 0.005$, $p \leq 0.008$ and $p \leq 0.02$, respectively.

The blood investigations, in particular calcium metabolism, did not show any significant aberration. No side-effects were seen during the study period. In none of the patients was any skin irritation noticed.

Flow cytometric results

Typical DNA histograms representative for untreated and treated psoriatic plaques are shown in Fig. 2. Fluorescence of TP3 (red signal) was measured of cells which were vimentin-negative (green signal) and keratin 10-negative (orange signal). By distinction of subpopulations in this way, DNA content of the basal keratinocytes could be assessed. In this example, in the psoriatic lesion before treatment, we found a percentage of cells (i.e. of basal keratinocytes) in S + G_2M phase of 17.5%. Eight weeks later this percentage was reduced to 12.8%. Data analysis was restricted to patients from whom all four epidermal cell suspensions contained a cell amount of at least 30%. In this way, paired data from 12 patients were obtained.

For placebo-treated lesions the average percentage of cells in S + G_2M phase was $17.8 \pm 1.8\%$ before treatment. After treatment a tendency of a reduction was seen to $15.1 \pm 1.2\%$ ($p \leq 0.35$, Wilcoxon test for matched pairs). In $1\alpha,24$ $(\text{OH})_2 D_3$ -treated lesions the mean pretreatment percentage of cells in S + G_2M phase was $20.0 \pm 1.9\%$. After 8 weeks of treatment a more substantial and significant decrease to 13.2 ± 1.1 ($p \leq 0.01$) was seen. The mean pretreatment percentage of vimentin-positive

cells proved to be $15.1 \pm 2.4\%$ for $1\alpha,24$ $(\text{OH})_2 D_3$ -treated lesions and $16.2 \pm 2.5\%$ for placebo-treated lesions. After 8 weeks a reduction was seen for both sides to $12.9 \pm 1.4\%$ ($p \leq 0.66$) and $13.2 \pm 1.8\%$ ($p \leq 0.14$), respectively. In neither case was this a significant reduction. With respect to keratin 10 expression, the mean pretreatment percentage of positive cells was $37.7 \pm 3.7\%$ for $1\alpha,24$ $(\text{OH})_2 D_3$ -treated lesions and $35.2 \pm 3.0\%$ for placebo-treated lesions. The mean posttreatment percentages showed an increase to $43.4 \pm 3.5\%$ ($p \leq 0.43$) and $47.9 \pm 3.7\%$ ($p \leq 0.02$), respectively.

DISCUSSION

In the present investigation we assessed the effect of topically applied $1\alpha,24$ $(\text{OH})_2 D_3$ ointment ($4 \mu\text{g/g}$) on proliferation, differentiation and inflammation in the psoriatic plaque using multiparameter flow cytometry. In $1\alpha,24$ $(\text{OH})_2 D_3$ -treated lesions we found a statistically significant reduction of the percentage of basal cells in S and G_2M phase (as marker for proliferation) from 20.0% to 13.2%. This reduction was less and not significant in placebo-treated lesions. The amount of keratin 10-positive keratinocytes (as marker for differentiation) increased after 8 weeks. The decrease of vimentin-positive cells (as marker for inflammation) was in the same range for both $1\alpha,24$ $(\text{OH})_2 D_3$ - and placebo-treated lesions.

The clinical efficacy of $1\alpha,24$ $(\text{OH})_2 D_3$ ointment ($4 \mu\text{g/g}$ once daily) as a topical antipsoriatic agent is confirmed in this study. The mean reduction of the PASI score, as a result of 8 weeks' treatment with $1\alpha,24$ $(\text{OH})_2 D_3$ proved to be 48% (present study). This reduction is in line with our previous report showing 49% reduction of PASI scores by $1\alpha,24$ $(\text{OH})_2 D_3$ (23). Following a 6 weeks' treatment with calcipotriol ointment ($50 \mu\text{g/g}$) twice daily and betamethasone valerate ointment twice daily, the mean reduction of PASI scores were 68.8% and 61.4%, respectively (11). So far, however, no comparative studies of calcipotriol and $1\alpha,24$ $(\text{OH})_2 D_3$ are available. The absence of skin irritation and hypercalcemia in all patients validates earlier statements on $1\alpha,24$ $(\text{OH})_2 D_3$ (15).

In vitro, $1\alpha,24$ $(\text{OH})_2 D_3$ has been shown to inhibit proliferation of keratinocytes (16, 19, 20). In the immunohistochemical study $1\alpha,24$ $(\text{OH})_2 D_3$ proved to inhibit recruitment of cycling epidermal cells profoundly (23). In the present study a reduction

of the percentage basal cells in S and G₂M phase of 34% was reached. In a previous flow cytometrical study from our department on the in vivo effect of calcipotriol, a 31% reduction of the percentage of cells in S and G₂M phase from 11.8% (before treatment) to 8.2% (after 6 weeks' treatment) was shown (36). An important methodological difference between the two studies is that the percentage of cells in S and G₂M phase was limited to the basal keratinocytes only in the present study, in contrast to all epidermal cells in the previous study. By using a triple labelling method, non-keratinocytes and differentiated keratinocytes were excluded, which permits a precise and specific assessment of epidermal cell cycle kinetics.

The absence of a significant change in the relative number of keratin 10-positive cells after treatment with 1 α ,24 (OH)₂ D₃ could suggest that basal and suprabasal compartments maintain the same relative size before and after treatment. Previous flow cytometric studies, however, have demonstrated the relative numbers of keratin 10-positive cells in untreated psoriatic plaques (46.6%) to be reduced compared to normal skin (57.2%) (24). This means that, though a normalization of keratin 10 expression can be produced by calcipotriol (37), 1 α ,24 (OH)₂ D₃ does not seem to influence this expression significantly.

In vitro studies have demonstrated a direct effect of 1 α ,24 (OH)₂ D₃ on inflammatory cells. A dose-dependent induction of differentiation and suppression of antibody response to T cell-dependent antigen by 1 α ,24 (OH)₂ D₃ has been shown (38). The functional properties of neutrophils in vitro were not modulated by 1 α ,24 (OH)₂ D₃ (15, 21). In a previous in vivo study, an 8-week treatment period of 1 α ,24 (OH)₂ D₃ had resulted in a significant reduction of the number of T cells, monocytes and neutrophils in the inflammatory infiltrate of the psoriatic lesion (23). However, in the present study only a tendency of a reduction of the relative number of vimentin-positive cells, i.e. all mesenchymal cells, including the infiltrate cells, was observed. This discrepancy might be explained by the fact that the total number of cells present per surface area (mesenchymal cells and keratinocytes) has decreased following treatment (35). This implicates that the decrease in the absolute number of vimentin-positive cells was in the same range as the decrease of all epidermal cells obtained from the biopsy. Further studies will unravel the relative changes in populations of infiltrate cells during treatment.

The present study lends support for the hypothesis that topical 1 α ,24 (OH)₂ D₃ exhibits its antipsoriatic potential mainly through an inhibiting effect on epidermal growth. The influence on epidermal inflammation and keratinisation is limited. Three-colour flow cytometry seems to be an attractive tool to make an objective assessment of the effect of antipsoriatic therapy on different cell parameters. Validation of the usefulness of this single measurement for proliferation, differentiation and inflammation can be reached by evaluation of other therapy modalities.

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