The Topical Treatment of Psoriasis;
Immunohistochemical and Clinical Aspects

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A sadder and a wiser man
He rose the morrow morn
S.T. COLERIDGE

Voor Roland

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CONTENTS

Chapter	**1**	**General introduction**	**1**
	1.1.	General aspects of psoriasis	**3**
	1.2.	Current treatments for psoriasis	**13**
	1.3.	Antipsoriatic mechanisms	**18**
	1.4.	Aims and questions	**24**
Chapter	**2**	**Immunohistochemical and clinical effects of the topical treatment of psoriasis**	**31**
	2.1.	Epidermal differentiation characteristics of the psoriatic plaque during treatment with calcipotriol	**33**
	2.2.	Epidermal differentiation characteristics of the psoriatic plaque during short contact treatment with dithranol cream	**49**
	2.3.	A novel dithranol formulation (Micanol): The effects of monotherapy and UVB combination therapy on epidermal differentiation, proliferation and cutaneous inflammation in psoriasis vulgaris	**63**
	2.4.	Clobetasol-17-propionate lotion under hydrocolloid dressing (Duoderm ET) once weekly versus unoccluded clobetasol-17-propionate ointment twice daily in psoriasis: an immunohistochemical study on remission and relapse	**75**
Chapter	**3**	**The UVB model: a new in vivo model to study topical treatments for psoriasis**	**85**
	3.1.	The immunohistochemical effects of a single challenge with an intermediate dose ultraviolet B on normal human skin	**87**
	3.2.	The effects of calcipotriol and clobetasol-17-propionate on UVB irradiated human skin, an immunohistochemical study	**101**
Chapter	**4**	**Efficacy and safety aspects of new topical treatments for psoriasis**	**115**

4.1. Therapeutic approach to erythrodermic psoriasis; The report of a case and a discussion of therapeutic options **117**

4.2. In-patient treatment with calcipotriol versus dithranol in refractory psoriasis **125**

4.3. Long-term efficacy and safety of once daily treatment with tacalcitol ointment in chronic plaque psoriasis **133**

Chapter 5 General discussion **147**

5.1 Introduction to discussion **149**

5.2 In vivo effects of topical therapy on the psoriatic plaque **150**

5.3 In vivo models for psoriasis **153**

5.4 The topical therapy of psoriasis: An update **155**

5.5 General conclusion and summary **160**

Samenvatting **165**

Dankwoord **169**

Curriculum Vitae **171**

Chapter 1

General introduction

1.1. GENERAL ASPECTS OF PSORIASIS

1.1.1. Definition & epidemiology

Psoriasis is a common and chronic skin disease characterised by epidermal hyperproliferation, premature terminal differentiation of the epidermal keratinocytes and cutaneous inflammation. The most characteristic lesions consist of sharply demarcated erythematosquamous plaques, particularly localised on the extensor prominences and the scalp. The prevalence of psoriasis in Caucasians varies between 1.5 and 3 %. Psoriasis is virtually absent in Eskimos and native American populations.[1] The age of onset of psoriasis is variable with two peaks, an early onset type (16-22 years) and a late onset type (57-60 years).[2] Psoriasis is equally distributed between both sexes but tends to evolve earlier in females.[3-5]

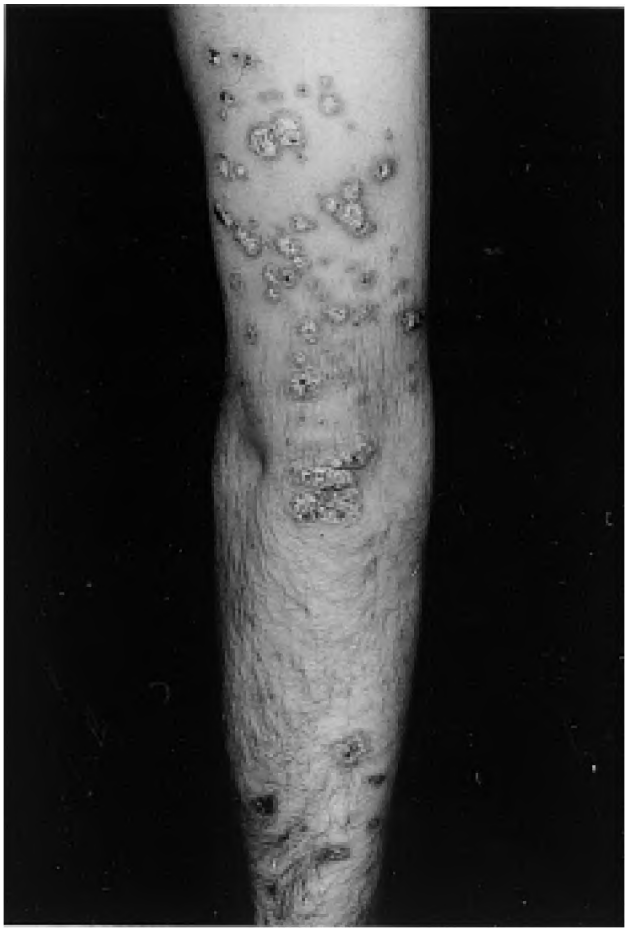

Fig. 1 a
Classical manifestations of psoriasis: sharply demarcated erythematous and squamous lesions

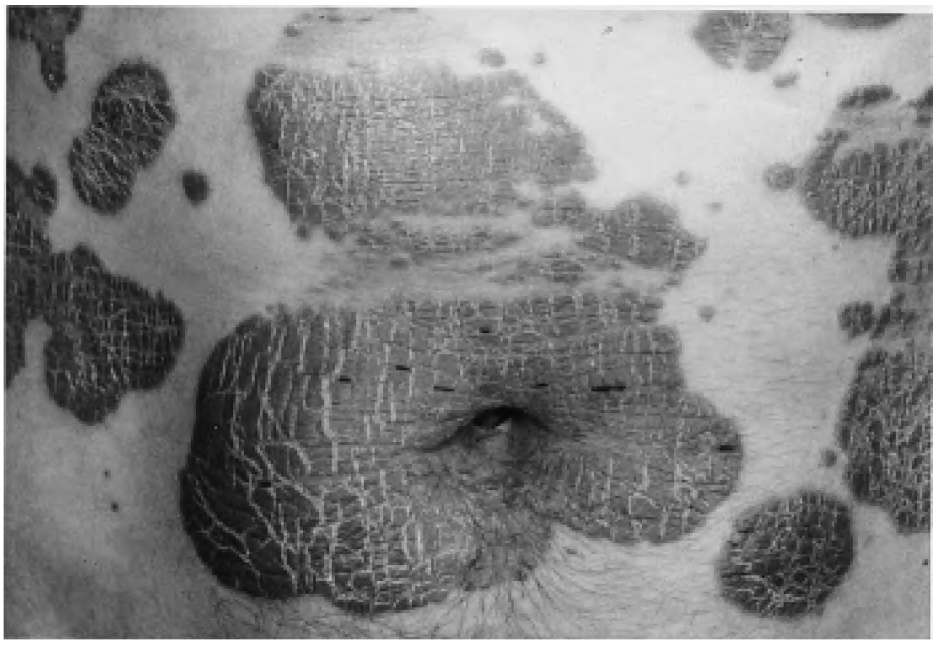

Fig 1 b Classical manifestations of psoriasis: sharply demarcated erythematous and squamous lesions

1.1.2. Clinical aspects

Psoriasis vulgaris is characterised by sharply demarcated nummular, palm-size and more than palm-size erythematosquamous plaques (figure 1). Its classical appearance permits a diagnosis 'à vue'. The efflorescences of the psoriatic plaque are erythema, induration and scaling; the intensity of these efflorescences may vary in severity depending on the stage of the lesions. Classical signs of psoriasis are the 'signe de la tache de bougie' (scratching the superficial scales of a lesion is like scratching a candle) and the 'signe d'Auspitz' (point-bleedings can be observed after removing the silvery scales). Sometimes, a clear peripheral zone, the halo of Woronoff, can be seen around a chronic psoriatic plaque. New lesions start as pin-point-lesions that may develop and coalesce together with other lesions into larger plaques. Psoriasis in general, is expressed as psoriasis vulgaris with a fairly symmetrical distribution pattern. The lesions can be localised anywhere, however, the face is usually spared whereas the extensor sites of elbows and knees and the scalp are preferentially involved with psoriasis.[1]

A particular form is guttate psoriasis in which numerous psoriatic plaques can be observed all over the body. Psoriasis guttata is the most frequent manifestation in children after a streptococcal throat infection. Systemic triggering factors are thought to play a major role in this psoriatic phenotype.[1]

Psoriasis can also be localised in the flexural areas like the armpits, genital region and groins. This manifestation is designated as psoriasis inversa and is seen in 2-6% of the patients with psoriasis. Psoriasis inversa is characterised by a sharply demarcated erythema; the scaling, which is characteristic for psoriasis vulgaris, is not observed.

Sometimes, erythroderma psoriatica may occur. This condition involves generalised erythema and scaling. The function of the skin is impaired and precautions have to be taken to protect the patient from dehydration and heath and protein loss. Therefore hospitalisation is indicated.

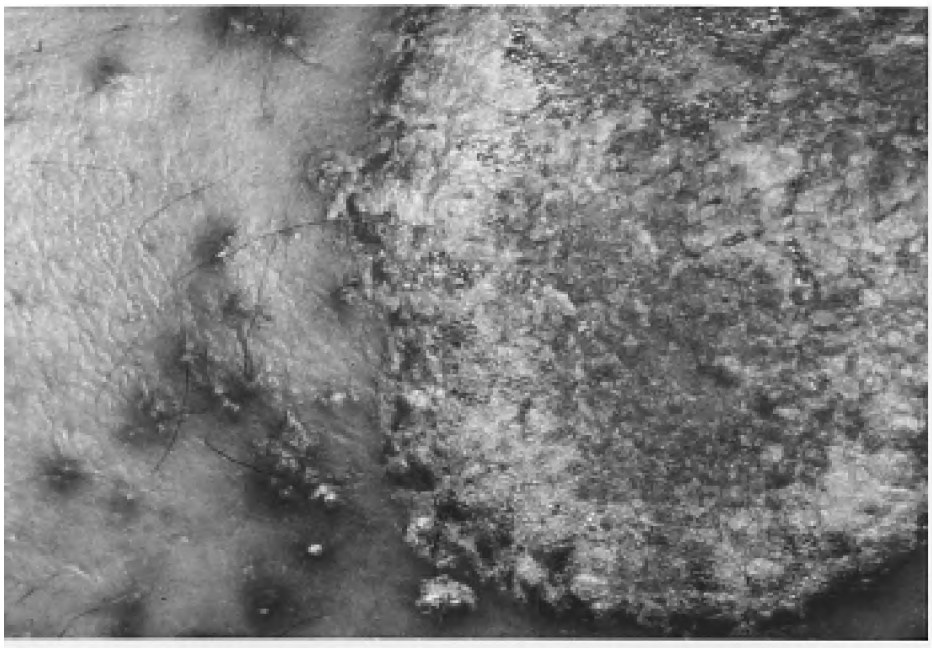

Fig. 2 Psoriatic lesion with pustules: psoriasis pustulosa

A manifestation of psoriasis with a marked expression of the inflammatory component is psoriasis pustulosa (figure 2). In this condition macroscopic pustules can be seen, either all over the body (Zumbusch type), at palms or soles (pustulosis palmoplantaris, Morbus Andrews-Barber) or at the acra of fingers and toes (acrodermatitis continua of Hallopeau, figure 3).

Fig. 3
Acrodermatitis continua of Hallopeau

Extracutaneous manifestations of psoriasis are involvement of nails and joints. Characteristics of nail involvement in psoriasis are seen in 10-50% of the patients and comprise pits of the nail plate, distal onycholysis, subungual hyperkeratosis and the 'oil-spot' phenomenon (figure 4).

Fig. 4 Nail psoriasis with distal onycholysis, pits and the oil-spot phenomenon

Psoriatic arthropathy is a complication of psoriasis that is seen in 5-10% of the patients and can also be observed in patients without cutaneous manifestations of psoriasis. The most frequent manifestation is arthritis with similar symptoms as occur in rheumatoid arthritis and M. Bechterew. The most pathognomonic manifestation is arthritis of the distal interphalangeal joints of the hands. Sometimes monoarthritis or polyarthritis of larger joints can be observed. Psoriatic arthritis is sero-negative. In patients with psoriatic arthropathy, an increased frequency of HLA-B27 and HLA-Bw38 has been found.[6,7]

Psoriasis is a chronic disease, the clinical course is characterised by remissions and exacerbations. Although severe complications such as erythroderma, generalised pustular psoriasis and psoriatic arthropathy may occur, in general the course is mild. Psoriasis may exacerbate due to endogenous and exogenous factors such as infections, various drugs (β-adrenergic antagonists, lithium, cyclo-oxygenase inhibitors and antimalarials), endocrine factors, hypocalciaemia and (psychogenic) stress. The elicitation of a psoriatic lesion by an injury is known as the isomorphic phenomenon and has been described for the first time by Heinrich Koebner.[1,8]

In classical psoriasis, the diagnose is easy. In case of an atypical presentation, the differential diagnosis may comprise all other erythematosquamous skin diseases: seborrhoeic dermatitis, pityriasis rubra pilaris, parapsoriasis, mycosis fungoides, dermatophytic infections and secondary syphilis.

Table I The family-related risk to get psoriasis[6]

Family members with psoriasis	Risk (%)
One parent, no siblings	10
No parents, one sibling	7
One parent, one sibling	16
No parents, two siblings	16
Both parents	50
Second degree relatives	4
Third degree relatives	1-2

1.1.3. Genetics

A genetic predisposition plays an important role in the pathogenesis of psoriasis. In monozygotic twins, a concordance of 73% has been reported.[9] The risk of getting psoriasis in case one of the relatives has the disease is shown in table I.[6] Nowadays there is consensus about the polygenetic inheritance of the disease and about the fact that the disease may come to expression due to multiple exogenous factors. Despite of the commonly accepted polygenetic inheritance, it was shown in one family that a single gene abnormality of chromosome 17 was associated with the psoriatic phenotype.[10,11] The number of psoriasis-associated genes and the identity of these genes are relevant to the subtype, extension and course of psoriasis in the individual patient and also determine the sensitivity for exogenous stimuli. In literature, some genes have been described which are supposed to be associated with a more severe course of psoriasis including some major histocompatibility complex antigens for instance the HLA class I (CW6) and HLA class II (Dr7) genes which underlay multiple HLA associations in psoriasis.[12,13] In patients with Morbus Bechterew, an association with HLA B27 has been found and in these patients, psoriasis is more frequently seen.[14] A polymorphism of the apolipoprotein E gene, and changes of the α-1-antitrypsine inhibitor gene and the

interleukin-1-receptor antagonist genes have also been demonstrated to be associated with psoriasis.[15-17]

1.1.4. Histopathological aspects

The histological picture of psoriasis vulgaris varies with the stage of the lesion but the general picture is characterised by parakeratosis, elongated rete-ridges, thinning of the suprapapillary epidermis and absence of the granular layer (figure 5). In the dermis, elongation and oedema of the papillae are seen with dilated and tortuous capillaries and an inflammatory infiltrate.

The dermal infiltrate is predominantly characterised by T-lymphocytes but polymorphonuclear leukocytes (PMN) are seen as well. The epidermis is infiltrated by T-lymphocytes. PMN aggregate in pustules and abscesses in the epidermis: micro-abscesses of Munro in the stratum corneum and spongiform pustules of Kogoj within the stratum spinosum which are highly diagnostic for psoriasis.[18]

Acute changes in a newly developing lesion are mainly characterised by dermal changes (capillary dilatation, dermal oedema and a mononuclear infiltrate around the capillaries) although controversy still exists whether the initiating events in early psoriasis are located in the dermal or epidermal compartment. Future studies at the transcriptional level will provide relevant information on the initial molecular event in the development of the psoriatic lesion.

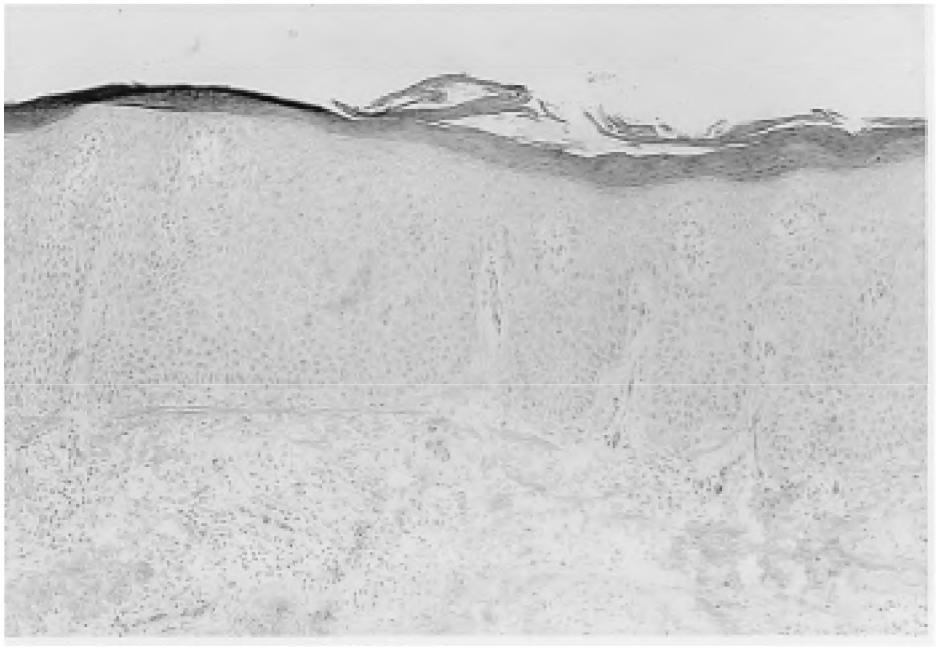

Fig. 5 Histology of a typical psoriatic lesion

Early in the evolution of a lesion, PMN are thought to move from the capillary loops in the tip of the dermal papillae into the epidermis; this is called the phenomenon of the squirting papillae. The acute psoriatic lesion is further characterised by thickening of the stratum corneum and parakeratosis.

In pustular psoriasis, besides micro-abscesses, large macro-abscesses are formed in the epidermis. In the upper dermis an infiltrate of lymphocytes and PMN migrating into the epidermis can be observed. Epidermal acanthosis, parakeratosis and tortuosity of the capillaries further complete the micromorphological picture of pustular psoriasis.

1.1.5. Pathogenetic aspects: epidermal growth and differentiation versus inflammation

Numerous factors are involved in the pathogenesis of psoriasis. Table II summarises the cutaneous processes studied so far in lesional and clinically uninvolved skin of psoriatics. The significance and interpretation of the aberrations in uninvolved skin is difficult and remains a matter of dispute. A biopsy of uninvolved skin can also be a 'preclinical lesion' and, on the other hand, abnormalities found in uninvolved skin may also be an effect of systemic dysregulations due to factors from active psoriatic plaques. Recently, these aspects have been reviewed by various authors.[19,20]

Table II Cellular and biochemical aberrations in different cell types in psoriasis

Cell type	Aberration	
	in lesional psoriatic skin	in uninvolved skin
Keratinocytes	• hyperproliferation • aberrant differentiation • abnormal cell signalling • aberrant production of cytokines	• normal or slightly increased proliferation • normal differentiation • abnormal cell signalling • normal production of cytokines
T-lymphocytes	• marked increased number of T-lymphocytes in dermis and epidermis • aberrant cytokine production • expression of activation markers • increased CD4/ CD8 ratio	• slightly increased number of T-lymphocytes in dermis; not in epidermis
PMN	• present in newly developing lesion • epidermal pustule formation	• not present
Monocytes	• expression of activation markers	• essentially normal
Langerhans cells	• decreased density	• essentially normal decreased density
Fibroblasts	• increased proliferation • altered extracellular matrix composition	• increased proliferation • altered extracellular matrix composition

Several groups regard an abnormality in inflammation control of primary importance in the pathogenesis of psoriasis. Several observations of the last decade, indeed, are in favour of such supposition. Here, the observations will be summarised, which are relevant to the dilemma whether psoriasis might primarily be an immunedisorder or a disorder of epidermal growth and differentiation:

- On the histological level, in pin-point lesions and in the margin zone of spreading psoriatic plaques, T-lymphocytes have been reported to accumulate in the dermis before the appearance of PMN (micropustules of Kogoj) and before epidermal hyperproliferation and abnormal differentiation. However, in these studies, relatively 'late' markers for altered proliferation and differentiation were used.[21,22] A further criticism to these studies is the feasibility that the sensibility of the method of assessment is critical for early or late detection of a phenomenon.
- Bacterial superantigens have been reported to play a major role in the appearance of new lesions:
  * Following streptococcal infections, flares of psoriasis occur.[1]
  * Treatment of children with tonsillectomy has resulted in decreased expression of psoriasis.[23]
  * Recently, it has been shown that intradermal injection of bacterial superantigens in uninvolved psoriatic skin transplanted on severe combined immunodeficient (SCID) mice results into the development of the psoriatic phenotype although the specificity of this effect has also been discussed since addition of serum to normal cultured keratinocytes is also able to induce a psoriatic phenotype in vitro.[24,25]
- Immunomodulation is important in the expression of psoriasis:
  * Patients with AIDS have more severe psoriasis.[26]
  * Treatment with the specific T-lymphocyte inhibitor, cyclosporin, a well-established treatment of psoriasis has been shown to leave epidermal growth and differentiation unaffected in vivo but to have a strong effect on immunomodulatory functions.[27]
  * Treatment with CD4 antibodies has been shown to improve psoriasis.[28,29]
  * Treatment with IL-2 (DAB) has been shown to improve psoriasis.[30]

Although these immunomodulations coincide with changes of the clinical expression of psoriasis, it remains to be shown whether coincidence also implies a

specific and direct causal relationship. On the other hand, several observations suggest that epidermal proliferation and differentiation are major determinants of the psoriatic process:

- The lesional skin of psoriasis is characterised by a massive thickening of the epidermis and it is the excessive scaling -the result of the process of keratinisation- which is a most significant problem for the patients affected with psoriasis.
- Calcipotriol, a topical antipsoriatic agent with an efficacy that is comparable to betamethason valerate, has been proven to exert its effect mainly by interfering with epidermal proliferation and keratinisation and less by modulating the inflammatory infiltrate. Although calcipotriol has some immunomodulatory effects,[31] calcipotriol so far, has not been shown to be effective in the well-established immunodermatoses (delayed type hypersensitivity, atopic dermatitis, lupus erythematosus).[32,data on file LEO Pharma]
- Recently, it was shown that psoriatic keratinocytes respond abnormally to humoral factors released by clones of T-lymphocytes derived from psoriatic lesional skin.[25,33,34] However, it remains to be demonstrated to what extent these effects are specific to psoriasis.
- The distant, clinically uninvolved skin does not show abnormalities with respect to immune mechanisms. However, important abnormalities have been shown in signal transduction pathways in the symptomless skin before any lesion has appeared.[20,35-37]

A joint venture of immunological mechanisms and epidermal processes seems a realistic supposition. In this thesis, attention is focused on epidermal growth and differentiation in psoriasis. The major target in this thesis will be the analysis of the modulation of epidermal growth and differentiation in psoriasis by antipsoriatic treatment.

1.2. CURRENT TREATMENTS FOR PSORIASIS

1.2.1. Introduction

Psoriasis is a chronic skin disease and can be treated, not cured. Therefore, long-term management is of utmost importance. In each patient with psoriasis, an individual therapy plan has to be made that may vary depending on the type of disease but also on the patient's health, time and expectations, taking efficacy and side-effects of the treatment into consideration. Often a combination of two or more therapies is used in order to enhance efficacy and to minimise side-effects. Topical therapies are the first choice of treatment;[38] phototherapy and systemic treatment are indicated if topical treatment is insufficient for reaching adequate control of psoriasis.[1]

1.2.2. Topical treatments

Emollients and keratolytics

Emollients and keratolytics are mainly fit for keeping the skin supple and for diminishing the effects of scaling. In general, a more active treatment is required.

Coal tar

Coal tar has been used as a safe and effective topical therapy for more than a century. Crude coal tar is a complex mixture of many hydrocarbons; it is not known what the exact active components are.[39] In daily practice, tar is chosen in the case of pruritic psoriasis. Many vehicles can incorporate tar, for instance creams, ointments and shampoos. Crude coal tar paste is most effective but it involves a messy regime that often requires hospitalisation. More refined tars can be used at home; they are less effective but can be used as adjuvans, in stead of bland emollients. Better results are reached with combinations of tar and other therapies for instance the Goeckerman regime in which crude coal tar is combined with ultraviolet light, and the Ingram regime which combines tar with dithranol and phototherapy.[40-42]

Dithranol

More than a century ago, a tree bark extract (chrysarobin), was discovered to have an important antipsoriatic potential. Dithranol (anthralin), a synthetic analogue of chrysarobin, has been used for over 80 years in the treatment of chronic plaque psoriasis.[43] Dithranol is a yellow powder that can be incorporated in a cream, ointment, paste or stick. Effects and side-effects seem to be closely connected: staining and irritation of the skin appear to be inseparable of the antipsoriatic activity. Dithranol applications can be carried out according to many different regimes for instance the Ingram regime. The time honoured 24 hour dithranol applications are supposed to be

most effective but, since this regime is messy and time consuming, it requires hospitalisation. Short contact applications and cream formulations have simplified dithranol treatment.[43]

Topical corticosteroids

For several decades, topical corticosteroids have been used extensively in the treatment of psoriasis. Topical corticosteroids are convenient to use and are able to induce fast remissions. But topical steroids also have disadvantages: discontinuation of topical steroids may result in a rebound phenomenon, resulting in a major relapse. Long-term use of corticosteroids can cause atrophy or striae of the skin, allergic contact dermatitis and habituation, so that more frequent or more potent corticosteroid applications are required. Systemic adverse events have been described following high doses of corticosteroids namely an inhibiting effect on the hypophysis-adrenal cortex axis. Rapid induction of clearance in recalcitrant localised psoriasis can be obtained using potent corticosteroids.[44] Low and medium potent steroids are used for maintenance therapy in mild disease. Intermittent therapy is often used to minimise adverse events.[1,44]

Hydrocolloids

It has been known for a long time that occlusion of psoriatic lesions has a beneficial effect.[45] Nowadays, hydrocolloid dressings (HCD) are available in all qualities and sizes with a remarkable adhering capacity and a great wearing convenience. Occlusion is thought to at least partly improve the impaired barrier function of the psoriatic skin. So far, no significant clinical effect of occlusion as a monotherapy has been established.[45,46] In combination with other therapies, HCD have a significant effect.[47,48] Topically applied corticosteroids under HCD are thought to increase the bioavailability of the corticosteroid.[49] Increased bioavailability, in combination with the occlusive-effect of the HCD, can induce rapid remissions in localised recalcitrant psoriasis.

Vitamin D_3 analogues

Vitamin D_3 analogues are the main achievement in the development of topical treatment of psoriasis of the last decade. The usefulness of active vitamin D_3 for psoriasis was established in the 1930's but the hypercalciaemic effects limited its use. In 1985, interest was reawakened by the report of a remarkable improvement of psoriasis in a patient during treatment of osteoporosis with oral 1-α-OH-vitamin D_3 (alphacalcidol).[50-53] Later, others found topical application of 1,25-$(OH)_2$-vitamin D_3 (calcitriol) to be beneficial for psoriasis as well. Calcipotriol, a novel vitamin D_3 analogue with a lower hypercalciaemic potential and an antipsoriatic activity comparable to medium strength

corticosteroids was developed as a safe and effective vitamin D_3 formulation for the topical treatment of psoriasis.[54-59] The most commonly encountered side-effect of calcipotriol is a transient irritant reaction, particularly of the facial skin.[54] Hypervitaminosis D_3 may lead to hypercalciaemia.[60,61] Calcipotriol ointment (50µg/g) up to 100 grams weekly is supposed to be safe. The use of larger quantities of ointment should occur under intensified supervision of blood parameters.[55,61]

1.2.3. Phototherapy

Sunlight and in particular ultraviolet radiation have a well-established effect on psoriasis. Phototherapy with UVB and photochemotherapy with topical or systemic psoralens in combination with UVA (PUVA) are mainly indicated in case of extensive psoriasis. Ultraviolet light induces a cascade of many biological processes that cause a proliferative and inflammatory response in normal human skin but in the psoriatic lesion, suberythematous doses of UV induce curative effects. Extensive use of UV sources accelerates ageing of the skin and PUVA increases occurrence of actinic keratoses and squamous cell carcinoma.[62,63] On the other hand, the fact that UV light has no systemic side-effects makes it a therapy that is especially suited for pregnant women with extensive psoriasis.

Phototherapy using narrow-band UVB with a maximum of energy at 311 nm is an optimised irradiation principle compared to conventional UVB phototherapy. In comparison to broad-band UVB therapy, narrow-band UVB is more effective and results in less erythema. The emission energy is lower which explains the relatively higher dose of narrow-band UVB irradiation required compared to conventional UVB irradiation for antipsoriatic treatment. This new UVB therapy is therefore relatively expensive. It is not yet clear whether the therapeutic benefit of narrow-band UVB therapy justifies this relatively expensive treatment.[64,65]

Topical PUVA with trioxalen and 8-methoxypsoralen is mainly a popular treatment modality in Nordic countries.[66,67] Bath-PUVA reduces the occurrence of nausea which is often encountered during treatment with systemic psoralens. Interestingly, a decreased risk for skin malignancies was documented in patients treated with bath-PUVA with trioxalen compared to oral PUVA with 8-methoxypsoralen.[67]

1.2.4. Systemic treatments

Methotrexate

Methotrexate is a very effective drug which has been used for a long time in the treatment of psoriasis. Its main side-effects are the suppression of the haemopoeitic system and liver damage. These side-effects limit the use of methotrexate.[68] However, provided that the guidelines for methotrexate treatment are taken into consideration, this treatment is a safe approach to recalcitrant psoriasis and psoriasis arthropathica.[69,70]

Retinoids

Retinoids are derivatives of vitamin A acid. Especially pustular psoriasis and active guttate psoriasis respond well to this treatment. Muco-cutaneous side-effects like dryness of the skin, reversible hairloss or hypertrichosis, generalised pruritus and paronychia are dose-dependent side-effects. Teratogenicity limits the use of retinoids in female patients of the childbearing age. Other side-effects are hypercholesterolaemia and hypertriglyceridaemia. The side-effect profile necessitates frequent follow-up visits with blood investigations.[71]

Cyclosporin

Cyclosporin is a relatively new therapy for psoriasis. Cyclosporin is a specific inhibitor of the T cell function and its mode of action is primarily via immunomodulation. Cyclosporin is primarily metabolised in the kidneys. The side-effects -mainly hypertension and renal impairment- limit the use of this antipsoriatic drug. Cyclosporin can be regarded as an important 'short period intervention' in very severe psoriasis.[27]

Other systemic drugs

Systemic corticosteroids are capable of causing rapid remissions in extensive psoriasis. But the well-known adverse events of systemic corticosteroids limit their use. Major relapses after discontinuation of this therapy make it a therapy that is obsolete nowadays.[1] Azathioprine,[72] fumaric acid,[73] sulphasalasine,[74] hydroxyurea[75] and FK506[76] are systemic medications which have not (yet) been registered for psoriasis. Occasionally these treatments might be of help in case of inefficacy of the other treatments or contraindications for the other treatments.

1.2.5. Treatments of focal interests in the present thesis

Recently, the systemic treatment of psoriasis was the objective for two theses in the Netherlands, Dr. R.J. van Dooren-Greebe evaluated the efficacy and side-effects of methotrexate and acitretin.[69] Dr. L. Witkamp evaluated efficacy and side-effects of cyclosporin and systemic tacrolimus (FK506).[76,77] For patients with severe psoriasis systemic treatment is of major importance. The focus of the present thesis is the topical treatment of psoriasis. The topical application of antipsoriatic compounds provides the advantage of restricting the availability of a compound to the target tissue: skin. However, systemic absorption through the skin remains an important aspect in regard to safety of a topical therapeutic agent.

The last decade, major progress has been made in the topical treatment of psoriasis:

- Dithranol based therapies have been popularised by the introduction of short contact application schedules, new vehicles which permit easy washing off and new principles of care which aim for patient care in conjunction with patient instruction.[43,78,79]
- Vitamin D_3 analogues have revolutionised topical treatment of psoriasis. Calcipotriol ointment was introduced as a treatment in most countries 1992.[80] A limitation of the treatment is irritation of the skin. Its efficacy is comparable with betamethason valerate.[54] Combination treatments with topical or systemic antipsoriatics and the search for analogues with a low degree of irritation are the most recent developments concerning vitamin D_3 analogues.
- Once weekly applications of topical corticosteroids occluded with hydrocolloid dressings is a 'high compliance approach' which is very effective.[48] Safety and post-treatment remission characteristics are current areas of research.

In fact, these new leads are the focal interest in the present thesis and new information on these areas will be presented in this thesis.

1.3. ANTIPSORIATIC MECHANISMS

1.3.1 Introduction

The aim of this section is to provide a review on the in vitro effect of established topical treatments for psoriasis on epidermal proliferation and differentiation. The cell-biological effects of most treatments have been studied on various in vitro models for inflammation, keratinocyte proliferation and differentiation. In the present thesis, focal attention is on epidermal proliferation and differentiation. Therefore the in vitro effects of treatments on keratinocyte growth an differentiation will be reviewed. For a review on effects of treatments on inflammation and immune mechanisms, the reader is referred to the literature.[19] As the present thesis is focused on topical treatments, for information on the effects of systemic treatments on epidermal proliferation and differentiation the reader is referred to the literature.[19]

1.3.2. Antipsoriatic mechanisms of topical treatments and photo(chemo)therapy

Tar

Data on the mode of action of this topical treatment are sparse. Tar is assumed to have an anti-mitotic effect in vivo.[81] In vitro research on keratinocytes and cell cultures with other cell types confirms this assumption.[82,83]

Dithranol

Auto-oxidation of dithranol induces free radicals in the skin and alters the cellular and subcellular redox potential with a diversity of antipsoriatic effects.84,85 Dithranol inhibits key enzymes in the metabolism of the cell, interacts with mitochondria, probably resulting in the inhibition of proliferation of the psoriatic keratinocytes, which has been proven to be inhibited up to 98%. Transforming growth factor α-mRNA expression and epidermal growth factor receptor binding are inhibited as well due to dithranol.[86]

Topical corticosteroids

Corticosteroids act via binding to a specific receptor, that is a member of the steroid-hormone receptor superfamily. The corticosteroid-activated receptor modulates the transcription of target genes.[87] Increased transcription of lipocortin, the inhibitor of the key enzyme in arachidonic acid metabolism phospholipase A2, results in reduced formation of arachidonic acid and its metabolites.[87] In keratinocyte cultures topical corticosteroids have been shown to inhibit proliferation.[88] To the best

of our knowledge no interference of corticosteroids with the keratinisation process has been reported so far.

Vitamin D_3 analogues

Calcipotriol is the vitamin D_3 analogue which has been introduced as a topical treatment of psoriasis five years ago.[89] Recently the modes of action of vitamin D_3 in the treatment of psoriasis have been reviewed.[90] At the molecular level, two principles are relevant. Vitamin D_3 binds to the vitamin D_3 receptor and the ligand-activated vitamin D_3 receptor transactivates various vitamin D_3 responsive genes, resulting in interference with epidermal growth and differentiation in many cell types including the keratinocyte.[91] The other principle is a direct effect of vitamin D_3 on opening of transmembrane calcium channels, resulting in increased calcium concentrations in the keratinocyte.[92] In keratinocyte cultures, it has been shown that calcipotriol, 1α,25-dihydroxy-vitamin D_3 (calcitriol) and 1α,24-dihydroxy-vitamin D_3 (tacalcitol) inhibit proliferation of keratinocytes, enhance involucrin transcription, increase activity of transglutaminase and enhance cornified envelope formation.[59,93-97]

Photo(chemo)therapy

Phototherapy (UVB) and photochemotherapy (PUVA) are well-established antipsoriatic treatments. Photo(chemo)therapy has a dual commitment to epidermal growth. On one hand, interference with DNA has been established, resulting in growth inhibition of keratinocytes in culture.[98,99] On the other hand, due to UV radiation, a diversity of mediators of inflammation is released into the culture medium by keratinocytes, such as IL-1, IL-6, TNF-α, NFκB and various arachidonic acid metabolites resulting in a diversity of effects on keratinocyte proliferation and differentiation.[100,101]

1.3.3. Models and phenomena of specific interest in this thesis

In the present thesis, the in vivo effects of topical treatments on epidermal growth and differentiation will be studied. The approaches for in vivo study and the markers of focal interest will be briefly introduced in this section.

Models of focal interest

The studies on the in vivo effects of antipsoriatic treatments can be carried out on repeated biopsies taken from the existing psoriatic lesions during treatment. Using this approach, the cell-biological processes underlying the clearing process of the psoriatic plaque can be studied.

However, in the long-term management of psoriasis the therapeutic approach of the reappearance of new lesions is of utmost importance. Therefore, the induction of psoriatic hyperproliferation -the recruitment process of cycling epidermal cells- and the accompanying processes in epidermal differentiation should be impersonated in an experimentally reproducible in vivo model. Using such a model, the effect of antipsoriatic treatments on the induction of the recruitment of cycling epidermal cells and abnormal epidermal differentiation can be qualified and quantified. In vivo skin models have been developed to mimic certain aspects of the origin of the psoriatic lesion. Epicutaneous application of leukotriene B_4 (LTB_4) elicits cutaneous inflammation.[102] The LTB_4 model mimics the movement of polymorphonuclear leukocytes (PMN) through the epidermis which is thought to be an early alteration in the formation of the psoriatic plaque.[103] The response to tape-stripping is an in vivo model which permits studies on the induction of epidermal proliferation and proliferation associated abnormal differentiation. The effect of systemic treatments on epidermal proliferation and differentiation have been studied using this model.[104-106]

For the studies on the in vivo effects of topical treatments, a new model is required which fulfils the following criteria:

i. Epidermal growth and differentiation characteristics should approach the situation in the psoriatic lesion.
ii. The trigger should not result in epidermal cell death or a major inflammatory infiltrate.
iii. The model should not damage the stratum corneum as topical drug availability is changed dramatically by stripping away the stratum corneum.
iv. The challenge can not be a topically applied chemical as the availability of the signal might be modulated by a topical treatment.

In the present thesis, besides the effects of different topical antipsoriatic therapies on lesional psoriatic skin, UVB challenged skin will be studied as a model for epidermal changes in a developing psoriatic lesion.

Markers of focal interest

To obtain insight into cell-biological phenomena that are important in psoriasis, immunohistochemistry was applied, using the indirect immunoperoxidase technique. A panel of monoclonal antibodies giving information on epidermal proliferation, epidermal differentiation and cutaneous inflammation was used:

Proliferation

- Ki-67

  The keratinocytes in the epidermis that actively participate in the cell cycle can be identified by the monoclonal antibody MIB-1, which is directed against the proliferation associated nuclear antigen, Ki-67.[107,108] Nuclear MIB-1 staining implies that a cell has escaped from the G_0 population, the population of cells that does not actively participate in cell division. The recruitment of cycling epidermal cells is the key-process in psoriatic hyperplasia. Ki-67 expression, assessed by MIB-staining is a most valuable approach to assess epidermal proliferation in psoriasis and the efficacy of antipsoriatic treatments on epidermal growth.

Epidermal differentiation

In human skin, the stratum corneum takes care of a major part of the barrier function of the skin. The keynote of the maturation of the keratinocytes from the basal layer to the corneal layer is the forming of a layer of corneocytes, the stratum corneum. This process is complex and involves the cross-linking of proteins and the formation of cornified envelopes. Epidermal differentiation is altered in the psoriatic skin.[109] To obtain information about this process, a set of monoclonal antibodies directed against several cellular markers for keratinocyte differentiation was selected to characterise different aspects of the epidermal differentiation process:

- Cytokeratin 16

  The cytoskeleton of the psoriatic epidermis expresses an alternative set of cytokeratins, different from normal human skin. In the basal layer of normal human skin, cytokeratin 5, 14 and 15 are expressed and in the suprabasal layer cytokeratins 1, 2, 10 and 11 are encountered. In hyperproliferative epidermis cytokeratin 6 and 16 are expressed.[109] Cytokeratin 16 is found in hyperproliferative skin but also in damaged and recovering skin and is, nowadays, thought to be associated with disturbance of the epidermal integrity.[110-112] In this thesis, the monoclonal antibody that was used to detect cytokeratin 16 is Ks8.12.[113]

Ks8.12 is not monospecific for keratin 16 and also reacts with keratin 13 which is not present in normal adult human skin.[114] Significant correlations between Ks8.12 staining and clinical scores in psoriasis have been demonstrated.[115] Therefore Ks8.12 is considered a valuable marker to study treatment related changes in psoriasis.

- Involucrin

  Involucrin is a soluble protein precursor of the cornified envelope.[116] The production of involucrin already starts in keratinocytes just above the basal layer of the epidermis.[109] At this point, the first steps in the organisation of the cornified envelope are taken. Using immunohistochemistry in normal human skin, only the fraction of the involucrin in the upper stratum spinosum and stratum granulosum is visualised. Since involucrin expression is not related to cytokeratin expression, involucrin is a distinct marker which is expressed in the phase which induces the final events of terminal differentiation. Involucrin is studied using the monoclonal antibody MON-150.[117]

- Filaggrin

  Filaggrin (**fil**ament **aggr**egating prote**in**), a histidine-rich protein, is present in the granular layer of normal human skin and one of its functions is the aggregation of cytokeratin filaments.[118] Filaggrin is precursed by profilaggin, which is deposited in keratohyalin granules and is broken down by proteolysis and dephosphorilation resulting in filaggrin. Breakdown products of filaggrin are urocanic acid and pyrrolidone carboxyl acid.[119,120] Urocanic acid is a physiological sun-protector and pyrrolidone carboxyl acid has a function in the hydration in the stratum corneum. In psoriatic skin, filaggrin is markedly decreased and only focally present. Filaggrin is a marker for late terminal differentiation and was studied to get insight into the complex process of altered differentiation in psoriasis.

- Keratinocyte transglutaminase

  Keratinocyte transglutaminase is a calcium-dependent enzyme that cross-links the constituents of the cornified envelope: involucrin, loricrin and keratolinin using ε-(γ-glutamyl)lysine isopeptide bonds.[116,121-126] Because of its calcium dependency, transglutaminase is marker that is directly influenced by vitamin D_3 analogues in vitro and in vivo.[95,127-129] Transglutaminase activity has been localised histochemically in the granular layer of the epidermis and the immunohistochemical expression has been found in the upper spinous and granular layers.[123,130] In psoriasis, transglutaminase and its activity already appear in the lower stratum spinosum.[131] Transglutaminase is studied using a monoclonal antibody against human keratinocyte transglutaminase.

Cutaneous inflammation

Increased accumulation of inflammatory cells in dermis and epidermis is an important and early characteristic of the histologic transition of clinically normal skin into a psoriatic plaque. In a chronic psoriatic plaque, the dermal inflammatory infiltrate mainly consists of mononuclear cells that are located diffusely in the stroma of the papillary and reticular dermis and around the dermal capillaries. In the epidermis, T-lymphocytes and PMN are the most important inflammatory cells.

- T-lymphocytes

  The mononuclear dermal infiltrate of the psoriatic lesion mainly consists of T-lymphocytes. Immunohistochemically, T-lymphocytes were visualised by the monoclonal antibody DAKO-T11 that is directed against the CD2 epitope.

- PMN

  One of the proteolytic enzymes of the polymorphonuclear leukocyte (PMN) is human leukocyte elastase. A monoclonal antibody against elastase (DAKO-elastase) was used to visualise PMN.

1.4. AIMS AND QUESTIONS

1.4.1. Immunohistochemical effects of topical antipsoriatic treatments

AIM 1: To study the in vivo effects of topical antipsoriatic treatments on epidermal proliferation and differentiation.

In particular the following questions were addressed:

- To what extent does calcipotriol interfere with differentiation, proliferation and inflammation? (chapter 2.1)
- To what extent do various vehicles of dithranol modify the response of the epidermis to dithranol treatment? (chapter 2.2 and chapter 2.3)
- To what extent is the response of the epidermis to a topical corticosteroid under a hydrocolloid dressing comparable to the response to a topical corticosteroid without a hydrocolloid dressing? (chapter 2.4)

1.4.2. Development of a new in vivo skin model

AIM 2: To develop a new model to study the induction of recruitment of cycling cells and proliferation associated differentiation characteristics in human skin in vivo and to study the effect of topical treatment on such a model.

In particular the following questions were addressed:

- Is the irradiation with an intermediate dose of UVB a model which permits the induction of recruitment of cycling epidermal cells and proliferation associated differentiation characteristics? (chapter 3.1)
- To what extent do topical corticosteroids and calcipotriol inhibit the UVB induced recruitment of cycling epidermal cells and proliferation associated differentiation? (chapter 3.2)

1.4.3. Clinical efficacy and safety

AIM 3: To study efficacy and safety aspects of topical treatments for psoriasis.

In particular the following questions were addressed:

- To what extent are new wash-off formulations of dithranol effective in the treatment of psoriasis and what about tolerance to these approaches? (chapters 2.2 and 2.3)
- To what extent is a topical corticosteroid under a HCD effective and safe in the treatment of psoriasis and to what extent is the post-treatment remission period comparable with the topical corticosteroid without occlusion? (chapter 2.4)
- To what extent does calcipotriol contribute to systemic treatment with cyclosporin in severe psoriasis? (chapter 4.1)
- How does calcipotriol treatment, carried out at the in-patient department, compare and contrast to classical in-patient dithranol treatment? (chapter 4.2)
- What is the long-term safety and efficacy profile of tacalcitol, a vitamin D_3 derivative with a low irritating potential? (chapter 4.3)

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Chapter 2

Immunohistochemical and clinical effects of the topical treatment of psoriasis

This chapter was based on the following publications:

C.J.M. van der Vleuten, E.M.G.J. de Jong, P.C.M. van de Kerkhof
Epidermal differentiation characteristics of the psoriatic plaque during treatment with calcipotriol. *Arch Dermatol Res* 1996; **288**:366-272.

C.J.M. van der Vleuten, E.M.G.J. de Jong, P.C.M. van de Kerkhof
Epidermal differentiation characteristics of the psoriatic plaque during short contact treatment with dithranol cream. *Clin Exp Dermatol* 1996; **21**:409-414.

C.J.M. van der Vleuten, M.J.P. Gerritsen, E.M.G.J. de Jong, M. Eibers, G.J. de Jongh, P.C.M. van de Kerkhof
A novel dithranol formulation (Micanol): The effects of monotherapy and UVB combination therapy on epidermal differentiation, proliferation and cutaneous inflammation in psoriasis vulgaris *Acta Derm Venereol (Stockh)* 1996; **76**:387-391.

C.J.M. van der Vleuten, I.M.J.J. van Vlijmen-Willems, E.M.G.J. de Jong, P.C.M. van de Kerkhof
Clobetasol-17-propionate lotion under hydrocolloid dressing (Duoderm ET) once weekly versus unoccluded clobetasol-17-propionate ointment twice daily in psoriasis: an immunohistochemical study on remission and relapse. (submitted)

2.1. EPIDERMAL DIFFERENTIATION CHARACTERISTICS OF THE PSORIATIC PLAQUE DURING TREATMENT WITH CALCIPOTRIOL

2.1.1. Summary

Treatment of psoriasis with vitamin D_3 analogues is well-established in the present dermatological practice. One of the clinical parameters of the psoriatic plaque that reduces early and markedly during treatment with the vitamin D_3 analogue calcipotriol (Daivonex®) is scaling. Since scaling is the clinical manifestation of epidermal differentiation, early changes in immunohistochemical markers for differentiation (transglutaminase, involucrin and filaggrin) were studied in patients that had been treated with calcipotriol for four weeks. Markers for proliferation (Ki-67 antigen) and inflammation (polymorphonuclear leukocytes and T-lymphocytes) were studied as well and correlated with the differentiation characteristics.

Clinically, a major improvement was seen in all patients. A significant decrease of the percentage transglutaminase positive cell layers was observed in the first week of treatment, whereas, in literature, an increase of transglutaminase activity in epidermal cell cultures following incubation with calcipotriol has been reported. Involucrin expression was only slightly modulated in vivo. However, a major restoration of the filaggrin positive cell layer and an important reduction of the recruitment of cycling epidermal cells characterised the epidermal response to calcipotriol treatment. Markers for inflammation (T11 positive cells and elastase positive cells) also reduced substantially in the first week of treatment with calcipotriol.

From this study it may be concluded that inhibition of epidermal growth and recovery of the filaggrin positive cell layer are targets for the in vivo effect of calcipotriol.

2.1.2. Introduction

Vitamin D_3 and its analogues have been shown to exert an important antipsoriatic effect.[1] In vitro and in vivo studies show a marked antiproliferative and keratinisation enhancing activity.[2-6] Inflammation is also affected by active vitamin D_3 derivatives.[6,7]

Calcipotriol (Daivonex®) is one of the first line treatments for psoriasis. While treating patients with this therapy, it is our impression that scaling is one of the clinical parameters that diminishes early and obviously. So one might speculate that normalisation of epidermal differentiation is one of the important antipsoriatic effects of active vitamin D_3. Indeed, in vitro studies have shown that proliferation of keratinocytes is inhibited and that the formation of the cornified envelope, transcription of involucrin and transglutaminase activity is enhanced by calcipotriol.[2,3,7-12] In vivo, inhibition of Ki-67 expression and a reduction of PMN are early effects, whereas a reduction of suprabasal expression of keratin 16 and T-lymphocyte accumulation are late effects.[6]

The aim of the present study was to find out the behaviour of the suprabasal compartment of the epidermis during treatment of the psoriatic plaque with calcipotriol.

The following questions were addressed:

i. Does calcipotriol ointment have a substantial effect on markers of epidermal differentiation during the treatment of chronic plaque psoriasis?
ii. What is the relationship between the effect on differentiation markers and other immunohistochemical effects of calcipotriol?

Immunohistochemistry on cryostat sections of skin biopsies with a panel of monoclonal antibodies during a four weeks clinical study with calcipotriol in patients with psoriasis was carried out to answer these questions.

2.1.3. Materials and methods

Patients

Six patients with chronic plaque psoriasis, five males and one female, participated in this study. Their age varied from 33 to 61 years with an average duration of their psoriasis of 17 ± 0.8 (mean ± SEM) years. They had used no systemic treatment for at least two months and no topical treatment for at least two weeks. In fact, the patients had hardly used any antipsoriatic treatment for periods of months or years before starting the present study. Patients were instructed to apply calcipotriol ointment (50 µg/g, Daivonex®, LEO Pharma, Denmark) twice daily on lesional skin with a maximum of 100 grams ointment weekly. No additional topical therapy was allowed. This study was approved by the local ethical committee. All patients gave their written informed consent prior to inclusion in the study.

Clinical assessments

Before therapy and 1, 2 and 4 weeks after therapy the severity of one target lesion was scored for each of the following clinical signs: erythema, induration and scaling. Each parameter was scored using a 5-point scale: 0 = complete lack of cutaneous involvement, 1 = slight involvement, 2 = moderate involvement, 3 = severe involvement, 4 = severest possible involvement.

Biopsy procedure

Punch biopsies of 3 mm were taken from the previously determined target lesion before therapy and 1, 2 and 4 weeks after therapy from all patients after local anaesthesia with xylocain 1% and adrenaline.

The biopsies were embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, USA), snap frozen in liquid nitrogen and stored at -80°C until use. Sections of 7 μm were cut, air dried and fixed for 10 minutes in acetone/ether (60/40%) (MIB-1 staining) or in acetone (other stainings) and again stored at -80°C.

Monoclonal antibodies

A panel of monoclonal antibodies was used.

To assess epidermal differentiation monoclonal antibodies against involucrin (MON-150[13], 1:25), anti-human keratinocyte transglutaminase (1:100, Mouse Monoclonal Antibody, IgG_{2a} Biomedical Technologies Inc.) and against filaggrin and profilaggrin (1:500, anti-filaggrin, BT576, Biomedical Technologies Inc.) were used.

To approximate the number of cycling epidermal cells in the basal layer an antibody directed against the Ki-67 antigen was used (MIB-1, 1:50, Immunotech, S.A., Marseilles, France).

Analysis of the inflammatory infiltrate was done by assessment of T-lymphocytes and Polymorphonuclear leukocytes (PMN) respectively using the monoclonal antibodies DAKO-T11 (1:100, Dakopatts, Copenhagen, Denmark) and DAKO-elastase (1:100, Dakopatts, Copenhagen, Denmark).

Staining procedure

For all monoclonal antibodies, except for T11, an indirect immunoperoxidase technique was used. For ten minutes the slides were fixed in acetone/ether (60/40%) in case of MIB-1 or in acetone for the other stainings. The slides were air dried and put in a phosphate buffer (PB) (72 mM Na_2HPO_4 and 28 mM NaH_2PO_4). Only the slides stained with anti-elastase were pre-incubated with methanol/ 0.1 % H_2O_2 (30 %) for 20 minutes. All antibodies were diluted in PB. The slides were incubated with the different primary monoclonal antibodies for 30 minutes except for the antibody against transglutaminase (60 minutes). After washing with PB the slides were incubated with the secondary antibody, rabbit-anti-mouse immunoglobulin conjugated with peroxidase (1:50, RAM-PO, Dakopatts, Copenhagen, Denmark) diluted in PB containing 5% human AB-serum for

30 minutes. After washing with PB and demineralised water a 3-amino-9-ethylcarbazole (AEC) solution was used for visualisation.

Staining with T11 was done with an indirect peroxidase-anti-peroxidase technique (PAP). The slides were put in PB for ten minutes and pre-incubated with 50 % Normal Rabbit Serum on PB for 20 minutes. After washing with PB the slides were incubated with the primary monoclonal antibody in a Miele Microwave at 80 Watt for 9 minutes. Then the slides were washed again in PB and incubated with rabbit-anti-mouse immunoglobulin (1:25, RAM-Ig, Dakopatts, Copenhagen, Denmark) in the microwave at 80 Watt for 9 minutes. After washing in PB the slides were incubated with PAP-complexes (1:100, Peroxidase monoclonal mouse antiperoxidase complexes, Dakopatts, Copenhagen, Denmark) in the microwave at 80 Watt for 8 minutes. After washing, this cycle was repeated. Visualisation of the complexes was done by the AEC-solution.

All slides were counter-stained with Mayer's Haematoxylin (Sigma, St. Louis MO, USA) and mounted in glycerol-gelatine.

Histological examination

The histological examination was performed blinded.

Epidermal proliferation was measured by counting the number of MIB-1 positive nuclei per mm length of the section.

The involucrin and transglutaminase expression were assessed by calculation of the ratio positive cell layers/ total cell layers of the viable epidermis. This was done at two sites: above the top of the dermal papilla and between two dermal papillae. The filaggrin expression was assessed by measuring the percentage of the length of the stratum corneum and stratum granulosum which was stained.

Inflammation (PMN and T-lymphocytes) was assessed separately for dermis and epidermis. Dermal inflammation was semi-quantitatively enumerated by expressing the number of positively stained cells as a percentage of the total number of infiltrate cells[14]: 0, no positive cells; 1, sporadic; 2, 1-25 %; 3, 26-50 %; 4, 51-75 %; 5, 76-99 %; 6, 100 %. Epidermal inflammation was assessed using a five-point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, pronounced staining.

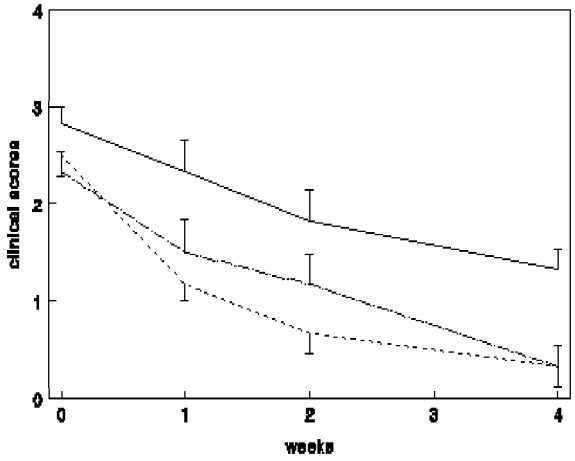
Statistical evaluation

For statistical analysis the t-test for paired values was used. A two-tailed hypothesis was employed to interpret data.

2.1.4. Results

Clinical response

In all patients the psoriatic lesions showed improvement. The course of the clinical scores of the target lesion is shown in figure 1.



Histological response

The transglutaminase expression (Figure 2a) on the top of the dermal papilla showed a significant decrease in the first week of therapy ($p = 0.03$); in the weeks following no significant changes were seen. At the interpapillary epidermis the transglutaminase expression (Figure 2b) slightly diminished resulting in a significant difference after four weeks of therapy ($p = 0.01$). After four weeks of treatment values for normal were reached.[15] In the involucrin expression (Figure 2c) at the top of the dermal papilla a significant increase ($p = 0.04$) after four weeks of therapy was observed.

The involucrin expression at the interpapillary epidermis (Figure 2d) did not show significant changes. The filaggrin staining (Figure 2e) in the stratum corneum significantly increased after two weeks ($p = 0.04$). In the stratum granulosum a significant increase was already seen after one week of treatment with calcipotriol ($p = 0.01$). Although 100% recovery of the stratum corneum was not reached, the recovery of the stratum granulosum layer was nearly complete. Pictures of the immunohistochemical staining for filaggrin before and after therapy are shown in Figure 3a+b.

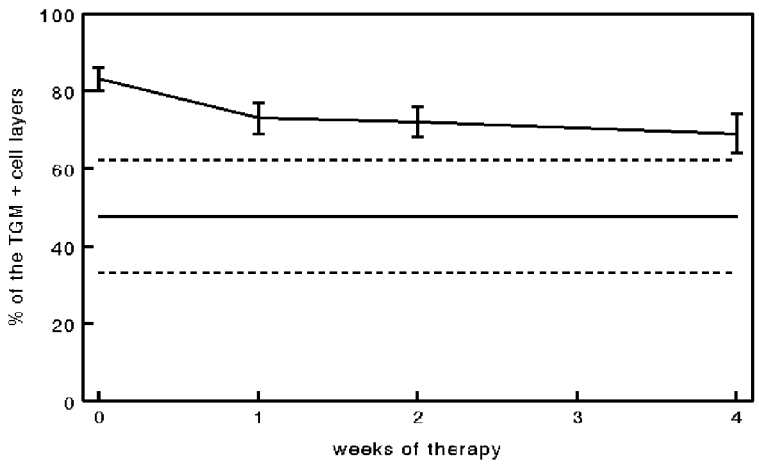


Fig. 2 a
Transglutaminase at the top of the dermal and during treatment with calcipotriol expressed as means ± SEM (values for normal human skin are represented by the horizontal bar as mean ± 2*SD[15])

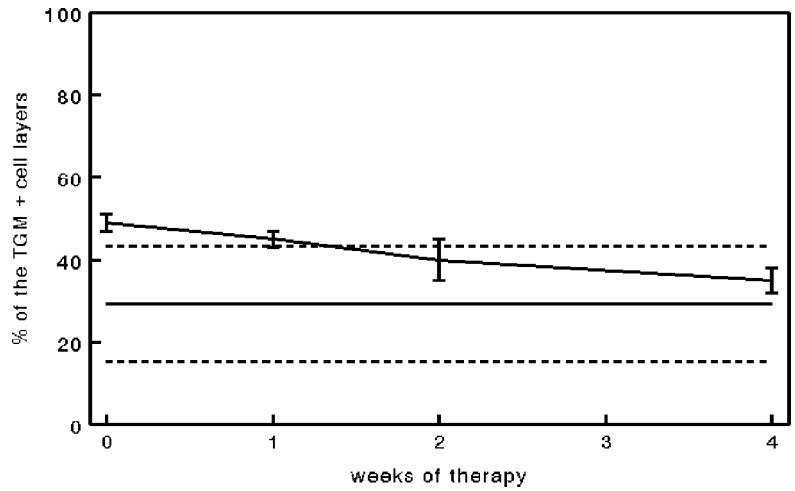


Fig. 2 b
Transglutaminase between two dermal papillae (——) before and during treatment with calcipotriol expressed as means ± SEM (values for normal human skin are represented by the horizontal bar as mean ± 2*SD[15])

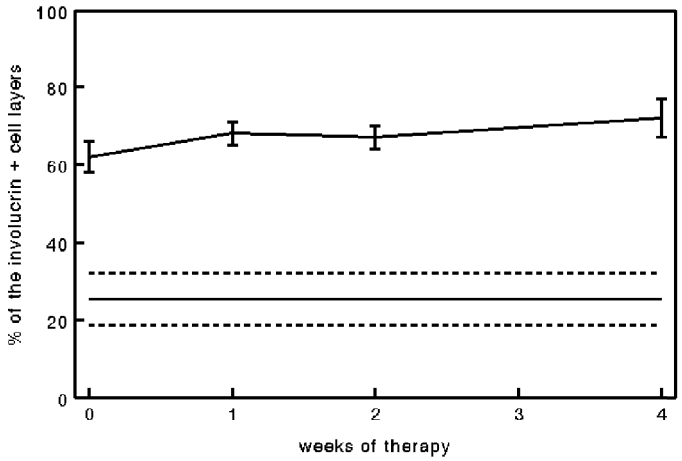


Fig. 2 c Involucrin at the top of the dermal papilla before and during treatment with calcipotriol expressed as means ± SEM (values for normal human skin are represented by the horizontal bar as mean ± 2*SD[33])

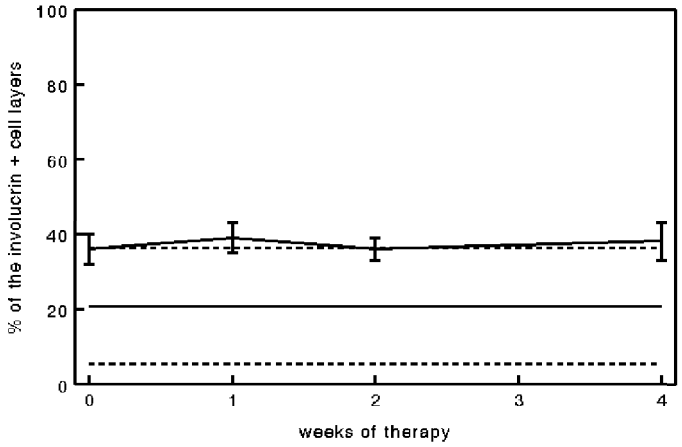


Fig. 2 d Involucrin between two dermal papillae before and during treatment with calcipotriol expressed as means ± SEM (values for normal human skin are represented by the horizontal bar as mean ± 2*SD[33])

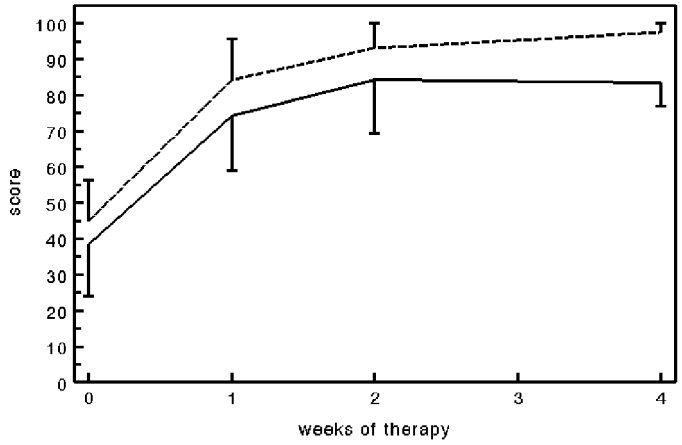


Fig. 2 e Filaggrin in stratum corneum (——) and stratum granulosum (- - - -) before and during treatment with calcipotriol expressed as means ± SEM

The number of Ki-67 positive nuclei per mm of the section (Figure 4) significantly decreased in the first week of therapy ($p = 0.04$). In the following weeks no significant changes were seen in the Ki-67 count. The number of Ki-67 positive nuclei approached but remained above the normal range.[16]

The staining of T-lymphocytes in the dermis (Figure 5a) altered significantly after the first week of therapy ($p = 0.003$), subsequently no significant changes were observed. T11 staining in the epidermis (Figure 5b) showed a tendency to decrease ($p = 0.08$) in the first week. In the weeks following no significant changes were seen. Anti-elastase staining (PMN)(Figure 5c) in the dermis significantly decreased in the first week of therapy ($p = 0.01$). No further significant changes were observed in the following weeks in the dermis. In the epidermis no changes in elastase binding were observed either. T-lymphocyte accumulation and PMN accumulation remained above the range for normal skin throughout the four weeks treatment period.

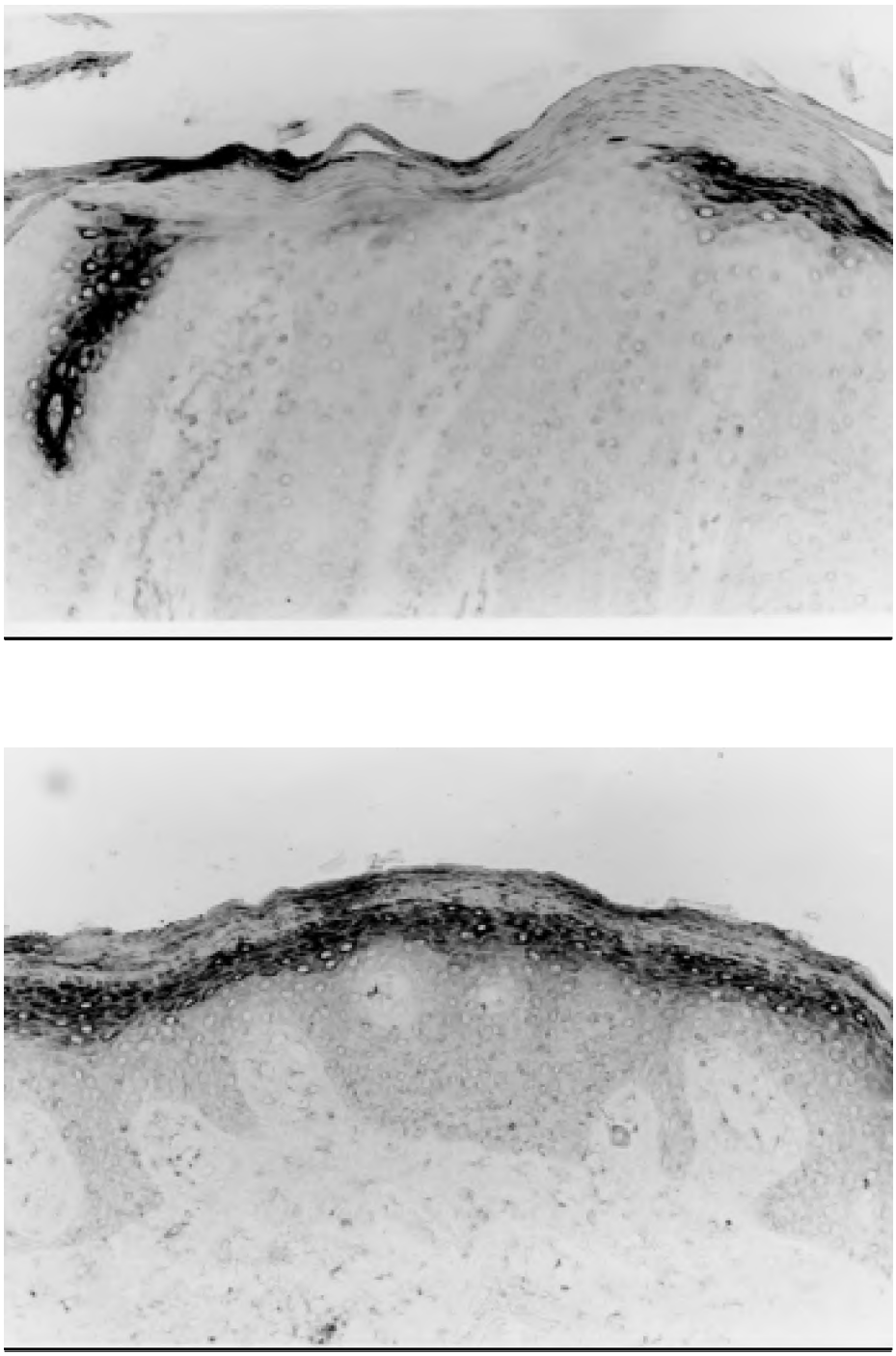


Fig. 3 Anti-filaggrin staining in one patient before (a) and after 4 weeks (b) of therapy

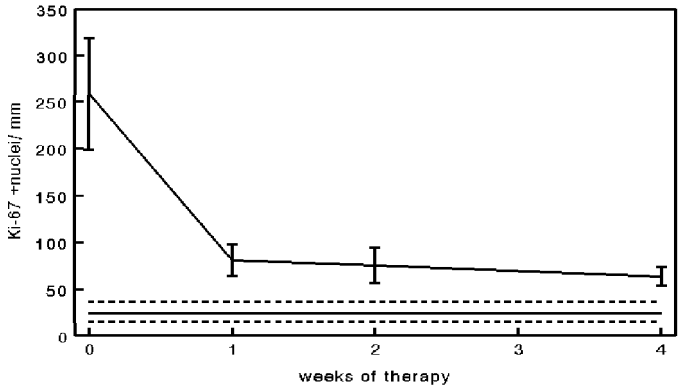


Fig. 4 MIB-1 staining before and during treatment with calcipotriol expressed as means ± SEM (values for normal human skin are represented by the horizontal bar as mean ± 2*SD[16])

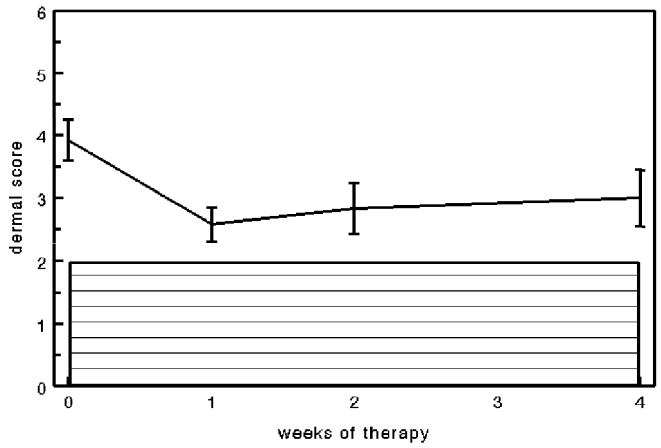


Fig. 5 a T11 (dermis) before and during treatment with calcipotriol expressed as means ± SEM (values for normal human skin are represented by the shaded area as mean ± 2*SD[15])

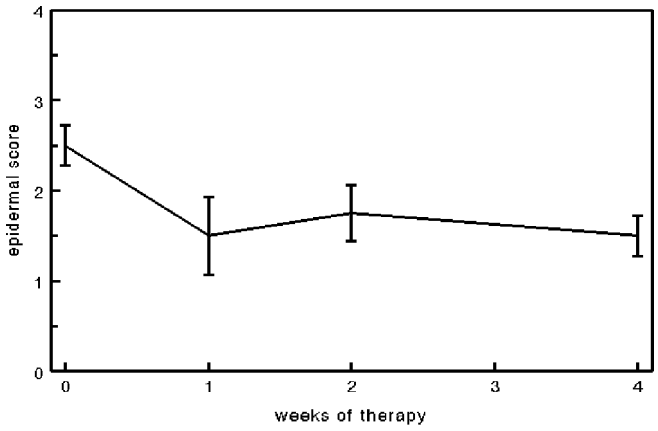


Fig. 5 b T11 (epidermis) before and during treatment with calcipotriol expressed as means ± SEM

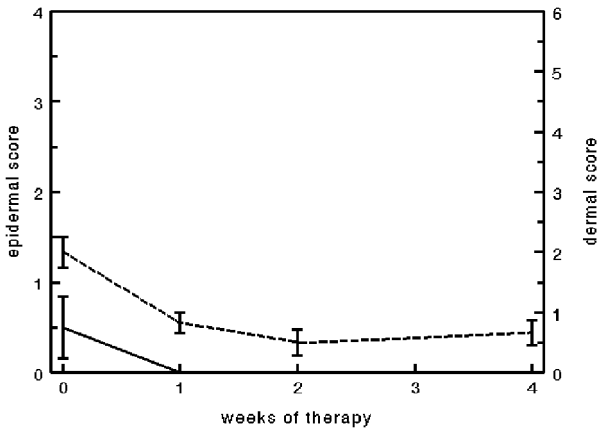


Fig. 5 c Anti-elastase in dermis (- - - -) and epidermis (——) before and during treatment with calcipotriol expressed as means ± SEM

2.1.5. Discussion

In the present study, a major clinical improvement was observed during a four weeks treatment period with calcipotriol. The present study comprises only the first phase of calcipotriol treatment since maximal reduction of severity scores is reached after an eight weeks treatment period.[17] Most studies agree that calcipotriol induces pronounced changes of epidermal behaviour, leaving the mononuclear infiltrate relatively

unaffected.[6,18-20] The present observation is in line with those studies and with the clinical impression of an early and relatively pronounced effect of calcipotriol on scaling, the clinical manifestation of epidermal differentiation.

Epidermal differentiation involves formation of cornified envelopes by cross linking (formation of the ε-(γ-glutamyl)lysine bonds) involucrin, loricrin and keratolinin by the calcium dependent enzyme membrane-bound transglutaminase (TGase 1).[21-24] In normal skin, staining with antibodies against TGase 1 and involucrin shows a band like pattern in the upper part of the epidermis representing the upper stratum spinosum and the stratum granulosum.[25] In situ hybridisation techniques demonstrate involucrin and TGase 1 mRNA at the transition to the stratum granulosum, being a very early event in differentiation.[26] Activity of TGase 1 was demonstrated in vivo in mice at the transition zone (one or at most two cell layers) from the stratum granulosum to the stratum corneum.[25] At this point the actual formation of the cornified envelopes takes place resulting in the stratum corneum in which antigenicity of involucrin is lost.[25] In psoriatic skin, the keratinisation process is disturbed. Large, clinically visible, histologically parakeratotic squames are formed in lesional skin.[26] Staining with monoclonal antibodies against involucrin and TGase 1 demonstrates far more positive cell layers, relatively as well as absolutely.[27] Also activity of TGase 1 and the number of produced cornified envelopes is greatly enhanced.[28] Messenger RNA of involucrin and TGase 1 is localised already in the lower cell layers of the suprabasal epidermis.[26] This is in contrast to normal skin.[26] Increased expression of TGase 1 protein and mRNA in psoriasis may partly explain the hyperkeratosis that is characteristic for psoriasis. The formation of a more highly cross-linked stratum corneum may lead to a greater retention of the squames observed in lesional skin.[29]

The present in vivo observations on epidermal differentiation during treatment with calcipotriol indicate that modulation of the process of keratinisation is a relatively early effect of this compound. However, these observations are in several respects at variance with in vitro observations. TGase 1 activity in cultured keratinocytes is enhanced by calcipotriol.[2] In the present in vivo study, however, TGase 1 positive cells decreased during treatment. In vivo, a similar decrease was observed during treatment of psoriatic plaques with the vitamin D_3 analogues 1,25-$(OH)_2$-D_3 and 1,24-$(OH)_2$-D_3.[27] Whereas vitamin D_3 analogues in vitro enhance the transcription of involucrin[11,12], only a minor modulation of the number of involucrin positive cell layers was observed in the present in vivo study. Treatment with 1,25-$(OH)_2$-D_3 and 1,24-$(OH)_2$-D_3 reduced the number of involucrin positive cell layers.[4,5] Despite the decrease in anti-TGase 1 binding, TGase 1 as well as involucrin remain high during this investigation. The newly formed keratinocytes in the psoriatic lesion keep on expressing the TGase 1 and involucrin mRNA too

early in their maturation process. So far, there is no convincing evidence that calcipotriol modulates epidermal differentiation in vivo via DNA transcription of involucrin or TGase 1.

A remarkable and consistent effect of calcipotriol (present study) and of 1,25-$(OH)_2$-D_3 and 1,24-$(OH)_2$-D_3[4,5] is the pronounced reformation of the filaggrin positive cell layer. Filaggrin plays a major role in the aggregation of keratin filaments, thereby forming the keratin configuration as can be seen in the lower stratum corneum.[30]

The present study demonstrates a potent reduction of the recruitment of cycling epidermal cells (Ki-67 positive nuclei). This result reconfirms the antiproliferative activity as observed in earlier studies.[6,18] The expression of keratin 16 and keratin 17 in the suprabasal compartment is considered to be related to epidermal recovery. During treatment with calcipotriol these hyperproliferation associated keratins proved to reduce.[6,18,31] The antiproliferative action of calcipotriol proved to be consistent in the in vitro and in vivo situation.

Vitamin D_3 analogues interfere with multiple parameters of cutaneous inflammation.[32] Modification of T-cell subpopulations and cytokine pattern and a reduction of PMN accumulation have been observed before.[32] The present study is at variance with a previous study[6] with respect to T-lymphocytes. In the present study the early reduction of the number of T-lymphocytes together with the reduction of PMN accumulation might be the result of the long periods (months-years) that patients had been untreated. In the previous study[6] showing only a modest effect on T-lymphocytes, up to two weeks before treatment the patients had been treated with calcipotriol or topical steroids already.

From this study it may be concluded that inhibition of epidermal growth, recovery of the filaggrin positive cell layer and a decrease of TGase 1 and involucrin positive cells are targets for the in vivo effect of calcipotriol on epidermal differentiation.

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2.2. EPIDERMAL DIFFERENTIATION CHARACTERISTICS OF THE PSORIATIC PLAQUE DURING SHORT CONTACT TREATMENT WITH DITHRANOL CREAM

2.2.1. Summary

Dithranol has been used successfully in the treatment of psoriasis for more than 75 years. Much in vitro and in vivo research has been done on the elucidation of the mode of action of this potent and safe antipsoriatic therapy. In vivo research revealed major effects of dithranol on epidermal proliferation and inflammation. Information on the in vivo effects on epidermal differentiation is limited. Therefore the dynamics of a set of differentiation markers (keratin 16, filaggrin, keratinocyte transglutaminase, involucrin) and markers for proliferation and inflammation (Ki-67, T-lymphocytes, Polymorphonuclear leukocytes) were studied in skin biopsies of six patients with psoriasis during four weeks of dithranol therapy. The treatment regime involved a short contact protocol at the unit for intensified out-patient treatment with an easily wash-off cream.

Treatment resulted in a decrease of the PASI-score of 48 % in four weeks. Immunohistochemically, a major decrease of keratin 16 content and virtually complete restoration of the filaggrin positive cell layer were seen. These changes proved to be significant comparing the markers in the group of six patients. Although many other topical treatments for psoriasis (occlusive therapy and vitamin D_3 analogues) result in a prominent reduction of the amount of transglutaminase and involucrin positive cell layers, the effect of dithranol on these markers is minimal.

2.2.2. Introduction

Although dithranol has been used for many decades in the treatment of psoriasis, the mode of action has not been established. Various authors have demonstrated that dithranol inhibits epidermal proliferation.[1,2] At the molecular level, various mechanisms have been hypothesised to be responsible for this antiproliferative effect: interference with DNA and mitochondria, inhibition of metabolic pathways, modulation of protein kinase C (PKC) and interference with arachidonic acid metabolism and cyclic nucleotides.[3,4] Other groups have demonstrated that dithranol interferes with several aspects of cutaneous inflammation.[5-7] So far, our information on the effect of dithranol on epidermal differentiation is very limited.[1,8]

The in vivo effect of dithranol on cutaneous inflammation and epidermal proliferation has been reported previously.[1,2] The aim of the present investigation was to study the dynamics of

dithranol induced changes with respect to differentiation of the psoriatic epidermis using a series of immunohistochemical differentiation markers (keratin 16, filaggrin, transglutaminase (TGase) and involucrin). These differentiation markers were related to the recruitment of cycling epidermal cells (Ki-67 positive nuclei) and the number of T-cells and polymorphonuclear leukocytes (PMN).

The differentiation characteristics and reference parameters for epidermal growth and inflammation were studied during a dithranol short contact regime. Dithranol was manufactured in a wash off cream. The treatments were carried out alternately at the unit for intensified out-patient treatment or at home after instruction and with twice weekly supervision of the patients. Punch biopsies were taken before and during treatment and processed for immunohistochemical analysis.

2.2.3. Materials and methods

Patients

Six patients with extensive chronic plaque psoriasis participated in this study, five males and one female. Their age varied from 26-61 years with an average duration of the psoriasis of 18 ± 8 years (mean ± SEM). They had used no systemic treatment for at least two months and no topical treatment for at least two weeks. No other medication was allowed that could influence the course of psoriasis.

Treatment protocol

Patients were treated with dithranol in a cream base using a short contact schedule. Dithranol creams at concentrations ranging from 0.1-5 % were manufactured by the hospital pharmacist according to a modified version of the protocol of Ros and Van der Meer.[9] The ingredients of the cream base are summarised in table I.

Table I Ingredients of the dithranol cream [9]

Dithranolum	1-50 gram
Cetiol V	202 gram
Cera cetomacrogolis	150 gram
Paraffinum subliquidum	150 gram
Acidum salicylicum	10 gram
Acidum sorbicum	1.5 gram
Acidum ascorbicum	0.5 gram
Aqua demi filtrata	ad 1000

Dithranol was applied once daily starting with a concentration of 0.1 % for 15 minutes. The cream was removed with water and detergents. No additional topical therapy was allowed except for 10 % liquor carbonis detergens in petrolatum/cremor lanette I ana and 10 % salicylic acid in axungia for treatment of the scalp. Treatments

were carried out at the department of intensified out-patient therapy. In the first week the patient visited the department daily and in the second and following weeks the patient visited the department twice a week. The dithranol concentration and/or application-time intervals were increased every three days if no burning or stinging was reported, under the supervision of a trained nurse and doctor. In the first week of this treatment regime, instruction and education of the patient by the nurse was intense to enable the patient to continue the dithranol applications at home. Treatment was continued until the psoriasis was cleared. The period of investigation was restricted to the first four weeks.

Clinical assessment

Before therapy and 1, 2 and 4 weeks after therapy the clinical response was monitored. Clinical monitoring was done using the PASI.

Biopsy procedure

Punch biopsies of 3 mm were taken of a representative psoriatic plaque before therapy and 1, 2 and 4 weeks after therapy from all patients after local anaesthesia with xylocain 1 % and adrenaline.

The biopsies were embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, USA), snap frozen in liquid nitrogen and stored at -80°C until use. Sections of 7 µm were cut, air dried and fixed for 10 minutes in acetone/ether (60/40 %) (MIB-1 staining) or in acetone (other stainings) and again stored at -80°C.

Monoclonal antibodies

A panel of monoclonal antibodies was used.

To assess epidermal differentiation monoclonal antibodies against keratin 16 and 13 (1:25, Ks 8.12, Sigma, St Louis, USA), anti filaggrin and profilaggrin (1:500, anti-filaggrin, BT576, Biomedical Technologies Inc.), anti-human keratinocyte transglutaminase (1:100, Mouse Monoclonal Antibody, IgG_{2a} Biomedical Technologies Inc.) and against involucrin, (MON-150[10], 1:25) were used.

To approximate the number of cycling epidermal cells in the basal layer an antibody directed against the Ki-67 antigen was used (MIB-1, 1:50, Immunotech, S.A., Marseilles, France).

Analysis of the inflammatory infiltrate was done by assessment of T-lymphocytes and Polymorphonuclear leukocytes (PMN) respectively using the monoclonal antibodies DAKO-T11 (1:100, Dakopatts, Copenhagen, Denmark) and DAKO-elastase (1:100, Dakopatts, Copenhagen, Denmark).

Staining procedure

For all monoclonal antibodies, except for T11, an indirect immunoperoxidase technique was used. For ten minutes the slides were fixed in acetone/ether (60/40 %) in case of MIB-1 or in acetone for the other stainings. The slides were air dried and put in a phosphate buffer (PB) (72 mmol/l Na_2HPO_4 and 28 mmol/l NaH_2PO_4). Only the slides stained with anti-elastase were pre-incubated with methanol/ 0.1 % H_2O_2 (30 %) for 20 minutes. All antibodies were diluted in PB. The slides were incubated with the different primary monoclonal antibodies for 30 minutes except for the antibody against TGase (60 minutes). After washing with PB the slides were incubated with the secondary antibody, rabbit-anti-mouse immunoglobulin conjugated with peroxidase (1:50, RAM-PO, Dakopatts, Copenhagen, Denmark) diluted in PB containing 5 % human AB-serum for 30 minutes. After washing with PB and demineralised water a 3-amino-9-ethylcarbazole (AEC) solution was used for visualisation.

Staining with T11 was done with an indirect peroxidase-anti-peroxidase technique (PAP). The slides were put in PB for ten minutes and pre-incubated with 50 % Normal Rabbit Serum on PB for 20 minutes. After washing with PB the slides were incubated with the primary monoclonal antibody in a Miele Microwave at 80 Watt for 9 minutes. Then the slides were washed again in PB and incubated with rabbit-anti-mouse immunoglobulin (1:25, RAM-Ig, Dakopatts, Copenhagen, Denmark) in the microwave at 80 Watt for 9 minutes. After washing in PB the slides were incubated with PAP-complexes (1:100, Peroxidase monoclonal mouse antiperoxidase complexes, Dakopatts, Copenhagen, Denmark) in the microwave at 80 Watt for 8 minutes. After washing, this cycle was repeated. Visualisation of the complexes was done by the AEC-solution.

All slides were counter-stained with Mayer's Haematoxylin (Sigma, St. Louis MO, USA) and mounted in glycerol-gelatine.

Histological examination

The histological examination was performed blinded; the scores for normal skin are shown in table II. Ks 8.12 staining in the epidermis was assessed using a semi-quantitative scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining;4,moderate-pronounced

staining; 5, pronounced staining; 6, whole epidermis stained. The filaggrin expression was assessed by measuring the percentage of the length of the stratum corneum and stratum granulosum which was stained. The involucrin and TGase expression were assessed by calculation of the ratio positive cell layers/total cell layers of the viable epidermis. This was done at two sites: above the dermal papilla and between two dermal papillae.

Epidermal proliferation was measured by counting the number of MIB-1 positive nuclei per mm length of the section throughout the whole length of the biopsy.

Inflammation (PMN and T-lymphocytes) was assessed separately for dermis and epidermis. Dermal inflammation was semi-quantitatively enumerated by expressing the number of positively stained cells as a percentage of the total number of infiltrate cells: 0, no positive cells; 1, sporadic; 2, 1-25 %; 3, 26-50 %; 4, 51-75 %; 5, 76-99 %; 6, 100 %. Epidermal inflammation was assessed using a five-point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, pronounced staining.

Table II Immunohistochemical sores $\pm$ 2 $\times$ standard deviation (2 $\times$ SD)[34-37]

Epitope	*Localisation*	*Mean* $\pm$ *2* $\times$ *SD*
Ks8.12	suprabasal	$\leq$ 1
Filaggrin	stratum	100 $\pm$ 0
	stratum	100 $\pm$ 0
Transglutami	tip of papilla	48 $\pm$ 15
	interpapillar	29 $\pm$ 14
Involucrin	tip of papilla	25 $\pm$ 7
	interpapillar	21 $\pm$ 15
Ki-67	stratum basale	27 $\pm$ 9
T11	dermis	$\leq$ 2
	epidermis	0 $\pm$ 0
Elastase	dermis	0 $\pm$ 0
	epidermis	0 $\pm$ 0

Statistical evaluation

For statistical analysis the t-test for paired values was used. A two tailed hypothesis was employed to interpret data.

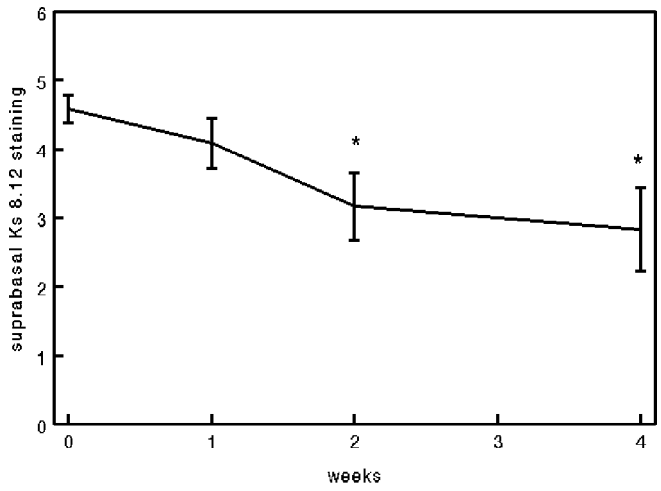

Fig. 1 a Ks8.12 staining in the suprabasal part of the epidermis during treatment with dithranol; values are mean ± SEM
(*: p< 0.05).

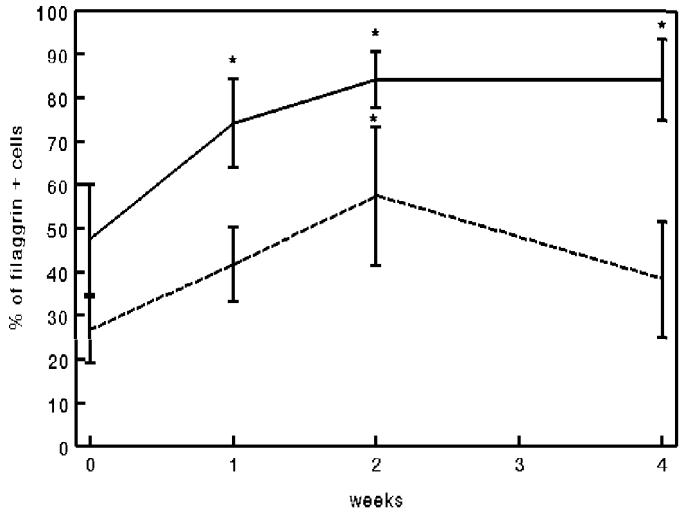

Fig. 1 b Filaggrin staining in stratum granulosum (——) and stratum corneum (- - - -) during treatment with dithranol; values are mean ± SEM
(*: p< 0.05).

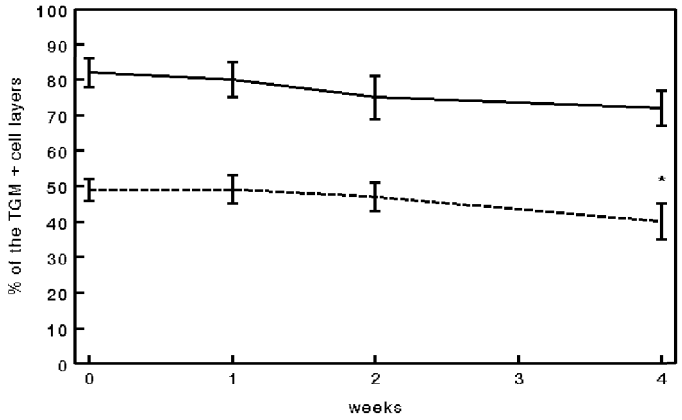

Fig. 1 c Transglutaminase staining above the dermal papilla (——) and between two dermal papillae (- - - -) during treatment with dithranol; values are mean ± SEM
(*: p< 0.05).

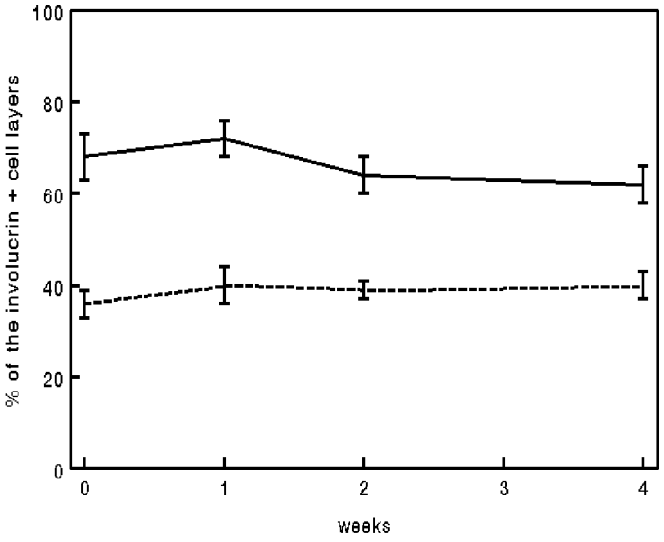

Fig. 1 d Involucrin staining above the dermal papilla (——) and between two dermal papillae (- - - -) during treatment with dithranol; values are mean ± SEM.

2.2.4. Results

Clinical assessment

After four weeks of treatment with dithranol the PASI-scores were reduced by 48 %.

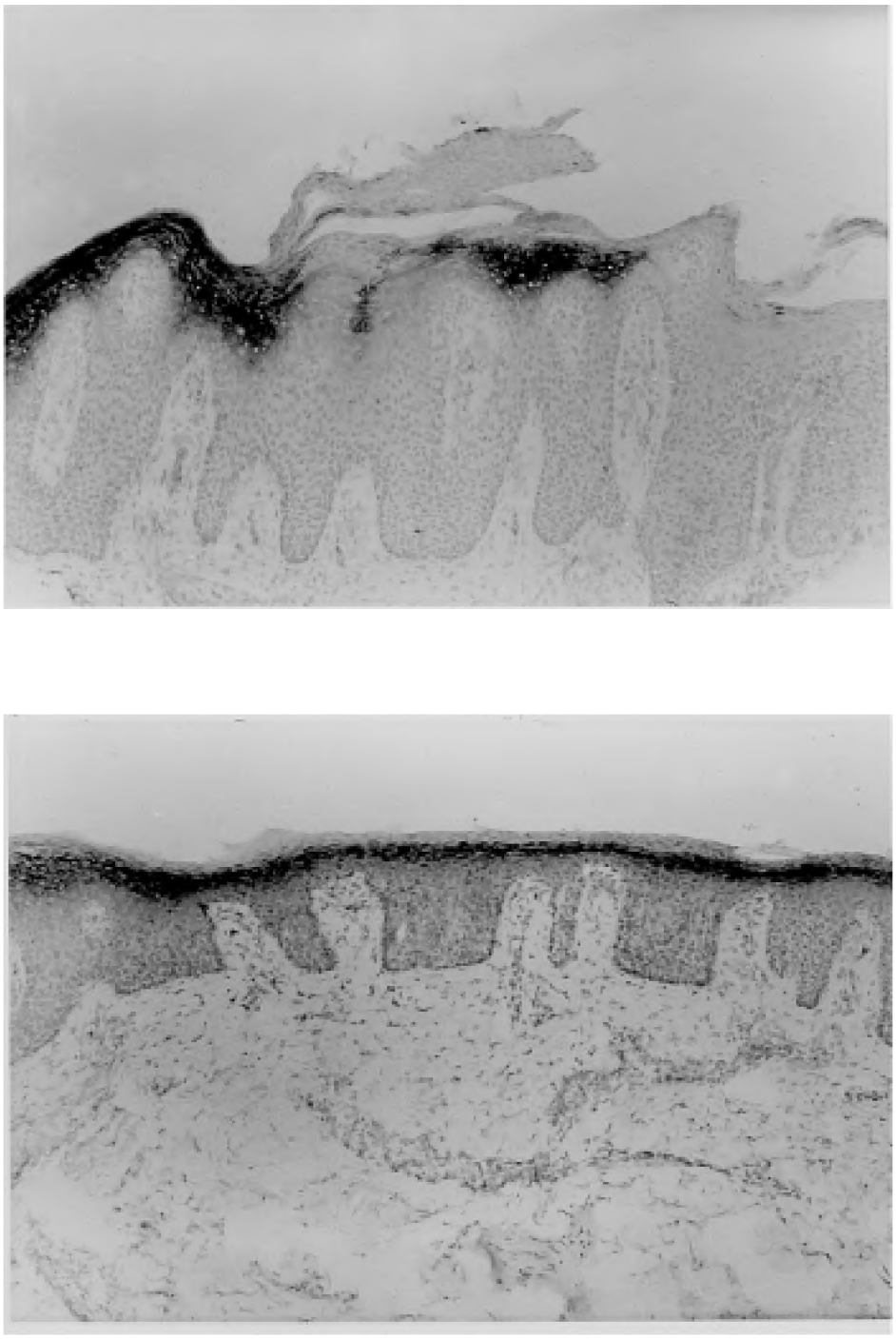

Fig. 2 Anti-filaggrin staining in one patient before (a) and after 4 weeks (b) of therapy.

Immunohistochemical assessment

The results of the immunohistochemical scores are presented in figures 1-4. Ks 8.12 staining (keratin 16, figure 1a) decreased substantially during the first two weeks of treatment ($p = 0.02$ after two weeks and $p = 0.02$ after four weeks). The filaggrin staining (figure 1b) increased substantially during the first two weeks of treatment ($p = 0.01$ after one week, $p = 0.006$ after four weeks for the stratum granulosum and $p = 0.03$ for the stratum corneum). Staining with anti keratinocyte transglutaminase (figure 1c) showed a slight and borderline significant decrease above the dermal papilla after two weeks ($p = 0.07$). TGase staining at the interpapillary epidermis did not reveal a significant reduction during the first two weeks; however a slight but significant decrease ($p = 0.03$) was observed after four weeks of dithranol. The involucrin staining (figure 1d) did not show any change. Immunohistochemical slides of the staining of filaggrin before and during therapy are shown in figure 2.

MIB-1 staining (Ki-67 positive nuclei, figure 3) revealed a significant decrease after two weeks of treatment with dithranol ($p = 0.05$) which became highly significant after four weeks ($p = 0.02$).

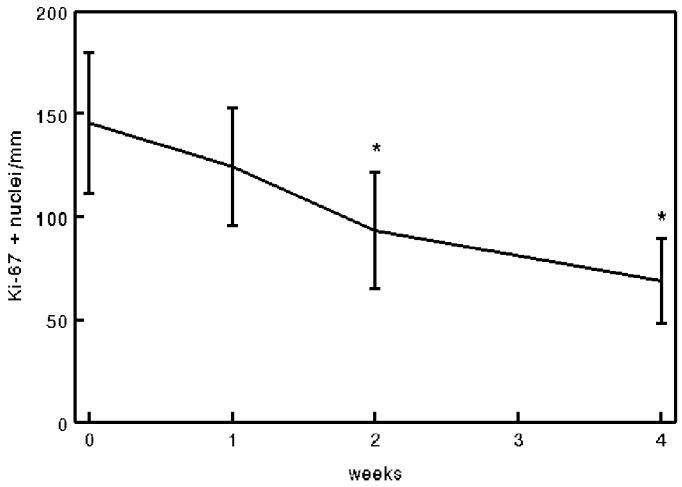

Fig. 3 MIB-1 staining during treatment with dithranol; values are mean ± SEM (* $p < 0.05$).

The percentage T11-positive T-lymphocytes (figure 4a) in the dermal inflammatory infiltrate decreased significantly ($p = 0.04$) in the time interval between one and four weeks. T11 staining in the epidermis and anti-elastase staining (figure 4b) in dermis or epidermis did not reveal significant changes.

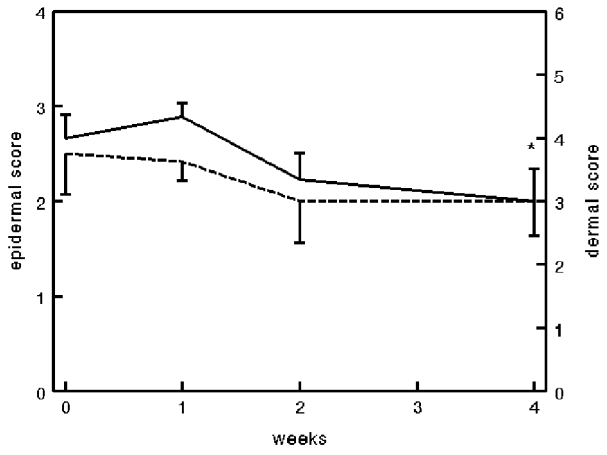

Fig. 4 a T11 staining in the dermis (——) and epidermis (- - - -) during treatment with dithranol; values are mean ± SEM (*: p< 0.05).

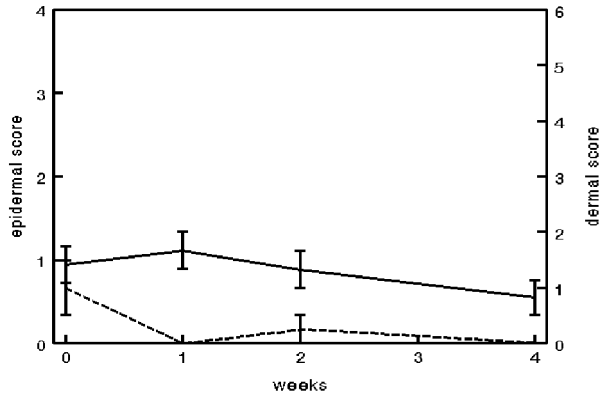

Fig. 4 b Elastase staining in the dermis (——) and epidermis (- - - -) during treatment with dithranol; values are mean ± SEM.

2.2.5. Discussion

Classical dithranol treatment with 24 hour applications has been the gold standard in the treatment of psoriasis for many decades. Side-effects like staining and irritation often limit its use. So far, no new analogues with a more advantageous efficacy/side-effect profile have been registered.[11] Alternative application schedules with cream formulations, on the other hand, do seem to reduce the discomfort of the classical 24

Hour dithranol applications.[3,12] Short application periods (minutes-hour) of dithranol increase bioavailability in the psoriatic plaque whilst decreasing its effects on the healthy skin surrounding the lesions.[13] Cream formulations, on the other hand, are often thought to be less effective than the classical formulations but supply more ease in rinsing the skin so that it takes only about an hour for the patient to carry out this treatment at home.[3,12,14] After four weeks of treatment, the clinical results in the present study were indeed inferior compared to classical in-patient treatment since clearing within 4 weeks is known from in-patient studies using classical 24 hours dithranol applications.[3]

The present study confirms earlier observations on the in vivo effects of dithranol on proliferation and inflammation of the psoriatic plaque[1] and extends our knowledge on the effects on epidermal differentiation characteristics during dithranol therapy. Already after two weeks of treatment the recruitment of cycling epidermal cells (Ki-67 positive nuclei) and keratin 16 expression are reduced and the accumulation of T-lymphocytes only diminishes slightly and relatively late during dithranol treatment. These changes proved to be statistically significant, already in six patients. The early reduction of PMN and the more impressive reduction of the proliferation markers as reported in a previous study might be due to a more aggressive schedule of dosage increments.[1] The changes proved to be statistically significant already in six patients.

The aim of this study was to determine the in vivo effects of dithranol on immunohistochemical markers for epidermal differentiation as previous studies have not addressed this issue. The pattern of interference of dithranol with these differentiation markers is intriguing. Whereas keratin 16 and filaggrin substantially change during treatment with dithranol, the number of TGase positive cell layers shows a significant but minimal decrease. The number of involucrin positive cell layers did not reduce at all during dithranol treatment. Many topical antipsoriatic therapies, for instance vitamin D_3 analogues, inhibit epidermal growth (recruitment of cycling cells and keratin 16 expression) and enhance filaggrin expression, just like dithranol.[15-20] However, in their effect on the number of TGase and involucrin positive cell layers, dithranol and the other local antipsoriatic therapies differ markedly: calcipotriol, tacalcitol, calcitriol and occlusive therapy, in contrast to dithranol, do have a substantial effect on TGase and involucrin.[16-18,20]

Epidermal differentiation and keratinisation are responsible for the maintenance of epidermal homeostasis. These processes are disturbed in psoriatic skin.[21,22] It has been suggested that terminal differentiation (forming of cross-linked envelopes) and keratin synthesis (and aggregation by filaggrin) are two processes that are independently regulated.[23] Such might explain the differential changes in the keratinisation related markers and terminal differentiation related markers during dithranol therapy. Multiple

factors regulate these processes. PKC, representing a family of different isozymes, is decreased or downregulated in psoriatic skin. Activation of PKC and subsequent down modulation of the enzyme is thought to play a major role in the modelling of the psoriatic phenotype and the regulation of terminal epidermal differentiation.[24-28] PKC has a direct effect on transcription of TGase, involucrin, filaggrin and keratins.[24,28-30] Dithranol decreases PKC activity in vitro and its therapeutic activity might at least partly be mediated by the inhibition of PKC.[4,11] In our in vivo study, normalised keratin and filaggrin expression and only slightly decreased TGase staining were observed. It is attractive to speculate that this differential reaction pattern of psoriatic skin to dithranol therapy might be a result of particular up or down regulation of a specific set of PKC isozymes each with differential effects on different markers for differentiation.[31] Vitamin D_3 analogues do also have effects on PKC.[32,33] Interference with other sets of PKC isozymes may probably explain the differences between dithranol and other local antipsoriatic therapies, such as vitamin D_3 analogues, on differentiation markers. Further studies are required before the role of PKC in the mode of action of dithranol can be established.

In conclusion, dithranol treatment, using short contact applications of a cream formulation under intensified clinical supervision, resulted in a substantial inhibition of epidermal growth, a substantial increase of the filaggrin positive cell layers, whilst interfering only slightly with the number of TGase positive cells and not at all with the number of involucrin positive cell layers. This might indicate that early differentiation and keratinisation are important targets for the in vivo action of dithranol.

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2.3. A NOVEL DITHRANOL FORMULATION (MICANOL): THE EFFECTS OF MONOTHERAPY AND UVB COMBINATION THERAPY ON EPIDERMAL DIFFERENTIATION, PROLIFERATION AND CUTANEOUS INFLAMMATION IN PSORIASIS VULGARIS

2.3.1. Summary

Micanol, in which dithranol is micro-encapsulated in crystalline monoglycerides, is easy to wash off and staining and irritation are inconspicuous. These features make it appropriate to use in an out-patient setting. In this study the immunohistochemical effects of this new dithranol formulation were studied and compared with UVB and the combination of these therapies in skin biopsies of eight patients with psoriasis. Markers for epidermal differentiation, proliferation and cutaneous inflammation were assessed.

The present study suggests that Micanol predominantly had diminishing effects on inflammation markers, hardly affecting the epidermis. UVB had a broad spectrum of reductions. It is feasible that the combination resulted in various synergistic effects.

Previous studies, however revealed a relative persistence of the inflammatory infiltrate with more effects on epidermal processes following dithranol treatment. Based on the present study and on previous studies it is hypothesised that Micanol delivers the active substance more directly in the dermal infiltrate, leaving the epidermis relatively unaffected. This might explain the low irritancy of Micanol treatment.

2.3.2. Introduction

Topical treatment of psoriasis with dithranol, either or not combined with UVB phototherapy, is a safe and effective approach for those patients who do not respond to first line treatments like vitamin D_3 analogues and topical steroids. Short contact schedules and new wash-off formulations have popularised the dithranol treatment of psoriasis vulgaris. Recently a new principle was introduced: dithranol micro-encapsulated in crystalline monoglycerides (Micanol).[1] The advantage of this new dithranol formulation is that it is very easy to wash off. In view of the relatively high melting point of the formulation, the cream has to be massaged into the skin. In our centre, a three-way comparative study was carried out in 36 patients, showing that Micanol has substantial clinical efficacy without significant irritation and staining of the skin and the patients' environment. Further, it was shown that the efficacy of dithranol in this formulation was

comparable with UVB phototherapy; the combination of the two therapies tended to be more effective than either monotherapy.[2]

The aim of the present study was to compare the immunohistochemical effects of dithranol in this new formulation with the changes due to UVB phototherapy and the combination of Micanol and UVB. These effects were compared and contrasted with studies previously carried out on the effects of dithranol in other formulations. Before and at the end of treatment, punch biopsies were taken from a group of eight patients who participated in a larger clinical study on efficacy and safety of these treatments. Assessments were carried out of immunohistochemical stainings with monoclonal antibodies against transglutaminase, involucrin, filaggrin, Ki-67, T-lymphocytes and polymorphonuclear leukocytes (PMN).

2.3.3. Materials and methods

Study design

Eight patients with extensive chronic plaque and/or guttate psoriasis were included in a partly open, partly double blind, placebo-controlled comparative study. The body of a patient was divided into two body-halves. Each body-half received one of the following treatments:

a) Micanol cream (Zyma SA, Switzerland) only.
b) Placebo cream combined with UVB.
c) Micanol cream combined with UVB.

The patients were treated during a maximum of eight weeks. The UVB treatment was the open part of the study. UVB was given three times a week; short contact dithranol therapy was given daily. Clinical assessments were carried out three times a week in the first two weeks and once a week during the following six weeks.

Patients

The group of eight patients consisted of two males and six females; their age varied from 24 to 65 years. These patients participated in a larger study on the clinical efficacy of Micanol, UVB phototherapy and the combination of UVB and Micanol. The psoriatic lesions were symmetrically distributed, chronic and in a reasonably stable phase. The percentage body involvement with psoriatic lesions was 5-35 %. Apart from psoriasis, the patients did not have other dermatological or internal diseases. Concomitant treatment was maintained only when it was not expected to interfere with the test medications. Before study initiation, no local antipsoriatic treatment had been administered for two weeks and the patients had not used systemic treatment for at least four

weeks. Permission of the local Ethics Committee and written informed consent from all patients were obtained.

Treatments

Dithranol/placebo treatment

The Micanol cream (Zyma SA Switzerland) and the placebo cream were supplied in concentrations of 0.25 %, 0.5 %, 1 %, 2 % and 3 %. The placebo cream consisted of the vehiculum of the Micanol without the active substance.

Daily applications of the dithranol and/or placebo cream were carried out on one or both halves of the body by the patient at home. The starting concentration was 0.25 % for 30 minutes. The cream was subsequently removed with water and detergents. After two days the dithranol concentration was increased if no stinging or burning occurred. At the highest dithranol concentration of 3 % the application period was lengthened at each visit with 30 min up to a maximum of 120 min.

UVB treatment

All patients received UVB treatment on one or both body-halves, three times a week during eight weeks. UVB exposure was carried out in a Waldman UV 1000 cabin (Waldman AG, Schwenningen, Germany), equipped with 26 Voltarc USA F71 T12/2072 bulbs with an irradiance of 1.9 mW/cm^2. The lamps had an emission spectrum of 285-350 nm, maximal at 310-315 nm. Before starting the minimal erythema dose (MED) was assessed. Half this dose was given to start UVB treatment. The doses were individually adjusted to cause suberythematous to slight erythematous reactions without burning.

Assessment of clinical efficacy

The patients were treated for eight weeks or shorter if lesions were cleared earlier. If only one body-half was cleared, all treatment of this side was stopped while treatment on the other half was continued. Clinical improvement was assessed using the PASI-score. The PASI-scores were calculated per body-side as half PASI-scores.

Biopsy procedure and immunohistochemical staining

In all eight patients punch biopsies of 3 mm were taken from a representative lesion before and after treatment. The biopsy procedure has been described before.[3] For immunohistochemical staining a panel of monoclonal antibodies was used.

To assess epidermal differentiation, monoclonal antibodies against involucrin (MON-150 [4], 1:25), anti-human keratinocyte transglutaminase (1:100, Mouse Monoclonal Antibody, BT621, IgG_{2a} Biomedical Technologies Inc.) and against filaggrin and profilaggrin (anti-filaggrin, 1:500, BT576, Biomedical Technologies Inc.) were used.

To approximate the number of cycling epidermal cells in the basal layer, an antibody directed against the Ki-67 antigen was used (MIB-1, 1:50, Immunotech, SA, Marseilles, France).

Analysis of the inflammatory infiltrate was made by assessment of T-lymphocytes and PMN respectively using the monoclonal antibodies DAKO-T11 (1:100, Dakopatts, Copenhagen, Denmark) and DAKO-elastase (1:100, Dakopatts, Copenhagen, Denmark).

Staining procedure

For all monoclonal antibodies, except for T11, an indirect immunoperoxidase technique was used. Staining with T11 was performed with an indirect peroxidase-anti-peroxidase technique (PAP). The staining techniques have been described previously.[3] The PAP procedure was carried out using the microwave method.[5]

Histological examination

The histological examination was performed blinded. These scoring methods have been performed and published before.[3,6]

Involucrin and transglutaminase expression was assessed by calculation of the ratio positive cell layers/total cell layers of the viable epidermis. This was done at two sites: above the dermal papilla and between two dermal papillae. Filaggrin expression was assessed by measuring the percentage of the length of the stratum corneum and stratum granulosum which was stained.

Epidermal proliferation was measured by counting the number of MIB-1 positive nuclei per mm length of the section.

Inflammation (PMN and T-lymphocytes) was assessed separately for dermis and epidermis. Dermal inflammation was semi-quantitatively enumerated by expressing the number of positively stained cells as a percentage of the total number of infiltrate cells: 0, no positive cells; 1, sporadic; 2, 1-25 %; 3, 26-50 %; 4, 51-75 %; 5, 76-99 %; 6, 100 %. Epidermal inflammation was assessed using a five-point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, pronounced staining.

Statistical evaluation

Data are reported as means ± SEM. Changes in PASI and immunohistochemical markers, due to therapy, were evaluated with the student t-test for paired values, in figures shown as *: $p \leq 0.05$; **: $p \leq 0.005$. A two-tailed hypothesis was employed to interpret data. A synergistic effect of two treatments was defined as an effect which proved to significantly exceed the sum of the effects of two individual treatments. In order to find out whether a synergistic effect can be validated in statistical terms, we performed a two way analysis of variance (2-way ANOVA).

2.3.4. Results

Clinical response

All patients showed a marked improvement on both body-halves. Two patients cured within eight weeks on one or both body-halves. The mean total UVB dose was 20 ± 3.0 J/cm^2 for the body-halves that were treated with UVB monotherapy and 18 ± 5.0 J/cm^2 (mean ± SEM) for the combination therapy treated body-halves. No statistically significant difference in UVB dose was seen between the body-halves that were treated with UVB monotherapy or combination therapy. The PASI-score decreased significantly for all treatments. The relative improvement with Micanol was 69 % ($p = 0.006$), UVB therapy resulted in a relative improvement of 71 % ($p = 0.04$) and the combination of both resulted in a relative improvement of 78 % ($p = 0.003$). The combination of Micanol and UVB revealed a significantly synergistic effect on the PASI-score ($p \leq 0.05$).

Histological results

In all the biopsies taken before treatment, psoriatic histological features were present: hyperkeratosis, acanthosis with thinning of the suprapapillary layer, pronounced elongation of the rete-ridges and in the dermis a marked cellular infiltrate. After treatment a diminution in epidermal thickness and dermal cellular infiltrate was seen in all biopsies, although hyperkeratosis and acanthosis were still present, also in the biopsies taken from the clinically cured body-halves.

The results of the immunohistochemical stainings are shown in figures 1-3. Micanol had mainly decreasing effects on inflammation markers: a highly significant reduction of the number of PMN ($p \leq 0.005$) and on the number of T-lymphocytes ($p \leq 0.005$) was observed. The epidermis

was not significantly affected by Micanol. UVB had an extensive profile of immunohistochemical reductions: highly significant reductions ($p \leq 0.005$) of the number of transglutaminase and involucrin positive cell layers, the number of Ki-67 positive basal keratinocytes and T-cells. The combination of dithranol and UVB resulted in various

synergistic effects. Statistically significant synergy was shown for the Ki-67-staining ($p \leq 0.05$), filaggrin-staining in the stratum corneum ($p \leq 0.05$), PMN in the dermis ($p \leq 0.05$) and in particular, of T-cells in dermis and epidermis ($p \leq 0.005$).

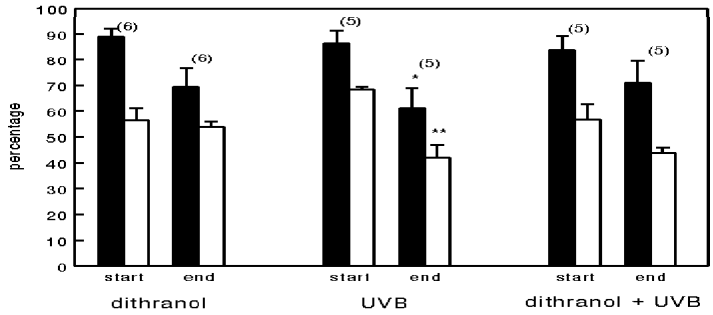

Fig. 1 a Transglutaminase above the dermal papilla (■) and interpapillar (□) before and after therapy (group comparison:
* = $p \leq 0.05$;
** = $p \leq 0.005$).

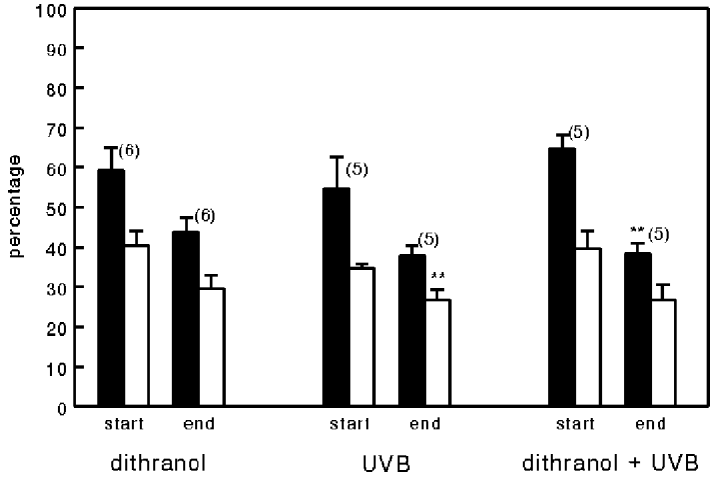

Fig. 1 b Involucrin above the dermal papilla (■) and interpapillar (□) before and after therapy (group comparison: ** = $p \leq 0.005$).

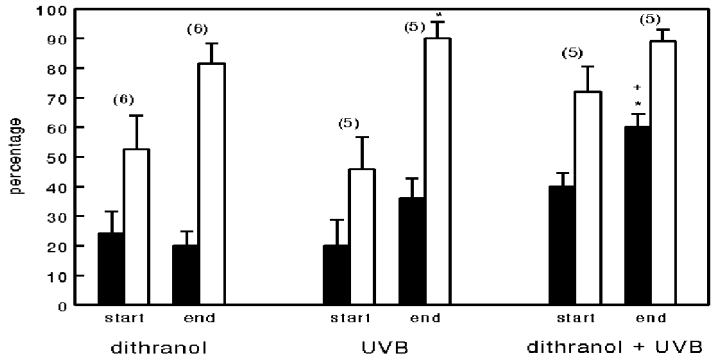

Fig. 1 c Filaggrin in the stratum corneum (■) and stratum granulosum (□) before and after therapy. (group comparison:
* = $p \leq 0.05$;
synergism:
+ = $p \leq 0.05$).

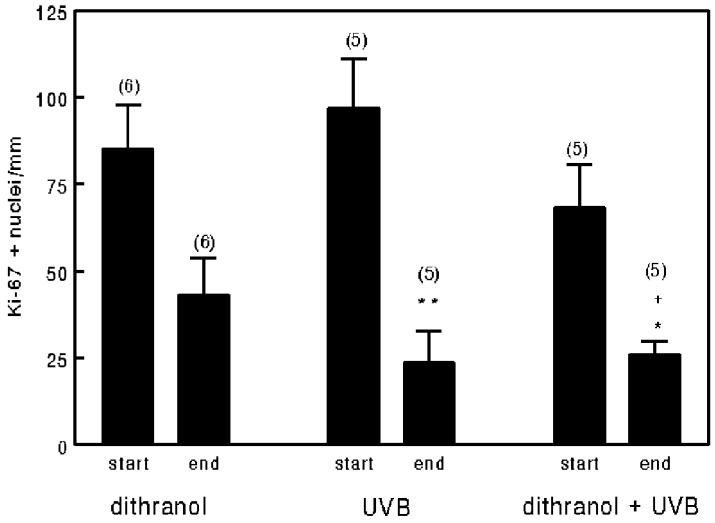

Fig. 2 Epidermal proliferation: Ki-67 staining before and after therapy. (group comparison:
* = $p \leq 0.05$;
** = $p \leq 0.005$;
synergism:
+ = $p \leq 0.05$).

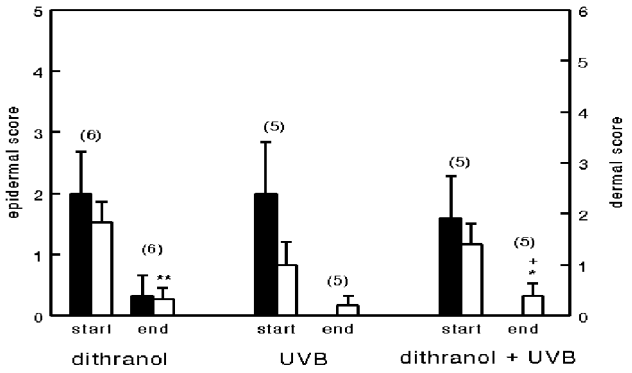

Fig. 3 a PMN in the epidermis (■) and dermis ( ) before and after therapy.

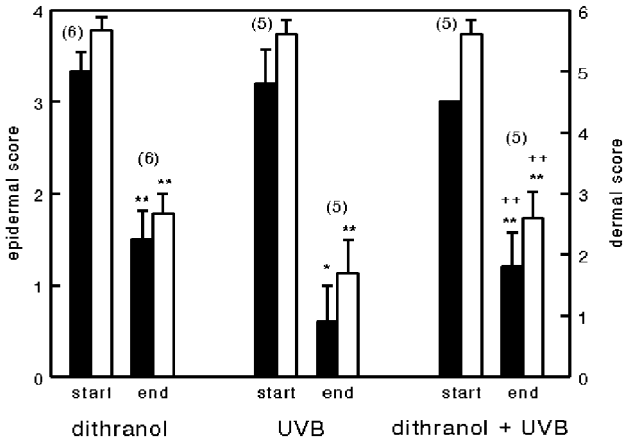

Fig. 3 b T11 in the epidermis (■) and dermis ( ) before and after therapy. (group comparison:
* = $p \leq 0.05$;
** = $p \leq 0.005$; synergism: + = $p \leq 0.05$; + + = $p \leq 0.005$).

2.3.5. Discussion

The design of the study was partly a left/right comparison, partly a parallel group study. The left/right analysis has the drawback that systemic effects of topical treatments might occur, resulting in a contralateral effect. Therefore it is remotely possible that the difference between the three regimes might have been expressed more evidently in case of a parallel group design. The clinical results observed in the present study are comparable with the results observed in the

plenary group.[2] In the present study of eight patients a statistically significant synergistic effect of the combination therapy was seen with respect to the PASI, indicating a more pronounced clinical effect of the combination therapy.

Our immunohistochemical data suggest that Micanol monotherapy had significant decreasing effects on dermal accumulation of PMN and dermal and epidermal accumulation of T-cells. In contrast, no substantial effects on epidermal growth and differentiation parameters were observed. These data are at variance with previous studies on dithranol incorporated in cream or petrolatum, which showed a relative persistence of the inflammatory infiltrate with more pronounced effects on epidermal growth and differentiation.[6,7] During treatment with dithranol in emulsifying ointment the accumulation of T-lymphocytes and CD14 cells persisted up to 8 weeks whereas the suprabasal expression of keratin 16 as well as the number of Ki-67 positive nuclei has diminished considerably during treatment.[6] The immunohistochemical results in this study were analysed using the same methodology as in the present study. It is of interest that the relative reduction of the PASI-score in the previous study[6] was 77 %; in the present study the reduction of PASI was 69 %. In a histochemical study on the effect of dithranol in petrolatum on psoriasis by Braun-Falco,[7] besides changes in parameters for differentiation, the persisting inflammatory reaction was a remarkable finding.[7] Although comparisons between different studies have their limitations, the statistical validation of the immunohistochemical studies demonstrates that Micanol base has a profile of immunohistochemical changes the is different from more traditional vehicles.

Phototherapy with UVB had substantial effects on expression of transglutaminase, involucrin, the recruitment of cycling cells and the accumulation of T-cells. Only a borderline significant effect was seen with respect to filaggrin expression. UVB phototherapy and dithranol treatment have markedly different profiles of effects on the skin compared to other local antipsoriatic therapies, for instance vitamin D_3 analogues (calcipotriol, calcitriol and tacalcitol).[3,8,9] These different histological changes are reflected in the different sequential clinical changes of the psoriatic plaque as a result of each therapy. UVB (λ = 290-320 nm) has major effects on the epidermis, resulting in DNA damage by formation of pyrimidine dimers and hence interfering with macromolecule synthesis and cell division.[10] The in vivo effect of vitamin D_3 analogues is primarily on epidermal proliferation and differentiation, partly via binding with the vitamin D_3 receptor, resulting in a cascade of nuclear mechanisms, and partly via non-genomic mechanisms.[11] Dithranol is thought to have its effects predominantly via auto-oxidation and the formation of free radicals, thereby causing a cascade of effects.[12] The so-called 'minimum structure for antipsoriatic activity' is responsible for the antipsoriatic effects, also causing irritation and staining of the skin.[13] Short

contact application schedules constitute one method of decreasing irritation and discomfort for the patient. Dithranol in the formulation of the present study is another method of decreasing the adverse events. One could speculate that dithranol in Micanol delivers the active substance more directly in the dermal infiltrate, where auto-oxidation starts, leaving the epidermis relatively unaffected. Such might explain the low irritancy of Micanol treatment.

In the combination therapy, a statistically significant synergistic clinical effect was demonstrated. The combination therapy also revealed evident immunohistochemical synergistic effects on the recruitment of cycling cells, involucrin above the dermal papilla, filaggrin in the stratum corneum, PMN and T-cells. It is intriguing that the immunohistochemical effects in the combination therapy can not always directly be predicted from adding up the effects in either monotherapy. The present study suggests that the immunohistochemical effects of both therapies are mingled and amplified, resulting in a new profile of immunohistochemical changes.

In literature, so far, no consensus has been reached as to the synergism of the combination of dithranol and phototherapy.[14-18] Biochemical studies revealed that UVB increases dithranol activity.[19] But it is well-established that during optimised dithranol therapy with the gold standard of 24 hour applications in an in-patient setting, UVB does not improve the antipsoriatic efficacy.[20,21] On the other hand, less optimised dithranol treatment (short contact treatment with dithranol cream at home) is enhanced by phototherapy as demonstrated in the present clinical study. It is a well-established fact that remissions last longer when UVB is added.[22] It is attractive to hypothesise that during less optimised dithranol treatment, in which less irritation is encountered, the effects of the dithranol therapy are enhanced by the effects of UVB on epidermal processes.

In conclusion, the present study suggests that Micanol predominantly has anti-inflammatory immunohistochemical effects. However, comparative studies with different dithranol formulations have to be carried out to prove this statement definitively. UVB had a broad spectrum of reductions in immunohistochemical parameters. The combination appeared to result in clinical and multiple immunohistochemical synergistic effects.

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2.4. CLOBETASOL-17-PROPIONATE LOTION UNDER HYDROCOLLOID DRESSING (DUODERM ET) ONCE WEEKLY VERSUS UNOCCLUDED CLOBETASOL-17-PROPIONATE OINTMENT TWICE DAILY IN PSORIASIS: AN IMMUNOHISTOCHEMICAL STUDY ON REMISSION AND RELAPSE

2.4.1. Summary

It is well-established that the efficacy of corticosteroids under occlusion of hydrocolloids (HCD) is superior compared to monotherapy with topical corticosteroids. However, following treatment with more potent corticosteroids, increased side-effects and a more pronounced rebound might be expected.

In the present clinical study efficacy, relapse and safety characteristics of two treatment modalities were compared: clobetasol-17-propionate lotion under a hydrocolloid dressing once weekly versus clobetasol-17-propionate ointment without a hydrocolloid twice daily. Clinical assessments were recorded and skin biopsies were taken before therapy, at clearance and six weeks after clearance. A panel of monoclonal antibodies to characterise epidermal proliferation, differentiation and inflammation was selected. In addition, clinical and histological assessments for skin atrophy were made.

Both therapies had a major therapeutic effect, which was reflected in the clinical and immunohistochemical parameters. The only difference between the two therapies was a faster remission induction time in patients treated with corticosteroids combined with HCD. Six weeks after discontinuation of treatment, similar clinical and histological signs of relapse were observed for both therapies. Epidermal thinning was observed to the same extent during either therapy but proved to be reversible within six weeks after discontinuation of treatment.

From this study it can be concluded that the combination of HCD and corticosteroids is able to induce relatively fast remissions compared to corticosteroid monotherapy but relapse and safety characteristics are comparable to the unoccluded corticosteroid therapy.

2.4.2. Introduction

Topical corticosteroids have been broadly utilised in steroid responsive dermatoses such as psoriasis and efficacy and safety have been studied extensively. Low and medium potent corticosteroids are used for maintenance therapy in mild and moderate diseases. Rapid remissions in more severely affected skin can be obtained using potent corticosteroids.[1] Adverse events of topical corticosteroids can be divided in local and systemic effects. Local adverse events like skin-thinning are frequently observed. Systemic adverse events like suppression of the adrenal cortex are more serious side-effects.[1-3]

Different therapeutic schedules and modes of application have been used in corticosteroid therapy. The last decade the treatment with corticosteroids under occlusive dressings has become a popular approach. Hydrocolloid dressings (HCD), used for occlusive therapy, are convenient to wear and have a beneficial effect on the psoriatic plaque even as monotherapy without corticosteroids.[4,5] In vivo studies show that monotherapy of psoriatic plaques with occlusive dressings decreases the number of involucrin and transglutaminase (TGase) positive cell layers,[6] whereas the mitotic activity, keratin 16 expression and dermal T-cell accumulation tends to decrease.[7] It has been established that the efficacy of corticosteroids combined with HCD is superior compared to corticosteroids without HCD occlusion but information on relapse and safety is sparse.[8-10] Recently, a multi-centre study was carried out to study clinical efficacy and safety of once weekly applications of clobetasol-17-propionate (clobetasol)[3] lotion under occlusion with the HCD Duoderm ET and to compare safety and efficacy of this approach with clobetasol ointment applied twice daily without occlusion.[11] Compared to unoccluded clobetasol treatment, clobetasol under HCD induced a faster remission whereas a 6 weeks post-treatment follow up revealed comparable relapse characteristics.[11]

In the patients, who participated in this study in the Nijmegen centre, skin biopsies for immunohistochemistry were taken before treatment, at clearance and six weeks after discontinuation of treatment. Immunohistochemical markers for epidermal proliferation, differentiation and inflammation were assessed (table I). Remission, relapse and safety characteristics of both treatments were compared and contrasted. In particular the following questions were addressed:

i. What are the differences with respect to immunohistochemical responses between treatment with a potent topical corticosteroid under a hydrocolloid and treatment with the corticoid without a hydrocolloid in chronic plaque psoriasis?
ii. In what respect do remission, relapse and safety characteristics differ between the two therapies used?

2.4.3. Materials and methods

Study design

Nineteen patients with chronic plaque psoriasis were included in an open, comparative study. One lesion of maximally 70 cm^2 was treated with either:

- clobetasol lotion (Dermovate lotion, Glaxo, Zeist the Netherlands) under occlusion of HCD (Duoderm ET, Convatec, Woerden, the Netherlands) with a change of the dressing and lotion once weekly (10 patients)
- clobetasol ointment (Dermovate ointment, Glaxo) applied twice daily (9 patients).

The patients were treated during a maximum of six weeks or shorter in case of clearance of the lesion. Clinical assessments were carried out every two weeks. After

clearance, no therapy was allowed and the patients visited the department every two weeks until the first signs of relapse of the psoriatic lesion were observed. The final investigation was three weeks after the first sign of relapse.

In all patients punch biopsies of 3 mm were taken from the target lesions before, immediately after treatment and six weeks after discontinuation of treatment. The biopsy procedure has been described before.[12,13]

Table I Monoclonal antibodies used in the present study.

Antigen	Antibody	Source	Dilution	Staining technique
Ki-67	MIB-1	Immunotech, France	1:50	indirect immunoperoxidase
Involucrin	Mon-150[16]	Dr. J van Duijnhoven, Holland	1:25	indirect immunoperoxidase
Transglutaminase	BT621	Biomedical Technologies, USA	1:100	indirect immunoperoxidase
Filaggrin	BT576	Biomedical Technologies, USA	1:500	indirect immunoperoxidase
CD2	DAKO-T11	Dakopatts, Denmark	1:100	avidin-biotin-complex method
Elastase	DAKO-elastase	Dakopatts, Denmark	1:100	indirect immunoperoxidase

Patients

The group of 19 patients consisted of 16 males and 3 females, with an age ranging from 32 to 79 years. Apart from psoriasis, the patients did not have significant other dermatological or internal diseases. No treatment for psoriasis was allowed other than the trial medication. Concomitant treatments which did not interfere with psoriasis or test medications were permitted to continue. Before study initiation, no topical antipsoriatic treatment had been administered for two weeks and the patients had not used systemic treatment for at least six weeks. Permission of the local Ethics Committee and written informed consent were obtained from all patients.

Assessment of clinical efficacy

Clinical efficacy was assessed using the sum-score: the sum of the three clinical severity parameters: erythema, induration and scaling, each of them scored on a 0-4 point-scale (0 = no involvement, 1 = mild involvement, 2 = moderate involvement, 3 = marked involvement, 4 = severe involvement). Photographs were taken at each visit. Remission was defined as no or only a

mild erythema without induration or scaling (sum-score ≤ 1). Relapse was defined as any increase of the sum-score. The length of the remission period and the sum-score at the moment of relapse were also recorded.

Immunohistochemical stainings

For the immunohistochemical stainings a panel of monoclonal antibodies (table I) was used. All antibodies were diluted in phosphate buffered saline (PBS). This immunoperoxidase technique was described previously.[12,13] Staining with DAKO-T11 was done with avidin-biotin complex method (ABC-kit (mouse), Vector Lab. Inc., Burlingame, USA). In brief, the slides were incubated with 20% normal horse serum and subsequently with the T11 antibody for 60 minutes. The slides were incubated with horse-anti-mouse-biotinylated IgG (1:200, Vector Lab. Inc., Burlingame, USA) for 30 minutes. After 2 washes with PBS an incubation of 30 minutes with avidin-biotin-peroxidase complex (1:50 Vector Lab. Inc., Burlingame, USA) was performed. Visualisation was done using a solution of 3-amino-9-ethylcarbazole (AEC). All slides were counterstained with Mayer's haematoxylin (Sigma, St Louis, MO, USA) and mounted in glycerol-gelatine.

Histological examination

The histological examination was performed blinded. The scoring methods used have been performed and published before.[12,13] Epidermal proliferation was measured by counting the number of Ki-67 positive nuclei per mm length of the section. The involucrin and transglutaminase expression were assessed by calculation of the ratio positive cell layers/total cell layers of the viable epidermis. This was done at two sites: above the dermal papilla and between the dermal papillae. In addition the total number of cell layers was recorded. The filaggrin expression was assessed by measuring the percentage of the length of the stratum corneum and stratum granulosum which was stained. Inflammation (PMN and T-lymphocytes) was assessed separately for dermis and epidermis. Dermal inflammation was semi-quantitatively enumerated by expressing the number of positively stained cells as a percentage of the total number of infiltrate cells: 0, no positive cells; 1, sporadic; 2, 1-25 %; 3, 26-50 %; 4, 51-75 %; 5, 76-99 %; 6, 100 %. Epidermal inflammation was assessed using a 0-4 point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, pronounced staining.

Assessment of skin atrophy

During the study clinical signs of atrophy were recorded. Histological atrophy was recorded by counting the total number of cell layers of the epidermis between the dermal papillae.

Statistical evaluation

Data are reported as means ± SEM. Changes in paired markers, due to therapy, were evaluated with the t-test for paired values. Unpaired data were analysed with a t-test assuming equal variances. A two-tailed hypothesis was employed to interpret data. A p-value ≤ 0.05 was regarded as statistically significant.

2.4.4. Results

Clinical response

Of the 19 patients who participated in the present study, 15 patients reached a clearance and completed the study. One patient, treated with clobetasol lotion and HCD, was excluded from the study because of impetiginisation during treatment. Three patients who were treated with clobetasol ointment did not reach a clearance after 6 weeks and therefore did not participate in the follow up period. The mean treatment time in the lotion+HCD group was 2.7 ± 0.3 weeks which was significantly shorter compared to the ointment group: 4.7 ± 0.7 weeks (mean ± SEM; p=0.02). The length of the remission was equal for both therapies (clobetasol lotion+HCD: 5.3 ± 1.1 weeks and clobetasol ointment: 5.3 ± 1.0 weeks (mean ± SEM)). The severity of the relapse was also similar following both treatments.

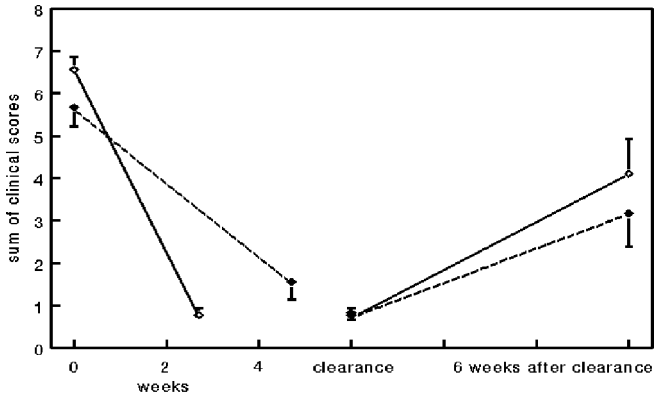

Fig. 1 The course of the sum of the clinical scores (erythema, induration and scaling) during treatment and after discontinuation of treatment with clobetasol lotion under occlusion with
hydrocolloid (——) and monotherapy with clobetasol ointment (- - - -)

The results of the clinical assessments, indicated by the sum-score, are shown in figure 1. During both treatments a statistically significant decrease of the sum-score was observed. (clobetasol lotion+HCD: $p<0.001$; clobetasol ointment; $p<0.001$) The scores in the two treatment groups at the start of therapy did not differ significantly. Neither did the scores at the end of the treatment period. Clearing was reached in 15 patients (clobetasol lotion+HCD: 9 patients; clobetasol ointment: 6 patients). Six weeks after discontinuation of treatment, 13 patients experienced a relapse with a substantial increase of the sum-scores (clobetasol lotion+HCD: $p=0.002$; clobetasol ointment: $p=0.08$) There was no significant difference between both therapies with respect to the sum-scores six weeks after discontinuation of treatment. The sum-score at the end of the six weeks post-treatment follow up was significantly lower than to the sum-score at the start of the study (clobetasol lotion+HCD: $p=0.02$; clobetasol ointment: $p=0.01$).

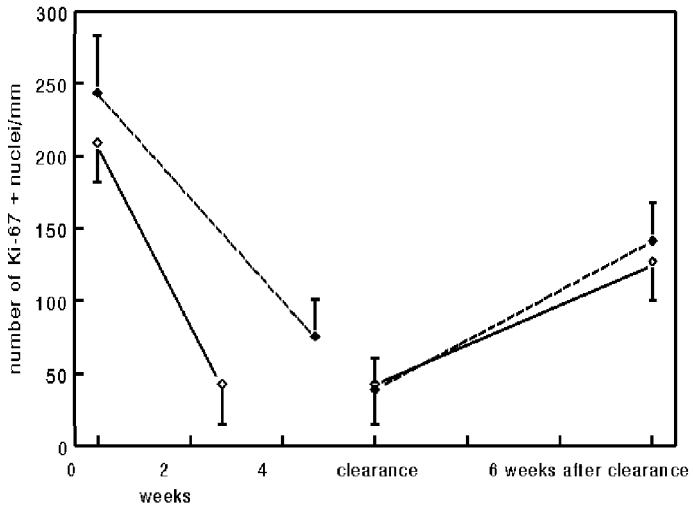

Fig. 2 The number of Ki-67 positive nuclei/mm of the epidermis during treatment and after discontinuation of treatment with clobetasol lotion under occlusion with hydrocolloid (——) and monotherapy
with clobetasol ointment (- - - -).

Immunohistochemical assessments

With respect to all immunohistochemical stainings, both therapies induced significant changes during treatment ($p\leq0.05$) (table II). At the start of therapy, both treatment groups did not show any statistically significant differences. No significant differences could be demonstrated between both treatment groups at clearance either. In figure 2, the response to both therapies is illustrated by the number of Ki-67 positive nuclei in the epidermis.

Table II Immunohistochemical sores, before clobetasol treatment, at clearance, and 6 weeks after clearance (mean ± SEM).

Antigen	Localisation	Before treatment lotion+HCD	ointment	Clearance lotion+HCD	ointment	6 Weeks after clearance lotion+HCD	ointment
Ki-67	basal layer	209 ± 27	243 ± 40	43 ± 28	39 ± 22	127 ± 27	141 ± 26
Involucrin	interpapillar	0.59 ± 0.05	0.53 ± 0.03	0.41 ± 0.04	0.40 ± 0.04	0.52 ± 0.05	0.50 ± 0.02
	above papilla	0.76 ± 0.06	0.89 ± 0.04	0.61 ± 0.03	0.66 ± 0.05	0.81 ± 0.03	0.81 ± 0.03
TGase	interpapillar	0.56 ± 0.03	0.63 ± 0.03	0.38 ± 0.03	0.41 ± 0.04	0.55 ± 0.05	0.56 ± 0.04
	above papilla	0.87 ± 0.02	0.90 ± 0.02	0.58 ± 0.04	0.60 ± 0.06	0.79 ± 0.04	0.78 ± 0.05
Filaggrin	granular layer	69 ± 7.3	65 ± 8.7	97 ± 1.5	94 ± 3.3	71 ± 10	82 ± 10
	corneal layer	44 ± 9.0	47 ± 12	97 ± 2.4	100 ± 0	64 ± 13	76 ± 13
CD2	dermis	4.8 ± 0.32	4.9 ± 0.4	2.4 ± 0.24	2.8 ± 0.5	4.1 ± 0.4	3.8 ± 0.7
	epidermis	3.3 ± 0.3	2.9 ± 0.4	0.56 ± 0.17	0.50 ± 0.34	2.7 ± 0.4	2.2 ± 0.6
Elastase	dermis	1.0 ± 0.4	1.1 ± 0.4	0.22 ± 0.15	0 ± 0	1.0 ± 0.4	0.67 ± 0.3
	epidermis	1.4 ± 0.4	2.2 ± 0.4	0 ± 0	0 ± 0	2.2 ± 0.5	1.2 ± 0.6

Six weeks following discontinuation of both treatments, all immunohistochemical markers changed substantially (table II) and these changes, indicating a relapse in psoriasis, were comparable for both treatment groups (table II). Six weeks after clearance, significant differences could be observed compared to the starting point of the study: a decrease of the Ki-67 count ($p=0.05$) and a decrease of the CD2 positive cells in the epidermis ($p=0.05$) for the patients treated with clobetasol lotion+HCD group compared to the pre-treatment values and a decrease of transglutaminase above the papilla in the patients treated with clobetasol ointment ($p=0.02$). The other parameters showed no statistically significant changes.

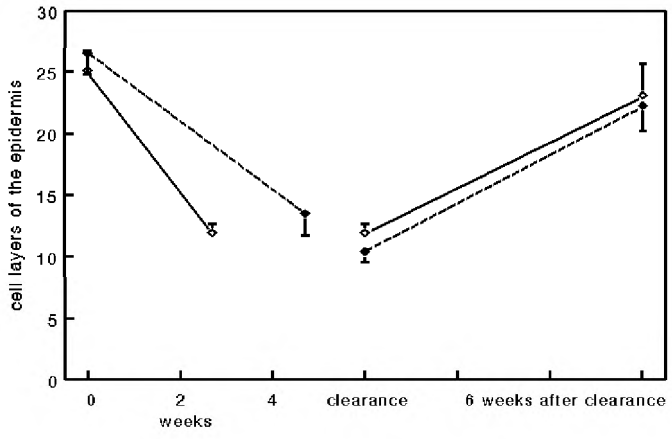

Fig. 3 The number of cell layers of the epidermis during treatment and after discontinuation of treatment with clobetasol lotion under occlusion with hydrocolloid (——) and monotherapy with clobetasol ointment (- - - -).

Assessment of epidermal atrophy

No clinical signs of atrophy were seen. The results of the counting of the number of cell layers are shown in figure 3. During both treatment modalities, a significant decrease of the number of cell layers was observed (clobetasol lotion+HCD: $p<0.001$; clobetasol ointment: $p<0.001$) but there was no difference between both therapies. After discontinuation of both therapies a similar and significant increase of the number of cell layers was observed (clobetasol lotion+HCD: $p=0.004$; clobetasol ointment: $p=0.05$). For both therapies, at the end of the six weeks follow up, the number of cell layers was not significantly different compared to the situation at the start of therapy.

2.4.5. Discussion

The clinical results of the present study are in line with the multi-centre comparative study[11]: a faster induction of remission in the clobetasol lotion+HCD group was observed compared to the clobetasol ointment group and equal clinical relapse characteristics were seen for both treatment groups.

In the present study, significant changes occurred during treatment with respect to all immunohistochemical markers which were analysed (table II). The degree of immunohistochemical changes did not differ significantly for both therapies. Despite of clinical clearance, only some of the immunohistochemical markers reached values of normal human skin.[14] Filaggrin, transglutaminase and elastase stainings reached the normal range during both treatment schedules.

Following discontinuation of corticosteroid treatment, all immunohistochemical markers changed substantially. These changes indicate a relapse of the psoriatic lesion (table II). The comparison of both therapies for the immunohistochemical markers six weeks after clearance, did not reveal any significant differences. This observation confirms and strengthens the conclusion of the clinical data of the multi-centre study[11], that both treatments have a similar post-treatment response pattern.

Clinically, in none of the patients, signs for skin atrophy were observed. Histologically, the number of epidermal cell layers demonstrated a significant decrease during therapy. Persisting local side-effects like striae and skin thinning after topical corticosteroids are mainly the result of dermal atrophy, but epidermal thinning may give insight into atrophogenecity as well.[15] The epidermal thinning observed in the present study proved to be reversible after discontinuation of either treatment within the six weeks post-treatment observation period. Some authors suggest that corticosteroids under occlusion should be regarded as a potentially more atrophogenic approach

than corticosteroids without occlusion and should therefore be avoided.[2] The present study, however, indicates that the combination of clobetasol and a HCD does not differ from monotherapy with clobetasol in inducing epidermal atrophy.

In conclusion, the present study demonstrates a similar immunohistochemical response of the combination of clobetasol lotion under HCD compared to monotherapy with clobetasol ointment. No immunohistochemical indication for a faster relapse following discontinuation of clobetasol lotion in combination with a HCD compared to monotherapy with clobetasol ointment was seen. With respect to the thickness of the epidermis the combined approach was not more atrophogenic compared to monotherapy. In addition, clobetasol lotion applied once weekly under HCD induced a faster clearing compared to clobetasol ointment twice daily. Therefore treatment with clobetasol lotion under HCD reduces the duration of treatment as well as the quantity of the topical corticosteroid required for reaching clearing.

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Chapter 3

The UVB model:

a new in vivo model to study topical treatments for psoriasis

This chapter was based on the following publications:

C.J.M. van der Vleuten, E.J.A. Kroot, E.M.G.J. de Jong, P.C.M. van de Kerkhof
The immunohistochemical effects of a single challenge with an intermediate dose ultraviolet B on normal human skin. *Arch Dermatol Res* 1996; **288**: 510-516.

C.J.M. van der Vleuten, C.G.E.M. Snijders, E.M.G.J. de Jong, P.C.M. van de Kerkhof
The effects of calcipotriol and clobetasol-17-propionate on UVB irradiated human skin, an immunohistochemical study. *Skin Pharmacology* 1996; **9**: 355-365.

3.1. THE IMMUNOHISTOCHEMICAL EFFECTS OF A SINGLE CHALLENGE WITH AN INTERMEDIATE DOSE ULTRAVIOLET B ON NORMAL HUMAN SKIN

3.1.1. Summary

Ultraviolet B irradiation (UVB) has extensively been advocated to investigate cutaneous inflammation in vivo. Mostly doses above the threshold of skin damage were used. Therefore it is not clear what changes were observed: specific effects of UVB or some extent of woundhealing?

In this study the dose-dependent effects of UVB on normal human skin were assessed using histology and immunohistochemistry. The dose of 1 MED was chosen as a dose inducing tissue changes with adequate morphology: no toxic but evident immunohistochemical changes. The sequential effects of this 1 MED of UVB were studied up to 14 days after UVB, using immunohistochemistry with a panel of monoclonal antibodies.

One MED of UVB resulted in substantial effects in human skin, mainly on proliferation and differentiation; the markers for inflammation did not reveal major changes. This model might provide a promising approach to evaluate the effect of drugs on epidermal proliferation and differentiation in vivo.

3.1.2. Introduction

To answer questions relating cutaneous inflammation, the response of normal human skin to ultraviolet B (UVB) has been used for decades by various groups as a classical approach in experimental dermatology.[1,2] Most authors evaluated the effects of three times the minimal erythema dose (MED) of UVB.[3,4] Following irradiation with three times the MED, however, a substantial damage of the epidermis occurs with cytolysis and sunburn cells. Therefore, the response to such a high dose UVB can be interpreted to a large extent as woundhealing. An alternative approach is to study the effect of an intermediate dose of UVB, which does not yet induce the gross histological changes that were mentioned above, but on the other hand does not have a substantial effect on epidermal behaviour.

The aim of the present study was firstly to determine this intermediate dose of UVB and secondly to elucidate the sequential changes due to this UVB dose. In particular the following question was addressed: what are the dynamics and characteristics in epidermal proliferation, epidermal differentiation and inflammation due to a single irradiation with an intermediate dose of UVB?

To assess these changes qualitatively and semi-quantitatively, immunohistochemical markers for epidermal proliferation, epidermal differentiation and inflammation were evaluated.

3.1.3. Materials and methods

Volunteers

Nine healthy volunteers, males and females (age 23-34 years), participated in this investigation. This study was approved by the local ethical committee. All volunteers gave their written informed consent prior to inclusion in the study.

UVB exposure

For UVB exposure a Waldman UV 7001 K light cabin, emitting broad-band UVB (285-310 nm) was used. In all volunteers the MED was determined on the non sun-exposed skin of the buttocks; MED is defined as the minimal dose yielding a sharply demarcated erythema after 24 hours.

Subsequently the first three volunteers were exposed to single doses of 0, ½, 1, 2 and 3 MED of UVB on three areas of 9 cm^2 at the skin of the buttocks. A challenge of 1 MED resulted in substantial changes with respect to epidermal differentiation without inducing histological damage. Therefore, the other six volunteers were exposed to 1 MED of UVB on three separate areas of 9 cm^2 at the skin of the buttocks.

Sample procedure

Punch biopsies of 3 mm were taken from the UVB exposed areas (one biopsy per area) after local anaesthesia with xylocain 1% and adrenaline. In the first three volunteers biopsies were taken 24 hours after UVB exposure. In the six other volunteers biopsies were taken 1, 4 and 14 days after 1 MED of UVB exposure (three volunteers) and 0, 2 and 10 days after 1 MED of UVB exposure (three volunteers).

The biopsies were embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, USA), snap frozen in liquid nitrogen and stored at -80°C until use. Sections of 7 μm were cut, air dried and fixed for 10 minutes in acetone/ether (60/40%) (Ki-67 staining) or in acetone (other stainings) and again stored at -80°C.

Monoclonal antibodies

A panel of monoclonal antibodies was used.

To approximate the number of cycling epidermal cells in the basal layer an antibody directed against the Ki-67 antigen was used (MIB-1, 1:50, Immunotech, S.A., Marseilles, France). Antibodies against tenascin (T_2H_5, 1:2000; obtained from AA Verstraeten, The Netherlands Cancer Institute, Amsterdam, The Netherlands) and against cytokeratin 13/16 (Ks8.12, 1:20, Sigma, St. Louis, USA) were used for proliferation associated dermal and epidermal changes respectively.

To assess epidermal differentiation monoclonal antibodies against involucrin, (MON-150[5], 1:25) and anti-human keratinocyte transglutaminase (1:100, Mouse Monoclonal Antibody, IgG_{2a} Biomedical Technologies Inc.) were used.

Analysis of the inflammatory infiltrate was done by assessment of T-lymphocytes, Polymorphonuclear leukocytes (PMN) and Langerhans cells respectively using the monoclonal antibodies against the CD2 antigen (DAKO-T11, 1:100, Dakopatts, Copenhagen, Denmark), against elastase (DAKO-elastase, 1:100, Dakopatts, Copenhagen, Denmark) and against the CD1a antigen (DAKO-T6, 1:100, Dakopatts, Copenhagen, Denmark).

Staining procedure

For all monoclonal antibodies, except for DAKO-T11 and DAKO-T6, an indirect immunoperoxidase technique was used. For ten minutes the slides were fixed in acetone/ether (60/40%) in case of Ki-67 staining or in acetone for the other stainings. The slides were air dried and put in a phosphate buffer (PB) (72 mM Na_2HPO_4 and 28 mM NaH_2PO_4). Only the slides stained with anti-elastase were pre-incubated with methanol/ 0.1 % H_2O_2 (30 %) for 20 minutes. All antibodies were diluted in PB. The slides were incubated with the different primary monoclonal antibodies for 30 minutes. After washing with PB the slides were incubated with the secondary antibody, rabbit-anti-mouse immunoglobulin conjugated with peroxidase diluted in PB containing 5% human AB-serum (1:50, RAM-PO, Dakopatts, Copenhagen, Denmark) for 30 minutes. After washing with PB and demineralised water a 3-amino-9-ethylcarbazole (AEC) solution was used for visualisation.

Staining with DAKO-T11 and DAKO-T6 was done with an indirect peroxidase-anti-peroxidase technique (PAP). The slides were put in PB for ten minutes and pre-incubated with 50 % Normal Rabbit Serum on PB for 20 minutes. After washing with PB the slides were incubated the primary monoclonal antibody in a Miele Microwave at 80 Watt for 9 minutes. Then the slides were washed again in PB and incubated with rabbit-anti-mouse immunoglobulin (1:25, RAM-Ig, Dakopatts, Copenhagen, Denmark) in the microwave at 80 Watt for 9 minutes. After washing in PB the slides were incubated with PAP-complexes (1:100, Peroxidase monoclonal mouse

antiperoxidase complexes, Dakopatts, Copenhagen, Denmark) in the microwave at 80 Watt for 8 minutes. After washing, this cycle was repeated. Visualisation of the complexes was done by the AEC-solution.

All slides were counter-stained with Mayer's Haematoxylin (Sigma, St. Louis MO, USA) and mounted in glycerol-gelatine.

Histological examination

The histological examination was performed blinded by two investigators. These scoring methods have been performed and published before.[6]

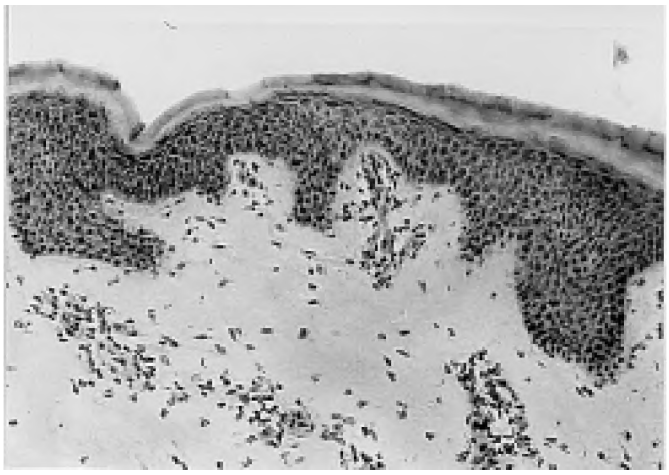
Epidermal proliferation was measured by counting the number of Ki-67 positive nuclei per mm length of the section. The distribution of tenascin in the papillary dermis was assessed using a 6 point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, moderate-pronounced staining; 5, pronounced staining. In addition it was recorded whether a staining pattern continuous or discontinuous adjacent to the basal lamina was seen. Cytokeratin 13/16 was scored separately for the basal and suprabasal compartment of the epidermis using a semi-quantitative 7 point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, moderate-pronounced staining; 5, pronounced staining; 6, whole epidermis stained.

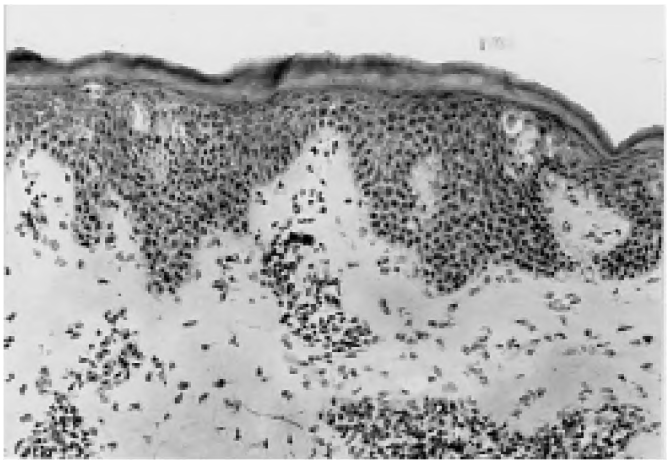
The involucrin and transglutaminase expression were assessed by calculation of the ratio positive cell layers/ total cell layers of the viable epidermis. This was done at two sites: above the tip of the dermal papilla and between two dermal papillae.

Inflammation (PMN, T-lymphocytes and Langerhans cells) was assessed separately for dermis and epidermis. Dermal inflammation was semi-quantitatively enumerated by expressing the number of positively stained cells as a percentage of the total number of infiltrate cells: 0, no positive cells; 1, sporadic; 2, 1-25 %; 3, 26-50 %; 4, 51-75 %; 5, 76-99 %; 6, 100 %. Epidermal inflammation was assessed using a five-point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, pronounced staining.

Statistical evaluation

Data are reported as means ± SEM. For statistical analysis the Mann-Whitney ranking test for unpaired data was used; a p-value < 0.05 was supposed to be statistically significant. In the figures, the asterisks above the error bars indicate the first statistically significant changes with regard to the starting point.





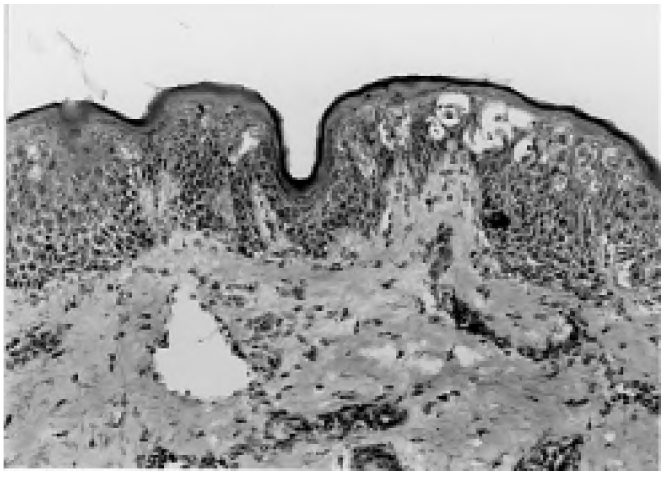


Fig. 1 H&E stained slides of normal human skin 1 day after UVB challenge with (a) 1 MED, (b) 2 MED and (c) 3 MED.

3.1.4. Results

Minimal erythema dose

The volunteers had skin types varying from 2 to 4 (Fitzpatrick-Pathak-classification); the MED ranged from 0.2 - 0.4 J/cm^2.

Dose finding study

In each volunteer the MED was determined. Biopsies of the skin exposed to 0, ½ ,1, 2, 3 MED of UVB were assessed histologically after 1 day. H&E staining revealed sunburn cells, cytolysis and intracellular oedema in the biopsies taken from the skin sites exposed to 2 and 3 MED as presented in figure 1. No toxic changes were seen on the H&E slides after ½ and 1 MED. Cytokeratin 13/16 staining revealed no changes in the skin exposed to ½ MED but an increased staining intensity in the skin exposed to 1 MED. Therefore, the dose of 1 MED was selected to evaluate the dynamics of the response to UVB challenge.

Clinical assessment of the time curve

At day 1 after 1 MED of UVB a sharply demarcated erythema was observed that faded and decreased with normalisation on day 10. Induration of the skin was visible after 1 day and also subsequently decreased with normalisation on day 10. Pigmentation was first observed on day 1 and gradually increased during the 14 days of observation. Scaling was first observed 2 days after UVB exposure and this increased during the 14 days of the investigation.

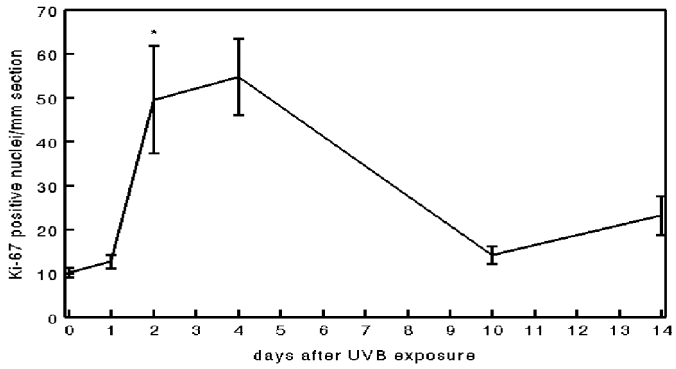


Fig. 2 a The number of Ki-67 positive cells per mm of the section in the basal layer of the epidermis following intermediate dose UVB challenge; data are reported as means ± SEM, the asterisks above the error bars indicate the first statistically significant changes ($p < 0.05$) with regard to the starting point.

Time curve of histological changes

H&E staining

The dermis as well as the epidermis were intact; no toxic changes, no cytolysis and no sunburn cells were seen in the H&E stained slides. On day 2 a slight increase of the dermal infiltrate cells was seen. On day 4-10 parakeratosis was observed. Acanthosis and elongated rete-ridges were seen on day 4 increasing up to day 14.

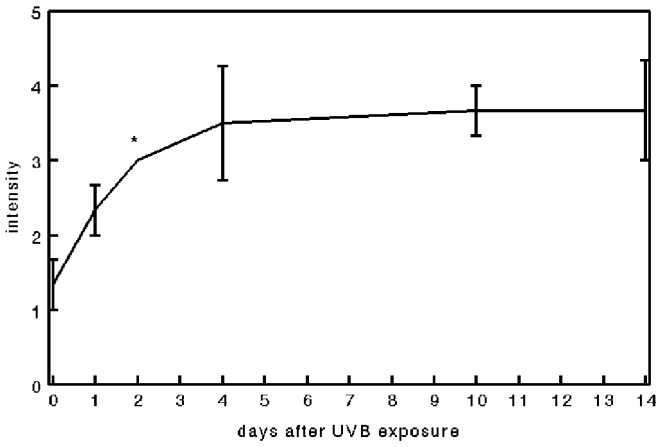


Fig. 2 b Tenascin expression in the dermis following intermediate dose UVB challenge; data are reported as means ± SEM, the asterisks above the error bars indicate the first statistically significant changes ($p < 0.05$) with regard to the starting point.

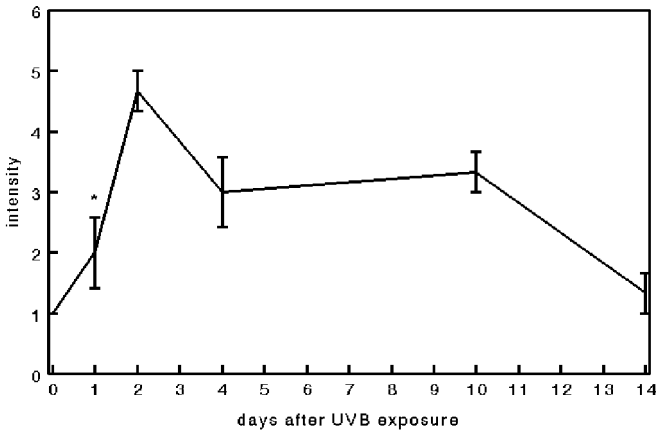


Fig. 2 c Cytokeratin 13/16 expression in the suprabasal cell compartment following intermediate dose UVB challenge; data are reported as means ± SEM, the asterisks above the error bars indicate the first statistically significant changes ($p < 0.05$) with regard to the starting point.

Epidermal proliferation and proliferation associated changes

Ki-67 expression (figure 2a) was seen in the stratum basale of the epidermis. After 1 day no change was seen in the number of Ki-67 positive cell nuclei per mm of the histological section. After 2 days a significant increase was observed ($p < 0.05$). After 4 days this increase had become more substantial. On day 10 the Ki-67 positive count equalled the density of day 0. However on day 14 again a tendency to increase could be observed.

A significant increase of tenascin expression was observed at day 2 ($p < 0.05$) which gradually increased and stayed high up to day 14 (figure 2b). At all time points either continuous or discontinuous tenascin expression in the dermis adjacent to the basal lamina was observed.

Ks8.12 staining (keratin 13/16 expression) (figure 2c) in the suprabasal epidermal compartment starts to increase significantly after 1 day after UVB ($p < 0.05$) with a maximum after 2 days. Subsequently the intensity decreased and tended to normalise at 14 days after UVB exposure. No significant changes were observed in the basal cell compartment with respect to cytokeratin 13/16.

Epidermal differentiation

Figure 3a illustrates the expression of involucrin. At day 2 a significant increase was seen ($p < 0.05$) that decreased significantly on day 10 ($p < 0.05$) tending to normalisation on day 14.

Transglutaminase expression (figure 3b) had a similar time course as involucrin. A significant increase was seen with a maximum on day 2 ($p < 0.05$). Afterwards on day 10 the expression had decreased and tended to normalisation on day 14.

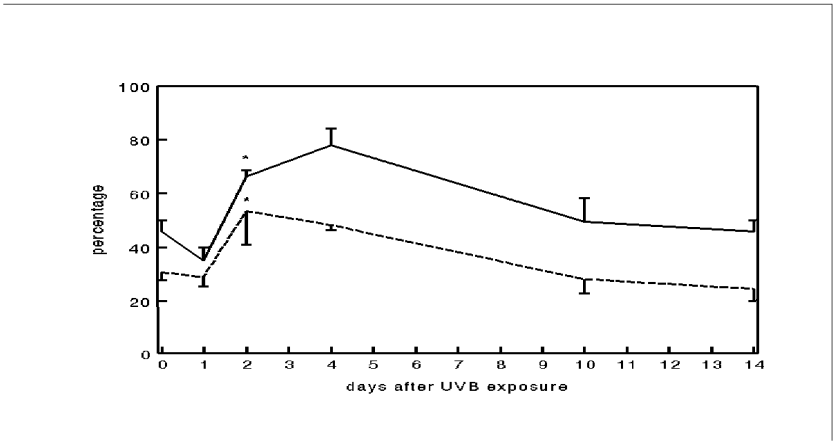


Fig. 3 a
The percentage of involucrin positive cell layers in the epidermis at the tip of the dermal papilla (——) and interpapillary (- - - -) following intermediate dose UVB challenge; data are reported as means ± SEM, the asterisks above the error bars indicate the first statistically significant changes ($p < 0.05$) with regard to the starting point.

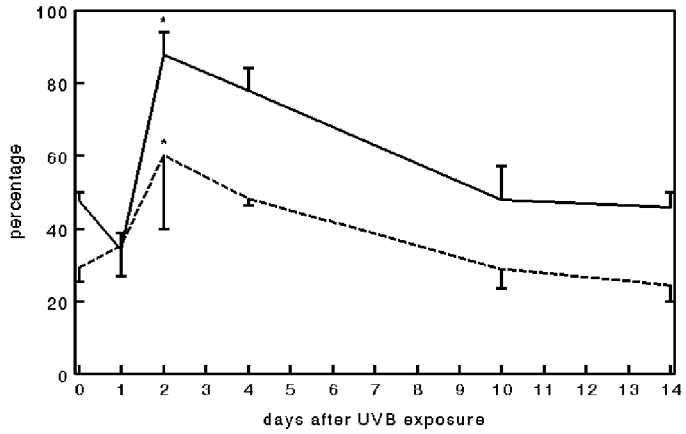


Fig. 3 b The percentage of transglutaminase positive cell layers in the epidermis at the tip of the dermal papilla (——) and interpapillary (- - - -) following intermediate dose UVB challenge; data are reported as means ± SEM, the asterisks above the error bars indicate the first statistically significant changes ($p < 0.05$) with regard to the starting point.

Inflammation

No significant changes were observed in either dermal or epidermal CD1a expression (figure 4a). No CD2 positive cells were seen in the epidermis in any of the histological slides. The percentage CD2 positive cells in the dermal infiltrate (figure 4b) increased significantly and was maximal on day 1 ($p < 0.05$). Afterwards there was a tendency towards a decrease and normalisation. No elastase positive cells were observed either in the dermis or in the epidermis following UVB challenge.

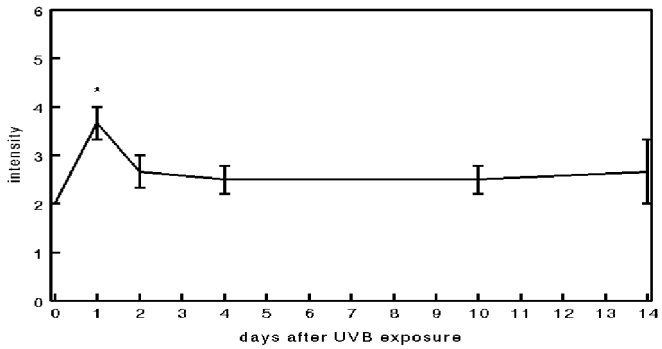


Fig. 4 a CD2 positive cells in the dermis; data are reported as means ± SEM, the asterisks above the error bars indicate the first statistically significant changes ($p < 0.05$) with regard to the starting point.

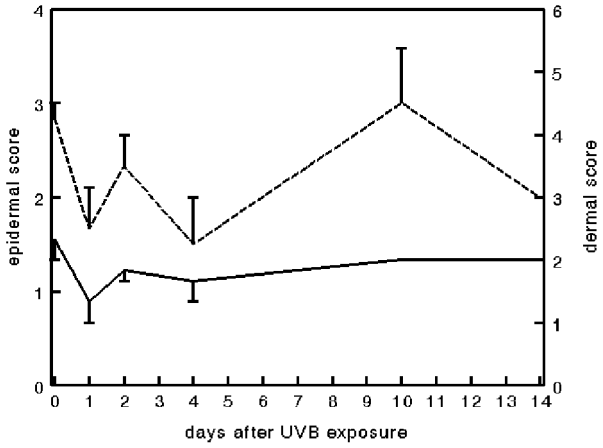


Fig. 4 bCD1a positive cells in dermis (——) and epidermis (- - - -); data are reported as means ± SEM.

3.1.5. Discussion

The present study clearly demonstrates that UVB using the maximal dose which did not yet induce obvious histological damage such as cytolysis and sunburn cells, profoundly modulates epidermal proliferation, epidermal differentiation and also interferes with inflammation. Many biochemical and histological changes due to high dose UVB have been described in literature.[1-4,7,8] The present study extends our knowledge on the response to intermediate dose UVB: a separation between UV damage and modulation of epidermal proliferation, epidermal differentiation and inflammation occurs following challenge with 1 MED of UVB.

The dynamics of the response to intermediate dose UVB are demonstrated in figures 2-4. At day 1 T-cell accumulation was observed in the dermis and the suprabasal epidermal compartment expressed cytokeratin 13/16. At day 2 the percentage of involucrin and transglutaminase positive cell layers had increased, dermal tenascin had increased and the recruitment of cycling cells was enhanced profoundly. No PMN accumulation was observed. In contrast to a study with higher doses of UVB[9], the number of CD1a positive cells was not modulated in our study.

An intriguing observation was the pronounced expression of the Ki-67 antigen 2 days after intermediate dose UVB. Autoradiographic studies also indicate hyperproliferation in response to high dose UVB.[1] But it can be argued whether ^{3}H-thymidine-incorporation always represents cell division. The mitotic index curve as shown by Mier et al.[10] does represent hyperproliferation and seems to precede Ki-67 expression

as observed in the present study. This can be explained by delayed expression of the Ki-67 protein.[11] In some communications the question is discussed whether it is proliferation or DNA repair due to DNA damage that is detected after UVB exposure. UVR causes formation of pyrimidine dimers.[12] The endogenous repair capacity of the DNA, also known as unscheduled DNA synthesis[13], repairs these defects. In this way the same proteins are involved which also are expressed during cell proliferation. PCNA is a marker for cell proliferation but is also expressed in DNA repair.[14] Therefore this marker does not provide the possibility to differentiate between both processes. Ki-67, on the other hand, is expressed exclusively in proliferating cells and is therefore an adequate tool to study proliferation in UVB exposed skin.[15] In a previous study concerning UVR exposure, however, an intermediate dose UV irradiation did not induce recruitment of cycling epidermal cells.[14] These discrepancies might be partially explained by the use of another light source and dose (UVB combined with UVA; 1½ MED), by another antibody recognising a different epitope of the Ki-67 molecule or by a different way of processing the biopsy material.[15] Hyperproliferation following high dose UVB is a well-established event after UVB.[1,10] The present study clearly demonstrates the substantial induction of epidermal proliferation following intermediate dose UVB challenge, suggesting that UVB induces epidermal proliferation directly and not as a result of skin damage.

Tenascin is an extracellular matrix protein, induced in hyperproliferative skin[16,17] and during remodelling of the epidermis.[18] Some studies suggest that tenascin acts as a local growth factor.[19] Fourteen days after UVB, tenascin was still high. There is no clearness about the half life time of tenascin. The persisting presence of tenascin might represent remodelling of the skin. To the best of our knowledge no data are available on the expression of tenascin following UVB challenge.

Due to UVB the differentiation pattern of the keratinocytes was changed. Keratin 16, a cytokeratin that is associated with hyperproliferative skin conditions like psoriasis, and is not expressed in normal human skin[20], was one of the first immunohistochemical changes in our study. Staining with Ks8.12, the antibody recognising keratin 16, 15 and 13[21], was predominantly seen in the basal layer of normal skin and was upregulated in the suprabasal layers of UVB challenged skin. Keratin 13 is not present in adult human skin[22] and keratin 16 is not expressed in basal keratinocytes. Therefore the staining in the stratum basale of the epidermis is thought to be due to keratin 15.[23] Since Ki-67 expression appeared after 2 days, cytokeratin 13/16 expression seems to precede proliferation and hence may not directly be related to hyperproliferation. An alternative explanation might be that expression of Ki-67 is delayed, so there is already proliferation but not yet Ki-67 expression. Indeed Gerdes et al.[11] demonstrated a specific Ki-67 negative G_1 phase for the

cells between the G_0 and S phase in peripheral mononuclear blood leukocytes. In human skin, tapestripping has been reported to induce a synchronised transition from G_0 to G_1 whilst Ki-67 expression is first seen after 40-48 hours.[24] Such a lag phase of Ki-67 expression might also explain the time gap between the appearance of cytokeratin 13/16 expression and increase of Ki-67 expression which we observed after UVB in the present study.

Transglutaminase and involucrin are markers for terminal differentiation. Functionally the enzyme transglutaminase is necessary for the formation of the cornified envelope by enhancing the crosslinking of involucrin and other proteins.[5,25] In normally differentiating skin these markers are only present as a band like pattern in the upper part of the stratum spinosum and the stratum granulosum. In hyperproliferative skin, terminal differentiation starts earlier and also a more substantial proportion of the epidermal keratinocytes expresses these markers for epidermal differentiation. UVB apparently initiates premature terminal differentiation in a specific cohort of the epidermal keratinocytes. This UVB challenged cohort moves upwards in the days after exposure to the upper part of the epidermis resulting in a band like expression at about 10 days after UVB.

Since exposure to 1 MED of UVB clinically results in erythema and induration, one might expect a pronounced inflammatory infiltrate. But after intermediate dose UVB the inflammatory changes are rather modest in contrast to the conspicuous epidermal changes. No infiltration of PMN was seen, neither in the dermis nor in the epidermis. A modest T-cell infiltration in the dermis occurred following UVB exposure. After 1 day there was a significant increase of the percentage of T-cells in the dermal infiltrate. From 2 days onwards the number of T-cells as a percentage of the total dermal infiltrate tended to normalise again. Langerhans cells are known to decrease due to higher doses of UVB.[9,26] In our study no significant changes in dermal or epidermal CD1a binding were seen as a result of 1 MED of UVB. Murphy et al.[9] described a dose dependent decrease of epidermal Langerhans cells, identified by the Leu 6 monoclonal antibody; the amount of Langerhans cells is reduced to approximately 50 % due to UVB doses of 1½ and 3 MED. A dose of 0.7 MED did not induce these changes.[9] The dose of 1 MED (present study) also failed to modulate the density of the Langerhans cell population.

A major question remains to be answered. To what extent are the changes described in the present study characteristic for UVB. Various models for the induction of cutaneous inflammation and epidermal proliferation have been characterised during the last decade: response to tape-stripping[20], response to the epicutaneous application of leukotriene B_4 (LTB_4)[27] or dithranol[6].

Whereas an intermediate dose of UVB only yields into a minimal inflammatory infiltrate, the other models for cutaneous inflammation show a moderate or marked accumulation of infiltrate cells.

In conclusion, the response to intermediate dose ultraviolet B radiation represents a response pattern characterised by a major induction of epidermal proliferation, enhanced epidermal differentiation and some T-cell accumulation without significant accumulation of PMN or modulation of Langerhans cells. In contrast to the in vivo models using high dose UVB, intermediate dose UVB challenge can be regarded as a model to investigate epidermal behaviour rather than inflammation. This model might provide a promising approach to evaluate the effect of drugs on epidermal proliferation and differentiation in vivo.

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3.2. THE EFFECTS OF CALCIPOTRIOL AND CLOBETASOL-17-PROPIONATE ON UVB IRRADIATED HUMAN SKIN, AN IMMUNOHISTOCHEMICAL STUDY

3.2.1. Summary

Corticosteroids and vitamin D_3 analogues inhibit proliferation, enhance normal keratinisation and interfere with cutaneous inflammation in in vitro systems. Both treatments are effective in psoriasis, although several reports suggest that vitamin D_3 is less effective in reducing the inflammatory changes compared to its potent effect on keratinocyte growth and differentiation. The aim of the present study was to compare and contrast the effects of the vitamin D_3 analogue calcipotriol, clobetasol-17-propionate (clobetasol) and the placebo (ointment base of calcipotriol) on immunohistochemical markers for epidermal growth, keratinisation and inflammation induced by a standardised single challenge with ultraviolet B (UVB) radiation in normal human skin.

Clobetasol proved to inhibit UVB induced proliferation of epidermal cells, tenascin induction, keratin 16 induction and the accumulation of T-lymphocytes and CD1a positive cells. Epidermal thinning due to clobetasol was also observed. No effect of clobetasol was shown on the enhanced terminal differentiation following UVB challenge. In contrast, calcipotriol reduced the number of transglutaminase positive cells following UVB challenge but increased the thickness of the epidermis without a significant effect on other markers for keratinisation, epidermal proliferation and inflammation.

The present study reconfirms the potent effect of topical corticosteroids on various aspects of UVB challenged skin. In contrast, calcipotriol interfered especially with one differentiation pathway (transglutaminase) without modulation of other UVB induced changes.

3.2.2. Introduction

Phototherapy with UVB is used frequently as a potent antipsoriatic therapy but in the healthy human skin, UVB irradiation is also known to induce erythema, induration and scaling.[1] The effects of UVB are dose-dependent. Higher doses are known to damage the skin resulting in cytolysis and oedema. Following lower doses more minute changes can be observed.[1]

Recently, the in vivo effects of an intermediate dose of 1 minimal erythema dose (MED) of UVB on normal human skin were characterised and it was demonstrated that such a UVB dose mainly influenced epidermal proliferation and differentiation, leaving markers for inflammation relatively unaffected.[1] In the present study, this UVB model was used to evaluate the effects of some well-established topical antipsoriatic therapies on UVB challenged skin: calcipotriol and

clobetasol-17-propionate (clobetasol).[2,3] Vitamin D_3 analogues as well as corticosteroids are members of the steroid hormone superfamily and both operate mainly via genomic mechanisms. In vitro, both drugs inhibit proliferation and have anti-inflammatory and immunomodulatory functions.[4-10] Therefore, the in vitro actions share important characteristics.

The aim of the present study was to investigate in what respect these antipsoriatic treatments were able to inhibit the UVB induced changes in normal human skin and to compare and contrast the in vivo actions of both compounds on UVB induced epidermal proliferation, keratinisation and cutaneous inflammation. In particular the following questions were addressed:

i. What are the effects of calcipotriol, clobetasol or the placebo on UVB irradiated skin?
ii. What are the effects of calcipotriol versus its placebo in UVB irradiated skin?
iii. What are the effects of calcipotriol versus clobetasol in UVB irradiated skin?

An immunohistochemical investigation of skin biopsies with a panel of monoclonal antibodies was carried out on cryostat sections to answer these questions.

3.2.3. Materials and methods

Volunteers

Six healthy male volunteers, (age 23-28 years), participated in this investigation. This study was approved by the local ethical committee. All volunteers gave their written informed consent prior to inclusion in the study.

UVB exposure

For UVB exposure a Waldman UV 7001 K light cabin, emitting broad-band UVB (285-310 nm) was used. In all volunteers the MED was determined on the non sun-exposed skin of the buttocks; MED is defined as the minimal dose yielding a sharply demarcated erythema after 24 hours. Subsequently the volunteers were exposed to single doses of 1 MED of UVB on four areas of $\pm$ 9 cm^2 of normal skin on the buttocks.

Ointment application

The volunteers were instructed to apply the three different ointments, on the corresponding areas, twice daily, starting one day before irradiation up to the moment of the biopsy, four days after irradiation. The ointments were calcipotriol ointment (Daivonex, Leo Pharmaceutical Products, Denmark), clobetasol-17-propionate ointment (Glaxo, Zeist, the

Netherlands) and the vehicle of the calcipotriol ointment (Leo Pharmaceutical Products, Denmark). On the fourth irradiated area, the control area, no ointment was applied.

Clinical assessment

Erythema of the four irradiated lesions was scored using a 0-4 point scale: 0 = no erythema, 1 = slight erythema, 2 = moderate erythema, 3 = severe erythema, 4 = severest possible erythema.

Sample procedure

Punch biopsies of 3 mm diameter were taken from the UVB exposed areas (one biopsy per area) after local anaesthesia with xylocain 1% and adrenaline. The biopsies were embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, USA), snap frozen in liquid nitrogen and stored at -80°C until use. Sections of 6 μm were cut, air dried and fixed for 10 minutes in acetone/ether (60/40%) (Ki-67 staining) or in acetone (other stainings) and again stored at -80°C.

Monoclonal antibodies

A panel of monoclonal antibodies was used. To approximate the number of cycling epidermal cells in the basal layer an antibody directed against the Ki-67 antigen was used (MIB-1, 1:50, Immunotech, S.A., Marseilles, France). Antibodies against tenascin (T_2H_5, 1:2000; obtained from AA Verstraeten, The Netherlands Cancer Institute, Amsterdam, The Netherlands) and against cytokeratin 13/16 (Ks8.12, 1:20, Sigma, St. Louis, USA) were used for proliferation associated dermal and epidermal changes respectively.

To assess epidermal differentiation monoclonal antibodies against involucrin, (MON-150[11], 1:25) and anti-human keratinocyte transglutaminase (1:100, BT621, Mouse Monoclonal Antibody, IgG_{2a} Biomedical Technologies Inc.) were used.

Analysis of the inflammatory infiltrate was done by assessment of T-lymphocytes, Polymorphonuclear leukocytes (PMN) and Langerhans cells respectively using the monoclonal antibodies against the CD2 antigen (DAKO-T11, 1:100, Dakopatts, Copenhagen, Denmark), against elastase (DAKO-elastase, 1:100, Dakopatts, Copenhagen, Denmark) and against the CD1a antigen (DAKO-T6, 1:800, Dakopatts, Copenhagen, Denmark).

Staining procedure

For all monoclonal antibodies, except for DAKO-T11 and DAKO-T6, an indirect immunoperoxidase technique was used. The immunoperoxidase technique has been described previously.[1,5]

Staining with DAKO-T11 and DAKO-T6 was done with the avidin-biotin complex method (ABC-kit (mouse), Vector Lab. Inc., Burlingame, USA). In brief, the slides were incubated with 20% normal horse serum and subsequently with the T11 or T6 antibody for 30 minutes. The slides were incubated with horse-anti-mouse-biotinylated IgG (1:100, Vector Lab. Inc., Burlingame, USA) for 30 minutes. After 2 washes with PBS an incubation of 30 minutes with avidin-biotin-peroxidase complex (1:100 Vector Lab. Inc., Burlingame, USA) was performed. Visualisation was done using a solution of 3-amino-9-ethylcarbazole (AEC).

All slides were counter-stained with Mayer's Haematoxylin (Sigma, St. Louis MO, USA) and mounted in glycerol-gelatine.

Histological examination

The histological examination was performed blinded, by two investigators. These scoring methods have been performed and published before.[1,5]

Epidermal proliferation was measured by counting the number of Ki-67 positive nuclei per mm length of the section. The distribution of tenascin in the papillary dermis was assessed using a 0-5 point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, moderate-pronounced staining; 5, pronounced staining. In addition it was recorded whether a staining pattern continuous or discontinuous adjacent to the basal lamina was seen. Cytokeratin 13/16 was scored separately for the basal and suprabasal compartment of the epidermis using a semi-quantitative 0-6 point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, moderate-pronounced staining; 5, pronounced staining; 6, whole epidermis stained.

The involucrin and transglutaminase expression were assessed by calculation of the ratio positive cell layers/ total cell layers of the viable epidermis. Besides this fraction, the absolute number of cell layers of the epidermis was recorded separately. This was done at two sites: above the dermal papilla and between two dermal papillae.

Inflammation (T-lymphocytes, PMN and Langerhans cells) was assessed separately for dermis and epidermis. Dermal inflammation (T-lymphocytes and PMN) was semi-quantitatively enumerated by expressing the number of positively stained cells as a percentage of the total number

of infiltrate cells: 0, no positive cells; 1, sporadic; 2, 1-25 %; 3, 26-50 %; 4, 51-75 %; 5, 76-99 %; 6, 100 %. Langerhans cells in the dermis were assessed on a 0-5 point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, moderate-pronounced staining; 5, pronounced staining. Epidermal inflammation was assessed using a 0-4 point scale: 0, no staining; 1, sporadic staining; 2, minimal staining; 3, moderate staining; 4, pronounced staining.

Statistical evaluation

Data are reported as means ± SEM. For statistical analysis the t-test for paired values was used. In principle, the statistical significance of the following comparisons were tested: calcipotriol, clobetasol or the placebo treated versus untreated UVB challenged skin (control), calcipotriol versus placebo and clobetasol versus calcipotriol. A two-tailed hypothesis was employed to interpret data. A regression analysis was performed to determine the significance of relations between different parameters.

3.2.4. Results

Clinical response

The volunteers had skin types II and III (Fitzpatrick-Pathak-classification); the MED ranged from 0.1 - 0.3 J/cm^2. Erythema scores that were recorded four days after irradiation are shown in figure 1. The erythema on the UVB irradiated skin areas that were treated with clobetasol appeared to be significantly less compared to the erythema at the calcipotriol treated sites (p=0.005) and the untreated sites (control sites) (p=0.009). Non of the other comparisons tested was significantly different.

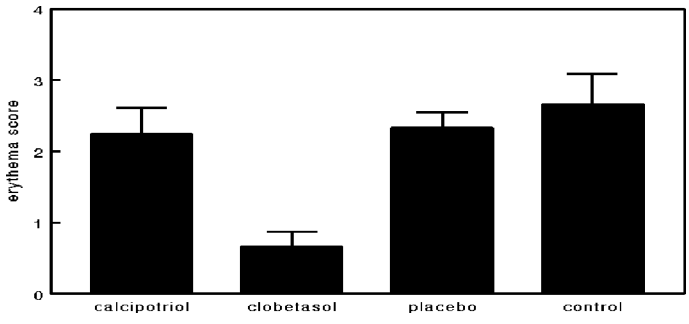

Fig. 1 Erythema at the UVB irradiated skin sites treated with calcipotriol, clobetasol, placebo and the untreated UVB challenged sites (control sites).

Immunohistochemical assessments

Epidermal proliferation and proliferation related changes

The results of the Ki-67 staining are demonstrated in figure 2a. A statistically significant decrease of the number of Ki-67 positive nuclei was observed at the clobetasol treated UVB challenged sites compared to the calcipotriol treated UVB challenged sites (p=0.0007) and the untreated UVB challenged sites (p=0.02). Calcipotriol did not significantly change the UVB induced hyperproliferation.

The results of the tenascin staining are shown in figure 2b. Decreased tenascin expression was seen at the clobetasol treated UVB challenged sites compared to the calcipotriol treated UVB challenged sites (p=0.02).

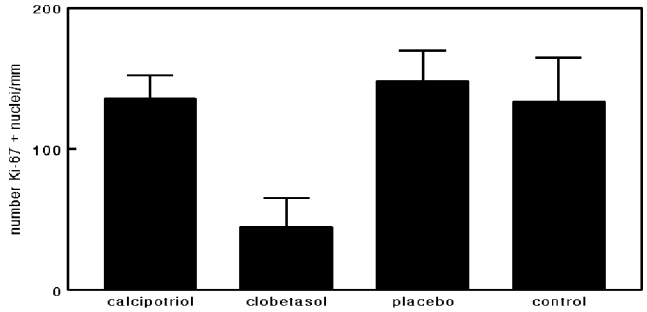

Fig. 2 a Ki-67 staining at the UVB irradiated skin sites treated with calcipotriol, clobetasol, placebo and the untreated UVB challenged sites(control sites).

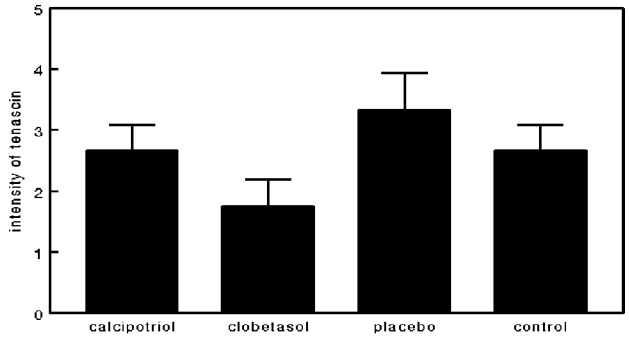

Fig. 2 b Tenascin staining at the UVB irradiated skin sites treated with calcipotriol, clobetasol, placebo and he untreated UVB challenged sites (control sites).

Staining with the Ks8.12 antibody can be divided in basal and suprabasal staining (figure 2c). For the basal compartment no significant differences were seen in the comparisons tested. In the suprabasal compartment the reduction of UVB induced Ks8.12 staining by clobetasol was more substantial compared to the reduction by calcipotriol (p=0.02) and compared to the untreated UVB challenged sites (p=0.08).

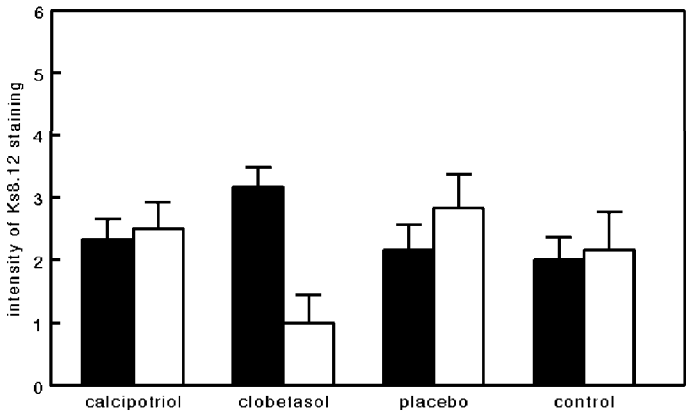

Fig. 2 c Ks8.12 staining in the basal layer (■) and suprabasal (□) at the UVB irradiated skin sites treated with calcipotriol, clobetasol, placebo and the untreated UVB challenged sites (control sites).

Epidermal differentiation

Staining for involucrin can be divided in staining between two dermal papillae and above the dermal papilla (figure 3a). Except for a significant reduction of involucrin expression at placebo treated UVB challenged sites as compared to the untreated UVB challenged sites (interpapillar: p=0.04; tip: p=0.006) no significant changes were observed comparing active drugs and placebo or control.

The transglutaminase staining is also displayed as staining between dermal papillae and above the dermal papilla (figure 3b). The only significant difference that was found was the decreased expression at the calcipotriol treated UVB challenged sites as compared to the placebo treated UVB challenged sites for the interpapillary region (p=0.06) as well as for the tip of the papilla (p=0.008).

The results of the counting of the total number of cell layers are shown in figure 3c. A significant increase of the number of interpapillary cell layers was seen in the calcipotriol treated UVB challenged skin areas versus the untreated UVB challenged sites (p=0.04) and a significant

decrease in the clobetasol treated UVB challenged sites versus the untreated UVB challenged sites (p=0.02). Above the papilla a significant difference was observed following UVB challenge between calcipotriol and its placebo with more cell layers at the calcipotriol treated site (p=0.05). Between clobetasol and calcipotriol at both UVB challenged sites a statistically significant difference was seen with substantially more cell layers following calcipotriol treatment (tip: p=0.004 and interpapillary: p=0.003).

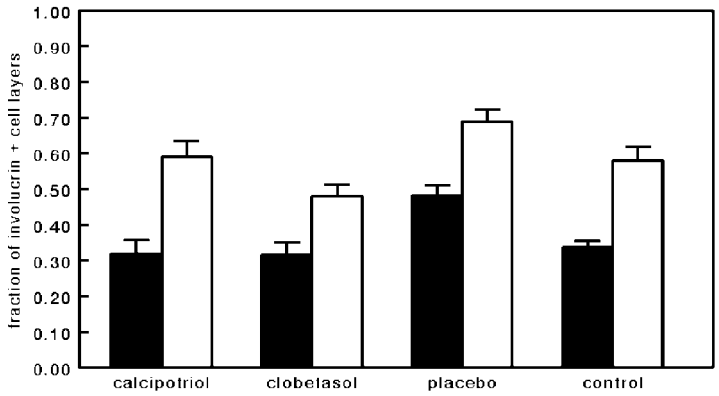

Fig. 3 a Involucrin staining, interpapillary (■) and above the dermal papilla (□), at the UVB irradiated skin sites treated with calcipotriol, clobetasol, placebo and the untreated UVB challenged sites (control sites).

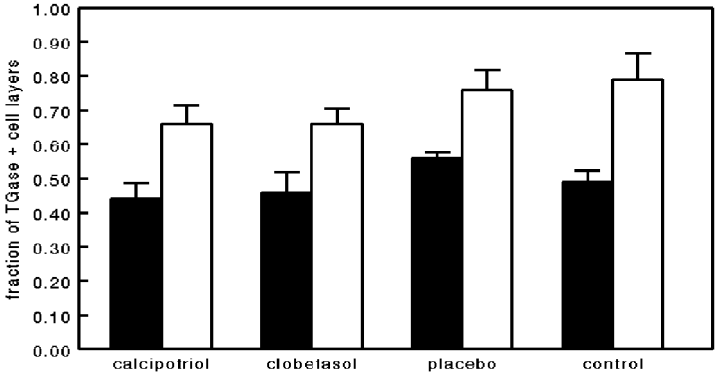

Fig. 3 b Transglutaminase (TGase) staining, interpapillary (■) and above the dermal papilla (□), at the UVB irradiated skin sites treated with calcipotriol, clobetasol, placebo and the untreated UVB challenged sites (control sites).

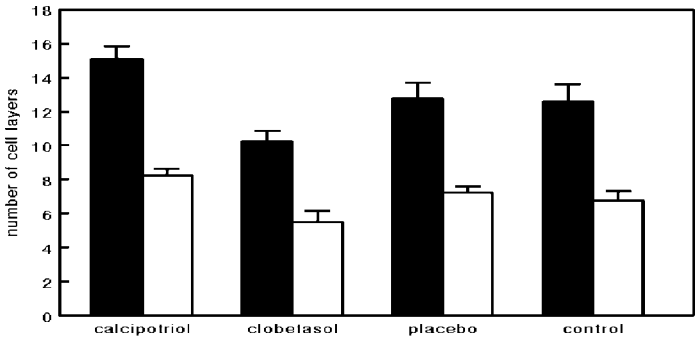

Fig. 3 c The count of the total number of cell layers, interpapillary (■) and above the dermal papilla (□), at the UVB irradiated skin sites treated with calcipotriol, clobetasol, placebo and the untreated UVB challenged sites
(control sites).

Inflammation

Staining for T-lymphocytes (figure 4a) demonstrated significant differences between calcipotriol and clobetasol with respect to modulation of UVB induced inflammation for both dermis and epidermis (dermis: p=0.03; epidermis: p=0.04) and a significant difference for T-lymphocytes in the dermis between clobetasol- and the untreated UVB challenged sites (p=0.02). A tendency of a reduction of T-lymphocytes in the epidermis was seen at UVB challenged placebo treated sites as compared to untreated UVB challenged sites (p=0.08). In contrast to the reduction of T-cells following clobetasol treatment, no significant effects were seen following calcipotriol treatment.

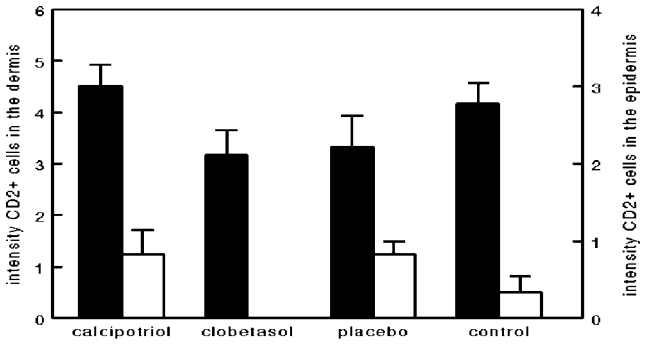

Fig. 4 a CD2 positive cells in the dermis (■) and epidermis (□) at the UVB irradiated skin sites treated with calcipotriol, clobetasol, placebo and the untreated UVB challenged sites (control sites).

Elastase staining, representing PMN, did not reveal any differences between the different treatments of the UVB irradiated areas. However, appearance of PMN following UVB was sparse.

Staining with the antibody against the CD1a epitope (Langerhans cells) (figure 4b) revealed significant differences in the dermis between calcipotriol and clobetasol treated UVB challenged sites (p=0.04) and between clobetasol treated and untreated UVB challenged sites (p=0.04). A reduction of UVB induced changes following clobetasol treatment and virtually no effects following calcipotriol treatment were observed.

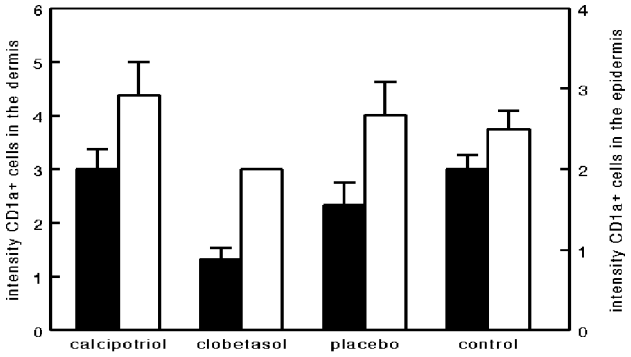

Fig. 4 b CD1a positive cells in the dermis (■) and epidermis (□) at the UVB irradiated skin sites treated with calcipotriol, clobetasol, placebo and the untreated UVB challenged sites (control sites).

Correlations

Statistically significant correlations were observed between the following parameters: Ki-67 and tenascin (p = 0.002), Ki-67 with Ks8.12 staining in the suprabasal compartment ($p = 2.6 \cdot 10^{-8}$), Ki-67 with erythema (p = 0.0005) and Ki-67 with the total amount of cell layers (p = 0.002). Correlations between erythema versus Ks8.12 staining in the suprabasal compartment (p=0.0003) and erythema versus T11 staining in the epidermis (p=0.04) were found.

3.2.5. Discussion

Calcipotriol and clobetasol both have a potent anti-inflammatory, immunomodularoty and proliferation inhibiting effect in vitro.[6,7,9,10] Both compounds are also well-established antipsoriatic treatments. However, it has been demonstrated in vivo during treatment of psoriatic plaques that calcipotriol has a limited effect on inflammation compared to its pronounced effect on epidermal

proliferation and differentiation.[4,5] The present study demonstrates a pronounced difference between calcipotriol and clobetasol with respect to UVB induced effects on epidermal proliferation and differentiation.

The response to an intermediate dose of UVB challenge has been characterised before and has proven to be consistent: a marked recruitment of cycling epidermal cells (Ki-67 positive nuclei), expression of the extracellular matrix protein tenascin, suprabasal staining of Ks8.12, reflecting cytokeratin 13 and 16 expression, an increased number of involucrin positive and transglutaminase positive cell layers, accumulation T-lymphocytes and a sporadic accumulation of elastase positive cells (polymorphonuclear leukocytes).[1]

In the present study, calcipotriol and clobetasol proved not to interfere with the expression of tenascin, involucrin and the number of PMN following UVB challenge. Clobetasol proved to inhibit the UVB induced recruitment of cycling epidermal cells (Ki-67 positive nuclei), the total number of cell layers in the epidermis and the suprabasal Ks8.12 staining, mainly reflecting keratin 16 expression. Also the UVB induced accumulation of T-lymphocytes and CD1a positive cells was reduced substantially by clobetasol. No significant effect of clobetasol was observed on UVB enhanced terminal differentiation (involucrin and transglutaminase positive cells). These results are compatible with earlier studies on the inhibition of the response to ultraviolet radiation.[12-15] The in vivo effects of corticosteroids in psoriasis are also compatible with the changes observed in the present model.[8,16]

Calcipotriol reduced the number of transglutaminase positive cell layers resulting from UVB challenge and increased the total number of cell layers in the epidermis without a significant effect on the number of involucrin positive cell layers. No significant effects could be shown with respect to the recruitment of cycling epidermal cells (Ki-67 positive nuclei), Ks8.12 staining of the suprabasal compartment or accumulation of infiltrate cells. This pattern, strikingly different from the response to clobetasol, is in line with the failure of calcipotriol to reduce erythema following UVB challenge in contrast to the pronounced reduction of erythema as reached by clobetasol. It is of practical relevance, however, that calcipotriol did not enhance the inflammation following UVB challenge. Indeed, in multi-centre studies the combination of UVB and calcipotriol has been shown to be effective and safe without a reduction of the minimal erythema dose.[17-19] To the best of our knowledge, no data are available on the effect of vitamin D_3 analogues on cutaneous inflammation.[20] In contrast to the failure of calcipotriol to inhibit cutaneous inflammation in this model, calcipotriol has a potent therapeutic effect in psoriasis and disorders of keratinisation.[2,21-23]

The interpretation of the difference between calcipotriol and clobetasol in UVB induced inflammation is difficult as many factors might be involved in the complex in vivo situation. Both calcipotriol and clobetasol are members of the steroid receptor superfamily and operate via genomic mechanisms. But diversity in both compounds with respect to intensity and velocity of the genomic and non-genomic mechanisms might explain the differences in modulation of UVB induced inflammation.

Intriguing is the discrepancy between the potent antiproliferative effect of calcipotriol during antipsoriatic treatment[5] and the absence of such an effect in the present study. In the present model, the absence of the antiproliferative effect of calcipotriol is reflected in an increased thickness of the epidermis whereas clobetasol decreased the thickness of the epidermis substantially. Previously it was shown that calcipotriol inhibits ornithine decarboxylase activity following tape-stripping, which demonstrates that calcipotriol interferes with growth control also in an acute model for cutaneous hyperproliferation.[24] The failure to inhibit recruitment of cycling cells in UVB induced inflammation suggests that it is not the recruitment process in itself that is the therapeutic target but that the reduction of epidermal hyperproliferation as reached in the treatment of psoriasis may result from other changes. The selective effect of calcipotriol on the number of transglutaminase positive cell layers and the total number of cell layers in UVB induced inflammation demonstrates that calcipotriol was available in sufficient quantities below the stratum corneum to have a cell-biological effect in the present study.

At the placebo treated sites UVB induced epidermal T-cell accumulation tended to be increased as compared to UVB induced epidermal T-cell accumulation at control sites. Although UVB induced erythema was not enhanced due to placebo ointment, increased epidermal T-cell accumulation might reflect an irritating effect of the placebo ointment, all the more because erythema and T-cell accumulation in the epidermis proved to be significantly correlated in this study. These findings are in line with the reported irritancy of the placebo ointment in calcipotriol studies.[2,21]

The use of potent corticosteroids in clinical practice is limited by their side-effects. Persisting local side-effects like striae and skin thinning are mainly the result of dermal atrophy but epidermal thinning may give insight into atrophogenicity in general.[25] In the present study clobetasol had a significantly decreasing effect on the total number of cell layers in the epidermis. This reconfirms our knowledge on the atrophogenicity of clobetasol. The significant increase of the number of cell layers in calcipotriol treated skin area versus the untreated and placebo treated skin has been documented before.[26] The occurrence of these effects, already within four days of treatment, indicates the rapidity of clobetasol and calcipotriol induced modulation of epidermal thickness.

Correlation analysis revealed that recruitment of cycling epidermal cells is a process that is closely associated with all parameters investigated in this model, which implies that recruitment of cycling epidermal cells is a general reflection of the response of the skin to UVB.

The present study reconfirms the potent effect of topical corticosteroids on UVB challenged skin and the failure of calcipotriol to modulate the cutaneous response to UVB.

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Chapter 4

Efficacy and safety aspects

of new topical treatments of psoriasis

This chapter was based on the following publications:

C.J.M. van der Vleuten, M.J.P. Gerritsen, P.M. Steijlen, E.M.G.J. de Jong, P.C.M. van de Kerkhof
Therapeutic approach to erythrodermic psoriasis; The report of a case and a discussion of therapeutic options. *Acta Derm Venereol* 1996; **76**: 65-67.

C.J.M. van der Vleuten, E.M.G.J. de Jong, H.F.C. Rulo, M.J.P. Gerritsen, P.C.M. van de Kerkhof
In-patient treatment with calcipotriol versus dithranol in refractory psoriasis. *Eur J Dermatol* 1995; **5**: 676-679.

P.C.M. van de Kerkhof, C.J.M. van der Vleuten, M.J.P. Gerritsen, C.P. Glade, Th. Luger, Th. Werfel, B. Klüß
Long-term efficacy and safety of once daily treatment with tacalcitol ointment in chronic plaque psoriasis. (submitted)

4.1. THERAPEUTIC APPROACH TO ERYTHRODERMIC PSORIASIS; THE REPORT OF A CASE AND A DISCUSSION OF THERAPEUTIC OPTIONS

4.1.1. Summary

In this case report a patient with therapeutically recalcitrant erythrodermic psoriasis is presented. After various attempts with several major therapies in this patient, the first substantial improvement was achieved using the combination of cyclosporin and calcipotriol, followed by the combination of UVB and calcipotriol.

In this paper the therapeutic options for severe psoriasis are discussed and since combined approaches seem to be an attractive alternative for severe psoriasis mechanisms of synergy of combined therapeutic approaches are hypothesised.

4.1.2. Introduction

Erythrodermic psoriasis is a rare but dramatic condition. As a result of a trigger of any kind, psoriasis can become unstable and can extend until the whole skin is erythematous and scaly. In general, systemic treatment of erythrodermic psoriasis is inevitable and patients should be admitted at the inpatient department.

Well-established therapeutic options for severe psoriasis, including erythrodermic psoriasis, are methotrexate, acitretin and cyclosporin. However, the therapeutic response in patients with the erythrodermic psoriasis may be variable and sometimes disappointing. The search for the appropriate therapy is time-consuming. Often a combination of systemic and local therapies will provide the eventual remedy for the patient after several weeks of intensified supervision.

The aim of this report is to present a case, indicating the therapeutic problems during treatment of erythrodermic psoriasis.

4.1.3. Case report

An 83-year-old erythrodermic man was admitted to our hospital. At dermatological investigation generalised erythema and extensive scaling was observed all over the body (figure 1), face, scalp, palms and soles. Histopathological investigation of the skin showed a chronic, non-specific dermatitis without signs of lymphoma or psoriasis. At general investigation we saw a dyspnoeic man with oedema on both lower legs. The body weight was 63 kg. No enlarged lymphnodes were palpable. No additional abnormalities were observed except for pre-existent gal-stones. Blood-tests, X-thorax, ECG, CT-scan of thorax and abdomen, X-colon did not reveal any internal pathology. Especially no evidence existed for malignancy. Serum $1,25(OH)_2$ vitamin D_3 and 25 OH vitamin D_3 were 70 pmol/l and 50 nmol/l respectively which were in the normal range.

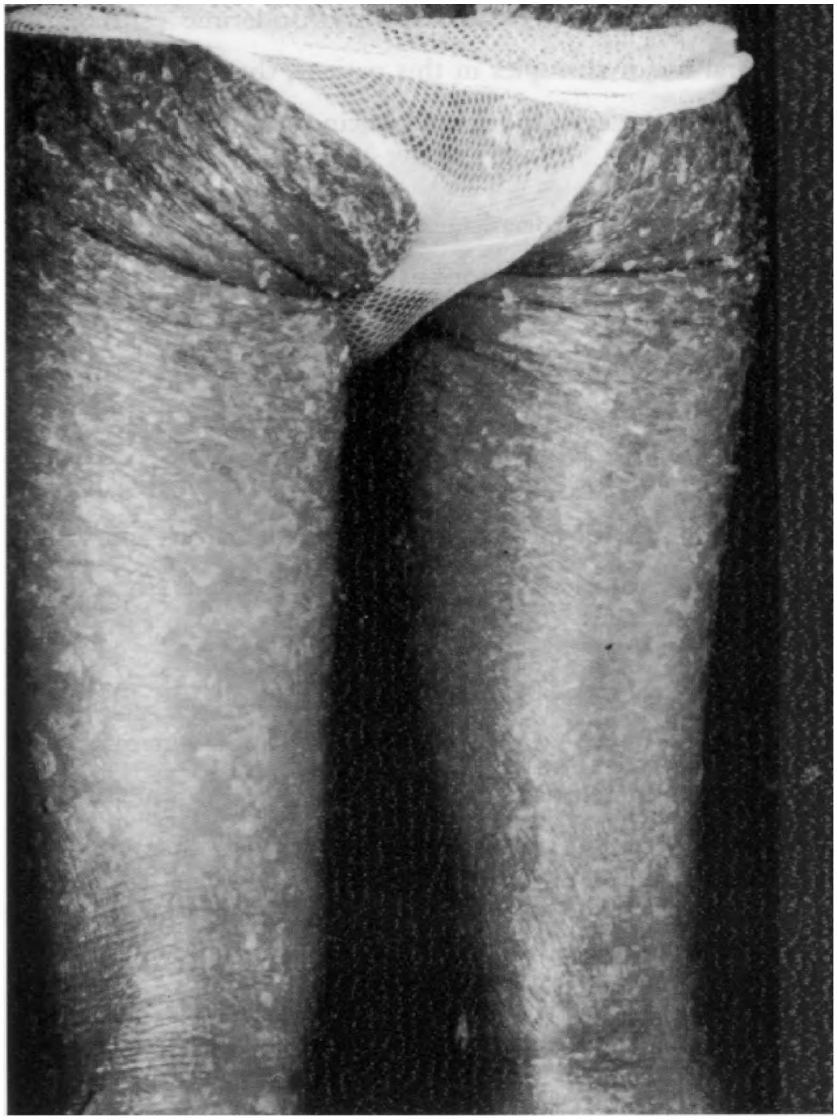

Fig. 1 Erythrodermic skin and extensive scaling all over the body, at the moment of admission to hospital.

The patient had had psoriasis vulgaris for five years. The condition could be controlled up to six months prior to admission. A first exacerbation was treated with tar-UVB and calcipotriol but four months later, the psoriasis flared again resulting in erythroderma. The patient was admitted to a hospital elsewhere and was treated with potent topical corticosteroids such as clobetasol 17-propionate and different systemic therapies; each of these only for a short period. Acitretin had been given for three weeks, methotrexate for two weeks and oral corticosteroids also

for two weeks without any substantial improvement. No factors were found that could have triggered this exacerbation of psoriasis. There was no history of infections or malignancy.

As the expression of psoriasis was extremely severe and unresponsive to various treatments, the patient was transferred to the university hospital. We started therapy with acitretin (20mg/day) and hydrocortisone (1% in petrolatum) topically. Water-salt-balance normalised; furosemide 40 mg daily was given to control oedema and dyspnoe. Protein loss due to scaling was compensated with the appropriate diet. After four days the dose of acitretin was increased to 30mg/day. Because of no improvement after another four days cyclosporin (3mg/kg/day) was added. This resulted only in a minor improvement after three weeks, subsequently the dose of cyclosporin was increased to 4mg/kg/day. After another week acitretin was stopped and cyclosporin was again increased to 5mg/kg/day. The skin condition in the patient still did not improve. Then it was decided to start local calcipotriol on the right side of the body whilst continuing cyclosporin. The calcipotriol-treated side showed a remarkable improvement compared to the other side which was treated with bland emollients (figure 2). After one week the whole body was treated on alternate days with calcipotriol twice daily up to 100 grams per week. On the remaining days of the week bland emollients were applied. As the quantity of calcipotriol ointment approximated 100 grams per week calcium and phosphate in the serum were measured at weekly intervals.

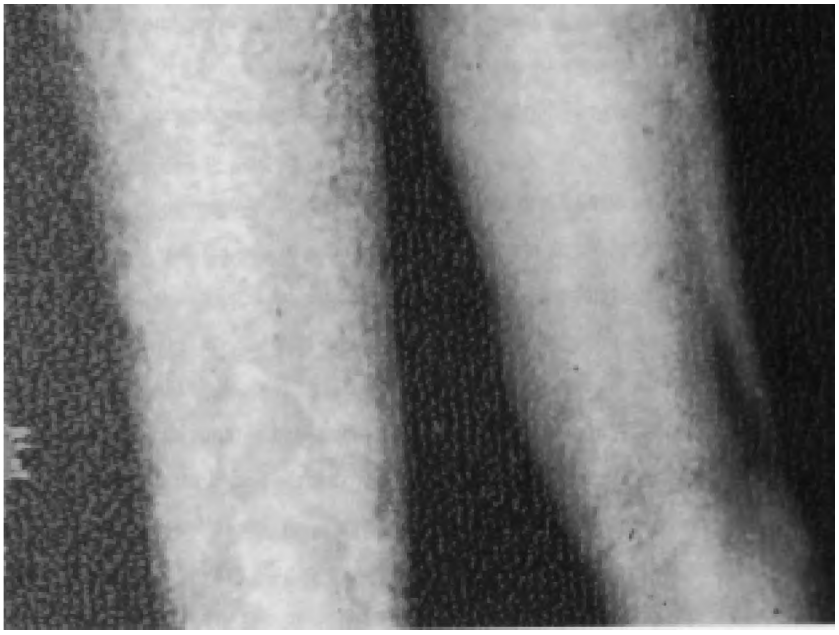

Fig. 2 Remarkable improvement on the right side of the body due to calcipotriol on this side combined with systemic cyclosporin.

The condition of the skin improved markedly within four weeks. Meanwhile, after treatment with cyclosporin for two months, the serum creatinin increased and the patient developed a tremor of an unknown origin that could have been a side-effect of cyclosporin. These symptoms necessitated discontinuation of cyclosporin. As an alternative to cyclosporin, phototherapy with a low dose UVB in combination with local calcipotriol was started. The patient responded well to this treatment and was discharged from hospital in a reasonable condition after one month phototherapy. Phototherapy in combination with local calcipotriol was continued at the out-patient department for about four months. So far the condition of the patient remains excellent without any psoriatic lesion up till six months after discharge from hospital.

During the various treatments, apart from the transient increase of serum creatinin and the temporary tremor during cyclosporin, no side-effects occurred. Serum calcium and phosphate remained in the normal range.

4.1.4. Discussion

Before deciding on the strategy of the treatment the underlying cause of the erythroderma has to be established. Histopathological investigation is not always specific.[1] The history of a previous skin disease is an important lead to the diagnosis; 25% of the cases is associated with psoriasis. Drugs, neoplasm and eczema account for the majority of the other known causes. In a substantial part of the cases no obvious cause is found.[2] In case the nosological identity of the erythroderma remains unknown, further internal investigation is required to exclude paraneoplasm.[3]

As erythroderma is a serious condition, fast improvement is urgently wanted. Topical treatments with potent corticosteroids may be useful, however, as the patient described in this report had been treated elsewhere already with potent corticosteroids (clobetasol 17-propionate), a weak steroid preparation was prescribed in order to prevent systemic complications. Systemic treatment is often necessary but is sometimes already changed if no response is observed after a few days. Also in the present case, various short treatments were initiated without allowing a sufficient treatment period for an antipsoriatic result. One may argue that the period of one week acitretin monotherapy might have been too short to induce a significant improvement. However the severity of the erythroderma required a fast therapeutic effect, hence the combined approach.

Combinations of the major therapies for psoriasis are an attractive option since some combinations allow a lower dose than used in monotherapy which reduces side-effects. The combination of methotrexate and etretinate is controversial in view of hepatotoxity;[4,5] the

combination of etretinate and cyclosporin has been used with success in psoriasis.[6,7] From a theoretical point of view the immunosuppressive effect of cyclosporin and the differentiation modulating effect of retinoids is a promising combination. Oral retinoids have also been combined successfully with UVB or PUVA (re-PUVA).[8,9] Combination of two immunosuppressive therapies like methotrexate and cyclosporin is not recommended.[10]

Another, practical approach is the combination of systemic and topical therapy. After various attempts with several major therapies in this patient including the combination of cyclosporin and acitretin, the first substantial improvement was achieved using the combination of calcipotriol and cyclosporin. In the past emollients, tars and topical steroids have been used in combination with systemic therapies.[11] Nowadays, the vitamin D_3 analogue calcipotriol is available. Its beneficial effect as a monotherapy in mild to moderate chronic plaque psoriasis has been well-established.[12] However, calcipotriol might irritate the skin in about 20% of the patients.[13] In particular patients with erythrodermic psoriasis are susceptible to low doses of irritants. On the other hand, patients with unstable and erythrodermic psoriasis have been reported to respond well to calcipotriol.[14,15] In the present case the maximum quantity of 100 gram calcipotriol ointment per week was not exceeded. As serum vitamin D_3 levels were normal it was excluded that this patient might have had a vitamin D_3 deficiency.

The skin is site of production of vitamin D_3 and target of its active metabolite: 1α,25-dihydroxyvitamin D_3.[16] Vitamin D_3 receptors, member of the steroid-hormone-receptor super-family, are found in the epidermis.[17] The therapeutic mode of action of vitamin D_3 and its analogues in psoriasis is partly via these receptors which regulate gene transcription and partly through non-genomic mechanisms.[18] Calcipotriol inhibits proliferation and induces terminal differentiation in cultured human keratinocytes.[19] In vivo these effects are observed as well.[20] Immunomodulating effects of calcipotriol are also described; inhibition of T-cell proliferation in response to interleukin 1 in vitro[21] and reduction of interleukin 6 in a psoriatic plaque in vivo in response to calcipotriol.[22]

In literature both cyclosporin[23] and UVB[24,25] have been combined successfully with calcipotriol in psoriatic patients. In particular low dose cyclosporin (2mg/kg/day) in combination with calcipotriol proved to be an effective and safe approach.[23] From a theoretical point of view it is attractive to speculate that calcipotriol-cyclosporin is a useful combination. The modes of action of cyclosporin and 1α,25-dihydroxyvitamin D_3 and its analogues are thought to be complementary.[26-28] Recently several investigators have demonstrated the synergistic effects of both antipsoriatic therapies. Calcipotriol can potentiate the immunosuppressive effect of cyclosporin in mixtures of human lymphatic and epidermal cells.[29] The effect of cyclosporin on interleukin 2 is increased by calcitriol.[26-28] On the other hand the differential effect of both treatments on epidermis

and immune system might explain the synergistic effect.[30,31] When cyclosporin treatment was not possible anymore in our patient due to increase of serum creatinin, UVB in combination with calcipotriol was applied successfully. In literature there is still no clearness about synergy of UVB and calcipotriol in psoriasis.[24,25] But the remarkable effect of the combination in this patient suggests that in some cases synergism might occur.

In case of erythrodermic psoriasis, the therapeutic strategy often includes systemic treatment. Options are monotherapy with acitretin, cyclosporin or methotrexate. The choice depends on indications and contraindications in the individual patient. In the present case of persisting erythroderma, combination therapy of cyclosporin plus calcipotriol and subsequently UVB plus calcipotriol proved to be a successful approach.

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4.2. IN-PATIENT TREATMENT WITH CALCIPOTRIOL VERSUS DITHRANOL IN REFRACTORY PSORIASIS

4.2.1. Summary

Calcipotriol (50 μg/g) ointment recently became available for the treatment of psoriasis. Calcipotriol has been shown to be superior to home treatment with dithranol. The time-honoured regime of topical treatment with dithranol in paste or petrolatum for 24 hours at the in-patient department is the golden standard of optimal efficacy of antipsoriatic therapy. This treatment regime is adopted in case of insufficient control of psoriasis by out-patient treatments.

The aim of the present study was to challenge the position of in-patient dithranol treatment. A left-right comparative case-control study was designed in ten patients with refractory psoriasis, comparing classical dithranol treatment and calcipotriol treatment at the in-patient department.

In contrast to what was expected, six of the ten patients showed a more pronounced improvement after two weeks at the calcipotriol treated sides. Irritation from calcipotriol was observed in four patients after one week and in two after two weeks treatment. At the dithranol treated sides three of the ten patients showed a better improvement. Four patients experienced irritation after one week and eight patients after two weeks. Irritation due to calcipotriol was not associated with an increased irritation due to dithranol, which implies that both treatments have a different mechanism of irritation.

The present case-control study indicates that calcipotriol has challenged the untouchable superiority of classical in-patient treatment with dithranol. Further studies are indicated to improve compliance of out-patient calcipotriol treatment by cream formulations and once a day schedules.

4.2.2. Introduction

Chronic plaque psoriasis can be treated with various out-patient therapies.[1] However, in patients with extensive, therapy resistant and severely disabling psoriasis it is our policy to admit the patient to the in-patient department. For this group of patients with refractory psoriasis dithranol so far has been the therapy of first choice at our in-patient department. In case lesions prove to be resistant against this treatment or in case new lesions continue to appear or in case of severely itching psoriasis various combination approaches are indicated.

Dithranol is a very safe and effective therapy.[2] Previously, dithranol has been used in petrolatum[3,4] and Lassar's paste.[5] Bioavailability varies significantly with its vehiculum.[6] Irritation

of the skin and discoloration of the skin and textiles have always been limiting its use.[7] For home treatment dithranol in a cream base has been developed which is easier to apply and wash off and therefore proved to be more acceptable for the patient to use at home.[8] A disadvantage is the lower efficacy of this mode of dithranol treatment.[2,8] In-patient treatment with dithranol in paste or petrolatum, however, is the golden standard of this time-honoured therapeutic approach, resulting in clearing of psoriasis in more than 90 % of the patients within three to five weeks time.[2] Dithranol in a cream base at the out-patient department only results in clearing in 10-35 % of the patients in seven to eight weeks time.[2]

The last decade vitamin D_3 analogues have been shown to have an important antipsoriatic effect.[9-12] Recently calcipotriol 50μg/g in ointment (Daivonex^R, LEO Pharmaceutical Products) became available as a routine treatment.[13] Several comparative studies on the efficacy of calcipotriol and the classical antipsoriatic therapies have been carried out to elucidate and establish the position of calcipotriol in dermatology. Betamethasone 17-valerate[14] and short contact dithranol treatment[15] have been compared to calcipotriol for its efficacy and safety. Calcipotriol proved to be equally effective compared to betamethasone 17-valerate. Remarkably, home treatment with calcipotriol appeared to be more effective compared to home treatment with short contact dithranol in a cream base.

In the present study the efficacy of treatment with dithranol and calcipotriol was compared in a group of patients with severe, therapy resistant and disabling psoriasis who were, for this reason, admitted to our in-patient department.

The aim of the present orientation is to challenge the common believe that dithranol is the treatment of first choice in severe psoriasis. In particular we addressed the following questions:

i) Does calcipotriol have a beneficial effect in severe psoriasis to the extent that dithranol is effective?

ii) Which aspects limit the use of calcipotriol in severe psoriasis?

As this study involves patients with severe psoriasis we set out a two week left-right comparative case-control study to explore the first two weeks of the in-patient treatment phase with calcipotriol and dithranol in Lassar's paste or in petrolatum.

4.2.3. Materials and methods

Patients

The investigation was carried out at the in-patient department. Patients with extensive and disabling plaque psoriasis, resistant to topical therapy, were admitted and included in the study.

Four males and six females were included with an age ranging from 20-72 years and with a duration of their psoriasis ranging from 3-53 years. The patients had used no oral treatment for psoriasis within six weeks prior to the study except for one patient who had used fumaric acid. Topical treatment was allowed until the date of submission to the hospital. Table I summarises the treatments for psoriasis of the patients during six weeks prior to admission. No oral medication was allowed that could influence the course of psoriasis. Hydroxyzine was allowed for those patients with severe pruritus. No additional topical nor systemic treatment for psoriasis was permitted during the trial except for corticosteroids at the scalp and the face.

Table I Previous therapy

Therapy 6 weeks prior to study	**#**
No therapy	1
Topical steroids	5
Calcipotriol	4
Tar	1
Fumaric acid	1
Phototherapy	1
Dithranol	1

Approach

Patients were treated during two weeks using a left-right within-subject comparison. One side of the body was treated with dithranol paste or petrolatum and the other side with calcipotriol. Which side of the body was treated with what therapy was randomly chosen. The regimen for dithranol consisted of 24 hour application of dithranol paste or petrolatum in increasing concentrations ranging from 0.05-4%. The concentration of dithranol was increased at alternate days. Calcipotriol was applied twice daily on lesional skin with a maximum of 100 grams per week. Individualisation of the treatment by adjunct therapies was postponed till after the two week's evaluation. The clinical scores were recorded before and after one and two weeks of therapy. Extent and severity of the disease were recorded as shown in table II.

Table II Scoring for extent and severity of disease

i) Extent of disease	**ii) Severity**
0 - no involvement	0 - no involvement
1 - <10 %	1 - slight
2 - 10-29 %	2 - moderate
3 - 30-49 %	3 - severe
4 - 50-69 %	4 - severest possible
5 - 70-89 %	
6 - 90-100 %	

The extent of the disease was scored in percentage of involvement of the skin. This percentage was transposed into an area score. Arms, trunk and legs were scored separately. The severity of erythema, induration, scaling and pruritus was assessed using a 5 point-scale (table II). After one and two weeks of treatment the skin-irritation as a result of therapy was recorded using the 5 point scale. PASI-scores were calculated according the formula in table III.

Table III PASI-score

Calculation of the PASI-score
PASI = 0.2 × A arms × Σ(E+I+S) + 0.3 × A trunk × Σ(E+I+S) + 0.4 × A legs × Σ(E+I+S)
A = Area score
Σ(E+I+S) = Sum of scores for Erythema, Induration and Scaling

Statistical analysis

Changes in clinical scores and comparisons between the two body-sides of the same patients were analysed using the Student t-test for paired values.

To obtain insight in correlation between different parameters which characterise the disease a regression analysis was performed (Pearson-r).

4.2.4. Results

At the start of the study severity-scores of both body-sides were comparable; the average PASI-score was 17.1 ± 2.1 (mean ± SEM) for the whole body. Both treatment regimens induced a statistically significant decrease in PASI-scores after one week of treatment ($p = 0.0005$ for calcipotriol and $p = 0.0003$ for dithranol). In the second week of treatment there was a significant further decrease of the PASI-score ($p = 0.03$ for calcipotriol and $p = 0.03$ for dithranol) compared to scores after one week. The calcipotriol treated side tended to respond slightly better to therapy than the dithranol treated side but this difference is not statistically significant ($p = 0.08$).

Figure 1 illustrates the difference (Δ) between the PASI-score before and after one and two weeks treatment of the calcipotriol and dithranol treated body-sides of the individual patients. Before treatment the PASI-scores at both body-sides were equal except in one patient (patient 8). After one week of therapy, however, three patients showed a therapy response in favour of dithranol and five patients showed a therapy response in favour of calcipotriol. Two patients showed no difference between the two therapies. After two weeks of therapy six patients responded better to the calcipotriol therapy and three patients responded better to the dithranol therapy. In one patient there was no difference in response to both therapies.

Pruritus was experienced in eight out of ten patients before treatment. Prior to therapy there was no difference between the two body-sides. The scores for pruritus are relatively high for psoriasis (2.0 ± 0.4)(mean ± SEM). After one week of therapy a significant decrease of pruritus was experienced at the calcipotriol treated sides ($p = 0.003$) as well as at the dithranol treated sides ($p = 0.005$). In the second week of treatment the scores for pruritus still tended to decrease. Calcipotriol was significantly better than dithranol in reducing pruritus ($p = 0.04$ after one week of treatment and $p = 0.04$ after the second week of treatment).

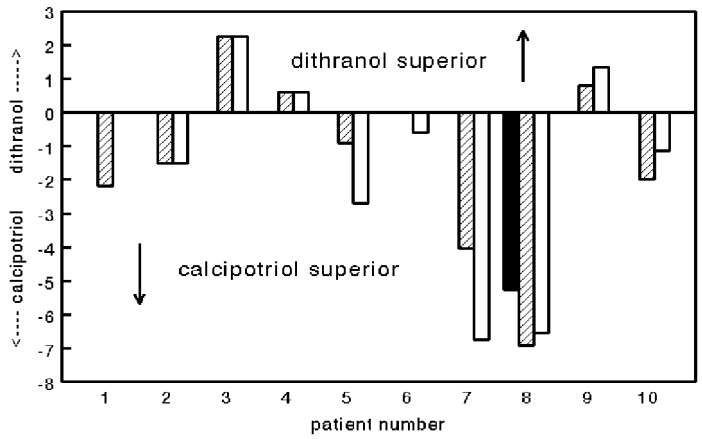

Fig. 1 Delta PASI-score (calcipotriol minus dithranol) before (▨), after one ( ) and two (□) weeks
treatment.

After one week of therapy irritation was seen on both body-sides due to both therapies, but there is no significant difference between the two therapies. The initial irritation of calcipotriol tended to decrease in the period between one and two weeks. Two out of four patients indicated that irritation had decreased during continued treatment with calcipotriol: the irritation as a result of dithranol treatment increased significantly in the second the week of therapy ($p = 0.01$). After two weeks of treatment the mean score for irritation due to dithranol was significantly higher than the score for treatment with calcipotriol ($p = 0.006$). There was no correlation between the scores for irritation on both body-sides in each patient after either one or two weeks of treatment. Regression analysis revealed that there was no association between pruritus before therapy and irritation as a result of two weeks of therapy for either calcipotriol or dithranol.

The mean duration of the in-patients treatment was 5.5 ± 0.7 (mean ± SEM) weeks. After the two weeks comparative study coal tar treatment was added in five patients, phototherapy with ultraviolet B was added in five patients and photochemotherapy in one patient. In three patients oral treatment with acitretin had to be added in order to enhance clearing. Out of these patients with difficult psoriasis four patients reached total clearing and the other six patients a substantial improvement.

4.2.5. Discussion

Most of the investigations on the efficacy and side-effects of calcipotriol deal with mild to moderate chronic plaque psoriasis.[1] However Beth-Jones et al.[16] reported a group of patients with extensive psoriasis; PASI: 18 ± 8.8 (mean ± SEM). A four week treatment period with calcipotriol ointment at home had resulted in a reduction of the PASI-score to 7.0 ± 2.0 (mean ± SEM)(61% reduction). Dubertret et al.[17] reported a reduction of the PASI-score from 14.2 ± 7.5 (mean ± SEM) to 8.6 ± 7.5 (mean ± SEM)(39% reduction) after two weeks treatment at home with calcipotriol ointment. In the present study the PASI-scores at the calcipotriol treated sides dropped from 8.3 ± 1.0 (mean ± SEM) to 2.9 ± 0.4 (mean ± SEM)(66% reduction). The difference between the present study and the other studies in extensive psoriasis[16,17] is the setting at the in-patient department in the present study and the setting of home treatment in the latter studies. Our figures are indeed analogous to another in-patient study on calcipotriol in which patients were treated during two weeks with a high dose calcipotriol and decrease in PASI-score of 71% was seen.[18]

Even a more pronounced discrepancy has been observed as to the efficacy of dithranol treatment carried out at in-patient departments and home treatment.[2] Dithranol treatment at the in-patient department has been shown to result into a clearing in 80-100% of the patients[19], whereas dithranol treatment at home resulted into a clearing in 6-56% of the patients.[20,21]

Four out of ten patients in the present study had been treated with calcipotriol before at home without a satisfactory improvement. In these patients in-patient treatment with calcipotriol resulted in a substantial improvement. The most feasible explanation for the pronounced discrepancy between the in-patient and out-patient setting is compliance.

A comparison between home treatment with short contact dithranol and calcipotriol revealed that calcipotriol is superior with respect to clinical efficacy compared to dithranol.[15] Comparing dithranol treatment at the in-patient department with calcipotriol at the in-patient department a tendency for superiority of calcipotriol above dithranol was observed in the majority of the patients in this case-control study. A statistically significant superiority of calcipotriol was

shown with respect to pruritus. These observations challenge the common believe that 24 hour application of dithranol in Lassar's paste or petrolatum is superior to any other topical treatment.

Irritation of lesional and perilesional skin was observed in four patients at the calcipotriol treated sides after one week's treatment. The irritation tended to decrease after two weeks treatment. In contrast to the habituation to calcipotriol[17], dithranol irritation tended to increase after two weeks of treatment. Irritation as a result of dithranol is supposed to be essential for its antipsoriatic effect but is also a less appreciated adverse event. The concentration of dithranol is increased by the tolerance of the individual patient. No correlation could be shown between dithranol irritation and calcipotriol irritation which suggest that the mechanism of irritation is different. It is of interest that those patients who experienced pruritus before treatment were not predisposed to develop irritation to calcipotriol or dithranol. It is striking to see that calcipotriol had a better effect on pruritus than dithranol in this group of patients.

In the present study the second phase of the in-patient treatment after the two-week investigation was difficult to evaluate as the strategy was to individualise treatment using combinations which were most fit for the individual patient.

From this study it may be concluded that calcipotriol treatment at the in-patient department, carried out with care and precision is highly effective to the extent that it challenges the time-honoured 24 hour applications of dithranol. It is attractive to hypothesise that the development of cream formulations and once a day schedules to improve compliance for calcipotriol might diminish the gap between efficacy of home treatment and treatment at the in-patient department.

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4.3. LONG-TERM EFFICACY AND SAFETY OF ONCE DAILY TREATMENT WITH TACALCITOL OINTMENT IN CHRONIC PLAQUE PSORIASIS

4.3.1. Summary

Tacalcitol (Curatoderm 4 μg/g ointment, once daily) has been shown to be effective and safe in the treatment of patients with chronic plaque psoriasis in a double-blind placebo controlled study. A group of 58 patients who had previously responded to tacalcitol, continued therapy with tacalcitol ointment. In the present communication, efficacy, safety and tolerance of this drug are reported during long-term application.

During a continuous treatment period of half a year (22 patients), during a treatment of one year (9 patients) and during a treatment of 60 weeks (3 patients) the degree of clinical improvement maintained comparable to the efficacy confirmed at the end of the double-blind short-term study. By 8 out of the 58 patients irritation of the skin or a burning, itchy sensation was experienced. However, no single patient had to discontinue treatment for this reason.

Safety parameters did not show clinically relevant changes. In particular serum calcium and phosphate and nocturnal urine alpha-1-microglobulin remained unaffected by prolonged treatment.

In conclusion, tacalcitol is a well-tolerated and effective antipsoriatic treatment for long-term control of psoriasis. It is indicated that further long-term efficacy and safety studies are carried out in patients with extensive psoriasis beyond the treatment period of 20 weeks.

4.3.2. Introduction

Vitamin D_3 analogues represent a major innovation of the treatment of psoriasis today.[1-4] During the last 5 years, the analogue calcipotriol (50μg/g) ointment has become a first line therapy for this indication. Calcipotriol treatment is well-appreciated, also for long-term management of psoriasis, although irritation of the skin has been experienced by 25% of the patients and required discontinuation of treatment in approximately 6% of the patients.[5,6]

In Japan, tacalcitol (2μg/g, twice daily) ointment is a vitamin D_3 analogue which has become the mainstay in the routine treatment of psoriasis.[7-10] Irritation following tacalcitol application was recorded in less than 1%.[7-10] The clinical efficacy of this formulation proved to be comparable to twice daily treatment with betamethason valerate 1mg/g ointment.[11]

Recently, an European dose-finding study was carried out to assess the optimal concentration and the clinical efficacy of a once daily application of tacalcitol (0.25-16 μg/g ointment).[12] Concentrations above 4 μg/g did not enhance the antipsoriatic efficacy, whereas lower concentrations

than the optimum 4 μg/g were significantly less effective. A multi-centre placebo-controlled within patient left-right comparative study was performed in 122 patients to investigate clinical efficacy, side-effects and tolerance of this dosage regimen in Caucasian patients with chronic plaque psoriasis.[13] The once daily application of tacalcitol ointment (4 μg/g) for 8 weeks proved to be an effective, safe and well-appreciated antipsoriatic principle.

The objective of the present study was to assess efficacy, safety and local tolerance of tacalcitol ointment 4 μg/g, applied once daily, in the long-term treatment. This study was carried out in patients who had previously participated in the placebo controlled study. In particular the following questions were addressed:

i. Can the clinical efficacy of tacalcitol ointment, as confirmed by the 8-week placebo controlled study, be maintained during a long-term study?
ii. Does long-term use of this vitamin D_3 analogue affect systemic safety parameters?
iii. What is the frequency and severity of irritation of the skin during long-term tacalcitol therapy?
iv. What is the overall judgement of patients and investigators regarding global improvement and usefulness of this treatment?

4.3.3. Materials and methods

An open, multi-centre prospective study was performed to assess efficacy, safety and local tolerance of tacalcitol (4 μg/g ointment, once daily) in the long-term treatment of psoriasis. All patients had been included before in a within-patient left-right placebo controlled study on efficacy and safety of a short-term treatment with the same tacalcitol formulation.[13] Upon completion of the double-blind phase and a follow up period of 4 weeks, each patient, who had responded to tacalcitol before was invited to enter the long-term phase.

Patient selection

Patients with chronic plaque psoriasis were enrolled at 3 academic centres. All subjects had already fulfilled the inclusion and exclusion criteria which were the same as for the 8-weeks placebo controlled study.[13] In brief, patients of either sex, aged between 15 and 80 years, females of childbearing age if they were using adequate contraception and patients with normal serum calcium or phosphate were included. Systemic or topical antipsoriatic therapy, other than tacalcitol treatment, over 2 months respectively 4 weeks prior to the start of the study, was an exclusion criterion. Other exclusion criteria were: serious diseases, known allergy for study medication, medication interfering

with the course of psoriasis or systemic calcium metabolism. All patients gave their written informed consent, prior to the study. Inclusion criteria for the long-term evaluation were: location of psoriatic lesions anywhere except the scalp for test-area, male and female, only post-menopausal females or females in fertile age giving written consent not to become pregnant during this study, patients who have given informed consent, patients who finished the right-left comparison on tacalcitol with a satisfactorily therapeutic result and who are interested in treating their psoriatic lesions with tacalcitol furthermore. Patients were permitted to treat the psoriatic lesions with a maximum of 20 tubes à 100 gr. per patient. The patients were permitted to discontinue treatment after a minimum of 12 weeks treatment. The maximum treatment period was 60 weeks. The patients had to be treated for at least 12 weeks; otherwise they were regarded as drop out. Patients discontinued treatment for reason of clearing, side-effects or on their own initiative.

Treatment

During the long-term phase all psoriatic plaques (except on the scalp) could be chosen as test areas. A maximum of 20 g tacalcitol ointment was allowed once daily.

Study Medication

The test drug tacalcitol ointment contains the active ingredient 1,24-dihydroxy-cholecalciferol (tacalcitol) at a 4 μg/g concentration and the inactive ingredients paraffin oil, di-isopropyl adipat and white petrolatum. The preparations were filled in 100 g-tubes. Patients received a 4-weeks supply of study medication at start of the long-term period and again every four weeks

Efficacy assessments

At the start of the study (week 0) and subsequently every fourth week, clinical severity parameters were assessed by the investigator. The extent of the psoriatic test area was recorded as percentage of the total body surface and assessed at the baseline-visit and at regular visits. The symptoms: erythema, infiltration and scaling were recorded using a 5-point scale from 0 = 'none' to 4 = 'extremely severe'. At each visit, the symptoms erythema and scaling were compared to their initial severity. This condition was rated as 'deteriorated', 'unchanged', 'slightly improved', 'moderately improved', 'markedly improved' or 'cured'. At the end of the study, a global assessment of efficacy was made by the investigator and by the patient. The assessment of efficacy was rated as 'very good', 'good', 'moderate' or 'insufficient'. An assessment of usefulness was given by the patients at the end of the treatment on a 10-point scale from the 1 = 'not useful at all' up to 10 = 'extremely useful'.

Safety assessment

The occurrence of any adverse event was recorded at each visit. These events were evaluated for duration, severity (slight, moderate or severe) and a possible relation to disease or drug. In particular, the investigator had to pay attention to any signs of irritation, skin rashes and their location, extent and severity.

A global assessment of tolerance was given at the end of the treatment by the investigator and by the patient, rated as 'very good', 'good', 'moderate' or 'insufficient'. The patient's general condition was recorded at start of and at each visit during the long-term study. The assessment was rated as 'very good', 'good', 'moderate' or 'insufficient'. Clinical laboratory evaluation was carried out at baseline and at each visit. The haematology comprised counts of erythrocytes, leukocytes, platelets, haemoglobin, haematocrit, the biochemistry, serum calcium, inorganic phosphate, creatinin, ASAT, alkaline phosphatase and LDH. In one centre nocturnal urine alpha-1-microglobulin was assessed additionally.

Statistical analysis

Changes from the beginning of the long-term study were given by P-values of the Sign Test and by the Mann-Whitney statistics. The Mann-Whitney statistics was used to measure the size of the magnitude of this change:

P (during treatment > before treatment) = 0.50 indicates equality of the two series, P (during treatment > before treatment) = 0.56 implies a small effect, P (during treatment > before treatment) = 0.64 a medium sized effect and P (during treatment > before treatment) = 0.71 indicates a large effect.[14]

4.3.4. Results

Demographic data

In total 58 patients, 18 women and 40 men, entered the long-term efficacy and safety study. The average age was 45 years, ranging between 19 and 78 years. Table I summarises demographic details in each of the centres.

Table I Demographic data

	Number of patients	male/female	Age (mean ± SD)	Weight (kg) (Mean ± SD)	Height (cm) (Mean ± SD)
Centre 1	42	29/13	45 ± 10.9	80.8 ± 13.4	176.3 ± 7.8
Centre 2	7	4/3	36.4 ± 13.9	76.1 ± 16.3	174.1 ± 12.4
Centre 3	9	7/2	47.1 ± 8.6	80.1 ± 13.4	177.1 ± 9.2
Total	58	40/18	44.5 ± 11.2	80.1 ± 13.6	176.1 ± 8.6

Patients details

All patients suffered from chronic plaque psoriasis. At the beginning of the previous double blind phase, the duration of psoriasis since the first outbreak was 225 ± 157 months (mean ± SD) and since the last attack 130 ± 80.4 months (mean ± SD). The time interval between the end of the double-blind phase and start of the long-term study was 4.4 ± 2.6 weeks (mean ± SD). The extent of the psoriatic lesions at the beginning of the double-blind phase was 9.7% ± 3.3 (mean ± SD) and at the beginning of the long-term study 8.6% ± 3.9 (mean ± SD). At the beginning of the double-blind phase the sumscore was 8.9 ± 1.5 (mean ± SD) and at the beginning of the long-term study 7.9 ± 2.1 (mean ± SD).

Disposition of patients

Out of the patients, who had been included in 14 centres during the double-blind phase, in total 58 were enrolled at the three centres for the long-term efficacy and safety evaluation.

Table II Reasons for termination of long-term treatment

Reason for termination	Number of patients (%)
According protocol	43 (74%)
Insufficient efficacy	7 (12%)
Prohibited concomitant therapy*	3 (5%)
Non-compliance	2 (3%)
Other	3 (5%)

*Topical corticosteroids (n=2) and non-steroidal anti-inflammatory drugs (n=1)

The duration of the long-term efficacy study for all patients was 24.8 ± 17 weeks (mean ± SD). Table II summarises reasons for termination of the long-term treatment. In no single patient, clearing was the reason for termination. In 5 patients the test drug was not applied permanently throughout the study and in 6 patients the last examination was 1-4 weeks following the discontinuation of the treatment. No correlation was observed between the severity scores before start and the subsequent duration of the long-term treatment.

Patients who had a more extensive involvement of body surface were treated relatively shorter compared to patients with a more moderate involvement (figure 1). The correlation was statistically significant ($r = 0.32$; $p < 0.01$).

Clinical efficacy

The percentage of bodysurface involved with psoriasis decreased during the first 36 weeks of the long-term treatment. Already after 4 weeks a highly significant reduction of the affected test areas

was reached compared to the baseline (p = 0.0013). It should be noted that the extent of psoriatic lesions at start of the long-term study was lower compared to the extent of lesions before start of the double blind phase. Figure 1 illustrates the reduction of the extent of lesions during treatment compared to the pre-treatment values for each subpopulation still on active treatment at the time of each visit.

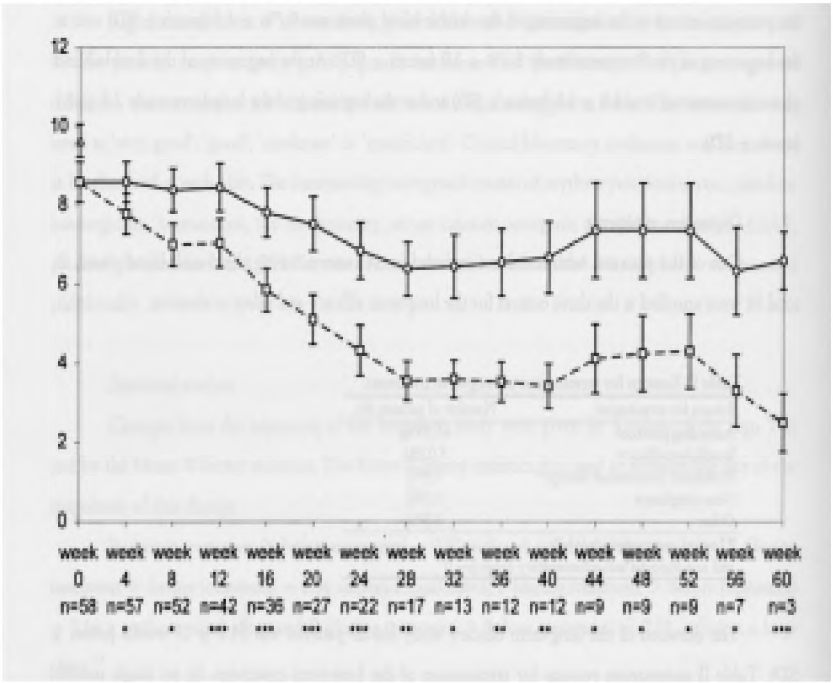

Fig. 1 The extent of psoriatic lesions before treatment (-O-), during treatment (- -□- -) and before the previous double blind phase (-Δ -) * P ≤ 0.005, ** P ≤ 0.0001, *** P ≤ 0.00001.

The sumscore defined as the sum of the scores for erythema, infiltration and scaling decreased throughout the long-term study by 2 score points. The reduction of the sumscore compared to the baseline sumscore at the start of the placebo-controlled study (8.9 ± 1.5) was 3 score points. After 8 weeks treatment the maximum improvement of the sumscore was reached and maintained during subsequent treatment. According to the Mann-Whitney statistics, this reduction of the sumscore can be considered as a large effect ($p \leq 0.0001$; P (during treatment > before treatment) ≥ 0.84). Erythema, induration and scaling all decreased during the treatment period.

Global assessment of efficacy by investigators and patients is summarised in table III. In 84% the assessments of investigator and patient were conform. The usefulness of the therapy, as assessed by the patients on an analogue 10-point scale (0-10) was assessed as 7 (median).

Table III Global assessment of efficacy by investigator and patient (total N = 58)

Efficacy Assessment	Assessment of efficacy by			
	Investigator	**%**	**Patient**	**%**
very good	6	10.3	9	15.5
good	28	48.3	24	41.4
moderate	21	36.2	19	32.8
insufficient	3	5.2	6	10.3
valid number	58	100	58	100

Adverse events/unwanted events

For 15 out of the 58 patients adverse events were reported. Table IV summarises these events. All extracutaneous adverse events were considered to be unrelated to psoriasis or study medication.

Table IV Adverse events in 58 patients (n.s. = not specified)

Unwanted event	Number of patients	Severity			
		mild	moderate	severe	n.s.
Headache	2	-	2	-	-
Abdominal pain	1	-	-	1	-
Myalgia	1	-	1	-	-
Costal confusion	1	-	1	-	-
Pain left hip	1	-	1	-	-
Common cold	1	1	-	-	-
Flu, sinusitis	1	-	-	1	-
Erysipelas	1	-	-	1	-
Burning sensation	3	-	3	-	-
Itching, pain	3	1	-	2	-
Irritation	2	1	1	-	-
Urticaria	1	-	-	-	1

Burning sensation (3), itching/pain (3) and irritation (2) were considered to be due to either psoriasis or the study medication. These symptoms did not increase in severity during treatment. In 2 patients these cutaneous adverse events were considered as severe, in 4 as moderate and in 2 patients as mild. In none of the patients treatment was discontinued for reason of these events.

Table V Global assessment of tolerance by investigator and patient (Total N = 58)

Tolerance Assessment	Assessment of tolerance by			
	Investigator	**%**	**Patient**	**%**
very good	36	62.1	34	58.6
good	21	36.2	22	38.0
moderate	1	1.7	1	1.7
insufficient	-	-	1	1.7
valid number	58	100	58	100

The global assessment of tolerance by the investigators and patients is summarised in table V. The investigators and patients considered tolerance as good or very good in 98% and 96% of the patients respectively. The general condition of the patients was considered as good throughout the long-term study.

Laboratory assessments

Laboratory investigations did not reveal any change of clinical importance. The sign test did not show a significant change of the haematology values compared to baseline except for a transient increase of platelets after 12 weeks ($p = 0.004$) and after 52 weeks ($p = 0.04$). With respect to biochemistry values, the sign test indicated no abnormalities except for an increase of ASAT after 20 weeks ($p = 0.03$). The levels of serum calcium, phosphate, creatinin and nocturnal urine alpha-1-microglobulin are illustrated in figure 2a-d. In no single patient hypercalcaemia was observed. Except for serum calcium after 8 and 20 weeks of treatment, no statistically significant change in serum calcium levels was demonstrated. The Mann-Whitney statistics indicated positive effects, i.e. a decrease of serum calcium during treatment compared to the levels at the beginning of the long-term study.

In one patient serum phosphate had increased up to 1.5 mmol/l. After 36 weeks, serum creatinin increased above baseline according to the sign test. In 8 patients, serum creatinin levels had been above normal between start and study week 12. The sign test had not revealed any change from baseline at any time of the long-term investigation with respect to nocturnal urine alpha-1-micro-globulin. In one patient, who suffered from hypertension since 1980, values above normal were seen throughout the long-term study, except at weeks 12 and 24.

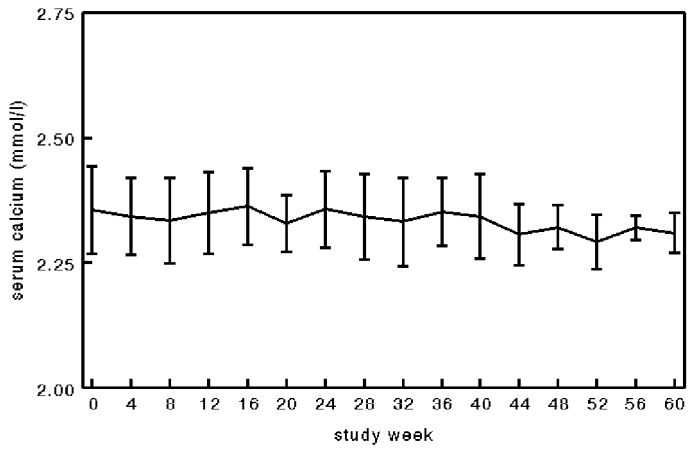

Fig. 2 a Serum calcium during treatment with tacalcitol.

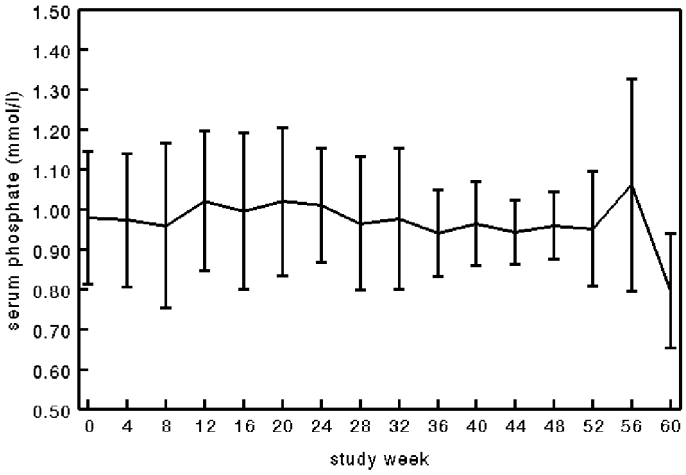

Fig. 2 b Serum phosphate during treatment with tacalcitol.

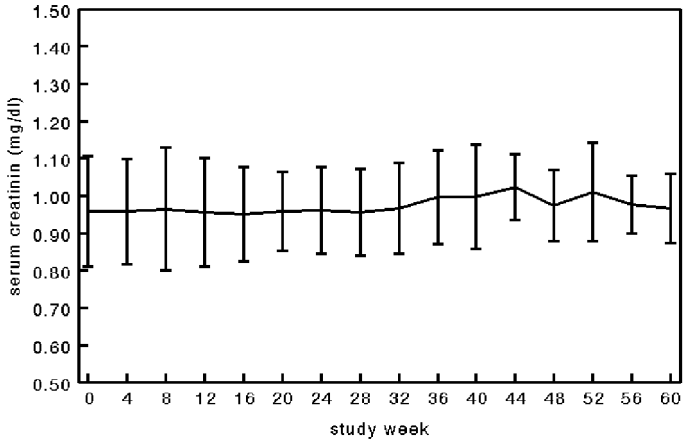

Fig. 2 c Serum creatinin during treatment with tacalcitol.

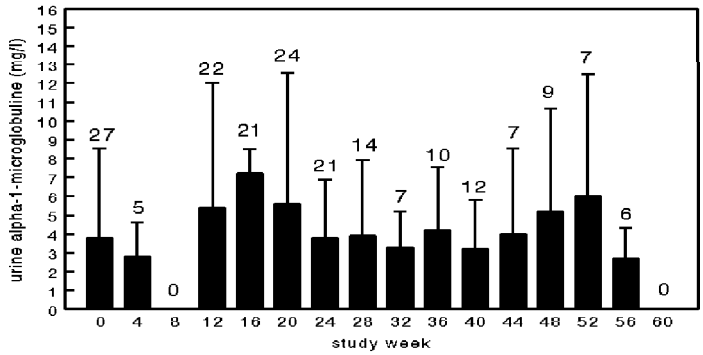

Fig. 2 d Nocturnal urine alpha-1-microglobulin during treatment with tacalcitol.

4.3.5. Discussion

The present study demonstrates that tacalcitol has a maintained efficacy antipsoriatic during long-term treatment and is well-tolerated.

Out of 14 centres which participated in the initial placebo controlled study, 3 centres carried out the long-term efficacy and safety study. Out of the patients who had previously been included in these three centres, 58 patients wanted to continue the treatment with tacalcitol. Such a desire is in line with the facts that 60% of the patients indicated that the efficacy was good to very good and that 95% of the patients indicated the tolerance as good or very good. Nearly half of the patients (n = 26) remained on continuous treatment for more than 20 weeks with a maximum treatment period of 62 weeks (n = 3). All but 5 patients were on continuous treatment without intermissions. A statistically significant correlation, was observed between the extent of psoriatic lesions before treatment and the duration of the treatment.

At week 28, the most pronounced reduction of the extent of psoriatic lesions was observed. However, the reduction of psoriatic lesions was statistically significantly less in those patients with a more extensive involvement. A possible explanation might be that accurate ointment applications in extensive psoriasis are more difficult and that the intensity of ointment applications is critical during treatment with tacalcitol.

The efficacy assessment during the present long-term study is uncontrolled. The placebo controlled efficacy assessment has been reported before.[13] The percentage of bodysurface involved

with psoriasis reduced by only 1% during the placebo controlled study. In the present study, however, the area of involvement reduced substantially, which reached statistical significance from week 4 onward ($p \leq 0.001$) and became more apparent after 12 weeks of treatment ($p \leq 0.00001$). The reduction of the sumscore compared to the baseline score before starting the double blind phase was on average 4 score points during the 8 weeks short-term study, whereas the reduction during the long-term study in comparison to the score at the start of the double blind phase was 3 score points. Therefore, prolonged treatment does not improve the sumscore of the individual beyond the reduction as reached during an 8 weeks' treatment. However, prolonged treatment causes a significant reduction of the body surface of psoriatic involvement. The maintained antipsoriatic efficacy without signs of habituation is in line with the efficacy characteristics of long-term administration of the vitamin D_3 analogue calcipotriol.[5,6] So far, a quantitative comparison of clinical efficacy and safety between different vitamin D_3 analogues is not possible as data are not available. Long-term treatment with tacalcitol ointment was judged by investigator and patients as good or very good in 58% and 57% of the patients respectively. Long-term treatment with this drug in the present series of patients can be regarded as a safe approach. There proved to be no interference with the general condition. Extra-cutaneous side-effects (table IV) were not considered to be drug related. Haematological and clinical biochemistry investigation did not reveal any clinically significant changes. In particular, serum calcium, phosphate and creatinin remained in the normal range. Recently nocturnal urine α-microglobulin (protein HC) proved to be a valuable marker for tubular dysfunction.[15,16] Tubular dysfunction is an early feature of calcium deposition in the parenchyma of the kidney. This parameter remained unaffected during the study. In conclusion, tacalcitol treatment in the present study was free of systemic side-effects.

Irritation of the skin has been reported regularly for various vitamin D_3 analogues.[17-19] However, irritation during treatment with low dose tacalcitol in psoriasis has been reported to be extremely rare in the Japanese psoriatic patients.[7-11] In the present study in Caucasian patients, irritation of the skin was observed in 12% of the patients. In no single patient, however, the irritation required discontinuation. No single patient experienced aggravation of irritation during continuation of tacalcitol treatment. The global tolerance of tacalcitol was judged as very good or good by 96% and 98% of the investigators and patients respectively. The convincing tolerance of tacalcitol ointment is in line with the Japanese experience.

The usefulness of tacalcitol ointment was judged to be 7 on a 10 point scale. This implies that tacalcitol is not the panacea of a highly effective antipsoriatic approach but rather represents a well-appreciated once daily treatment with a medium, for long-term control maintained, antipsoriatic

efficacy. The excellent local tolerance suggests that tacalcitol treatment might be indicated for the treatment of the face and flexures. Further studies are indicated to compare and contrast the clinical efficacy and safety profile of tacalcitol with other vitamin D_3 analogues and to study the safety in patients with extensive psoriasis during a long-term study.

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Chapter 5

General discussion

5.1. INTRODUCTION TO DISCUSSION

In the general introduction, three aims were defined in order to further develop our knowledge on the in vivo mode of action and clinical efficacy and safety of topical treatment of psoriasis. These aims were:

I. To study the in vivo effects of established topical antipsoriatic treatments on epidermal proliferation and differentiation in the psoriatic plaque.
II. To develop a new model to study the induction of recruitment of cycling cells and proliferation associated epidermal differentiation in human skin in vivo and to study the effect of topical antipsoriatic treatment on such a model.
III. To study efficacy and safety aspects of new and existing topical treatments for psoriasis.

The various questions were dealt with in the individual chapters. Here we will integrate these findings with the knowledge available in literature.

5.2. IN VIVO EFFECTS OF TOPICAL THERAPY ON THE PSORIATIC PLAQUE

Various studies have been undertaken to study cell-biological changes in the psoriatic lesion during treatment. In a previous thesis, epidermal growth (Ki-67 staining and keratin 16 expression) and inflammation characteristics (T-lymphocytes, elastase positive cells, Langerhans cells and monocytes/macrophages) were assessed during treatment with the vitamin D_3 analogue calcipotriol, the corticosteroid budesonide and dithranol in an emulsifying ointment.[1] The thesis 'Dynamics of epidermal growth and keratinization in psoriasis' comprised the study of differentiation markers filaggrin, involucrin and transglutaminase during monotherapy with hydrocolloids and experimental vitamin D_3 analogues.[2]

The present thesis comprises the effects of available and some new topical treatments and combinations of treatments on differentiation markers and in addition, the proliferation marker Ki-67, T-lymphocytes and polymorphonuclear leukocytes (PMN). The results of these and previous studies are summarised in table I.

Table I Simplified presentation of the in vivo effects of topical antipsoriatic treatments (↓ or ↑: < 10 % change; ↓↓ or ↑↑: 10-50 % change; ↓↓↓ or ↑↑↑: > 50% change; S: synergistic response (more than the sum of the individual treatments); =: no effect; nd: not done; HCD: hydrocolloid dressing. [1-3,present thesis]

	Ki-67	Ks8.12	involucrin	filaggrin	TGase	T-cells		PMN	
						dermis	epidermis	dermis	epidermis
dithranol (classic)	↓↓	↓↓↓	=	↑↑↑	↓	↓	↓↓	↓↓	↓↓↓
dithranol (Micanol)	=	nd	=	=	=	↓↓↓	↓↓↓	↓↓↓	↓↓↓
calcipotriol	↓↓↓	=	↑	↑↑↑	↓	↓	↓↓	↓↓	↓↓↓
calcitriol	↓↓↓	nd	↓	↑↑↑	↓	↓↓	↓↓	↓↓↓	↓↓↓
tacalcitol	↓↓↓	↓↓↓	↓↓	↑↑↑	↓↓	↓	↓↓	↓↓↓	↓↓↓
HCD	=	↓	↓	↑↑↑	nd	↓	=	=	=
corticosteroids	↓↓↓	↓↓	↓	↑↑	↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓
UVB	↓↓↓	nd	↓	↑↑↑	↓↓	↓↓↓	↓↓↓	=	=
Micanol+UVB	↓↓↓-S	nd	↓	↑↑-S	=	↓↓↓-S	↓↓↓-S	↓↓↓-S	=

Most topical treatments proved to decrease the number of Ki-67 positive keratinocytes, indicating a decreased proliferation rate. A consistent change in the differentiation pattern was seen during all treatments: an increased filaggrin content, a decrease of the percentage of transglutaminase positive cell layers and a decrease of the number of keratin 16 positive cells. Differences between different therapies were predominantly observed with respect to the reduction of the percentage of T-lymphocytes and PMN in the dermal infiltrate and in the epidermis.

Dithranol

Treatment with dithranol in the classical vehicles had major effects on epidermal proliferation and differentiation and only minimal effects on inflammation markers. Therefore dithranol can be regarded as a treatment that affects the epidermis more selectively. However, the anti-inflammatory potential seems to be influenced by the formulation of the dithranol preparation to a large extent. The classical vehicle of petrolatum,[4] the Dithranol Hermal-AW ointment[5] and the cream base used in the department for intensified out-patient therapy (chapter 2.2) proved to have minimal anti-inflammatory effects, whereas dithranol in the monoglyceride formulation had a profile of changes with a predominantly anti-inflammatory effect (chapter 2.3).

Vitamin D_3 treatments

Treatment with calcipotriol (chapter 2.1) and other vitamin D_3 analogues[2] had prominent effects on epidermal proliferation and differentiation. With respect to cutaneous inflammation, an important reduction of the number of PMN was observed during treatment with only minimal effects on the percentage of T-lymphocytes in the dermal infiltrate. This therapeutic response pattern suggests that a reduction of the number of T-lymphocytes is not a 'conditio sine qua non' for a therapeutic effect in psoriasis.

Hydrocolloid dressings

Hydrocolloid dressings as a monotherapy have a modest effect on cell-biological parameters of the psoriatic plaque: a reduction of keratin 16 expression and involucrin and an increase of filaggrin, although clinical parameters hardly indicate any clinical improvement.[3,6,7] Therefore, hydrocolloids are not suitable as a monotherapy. In combination with topical antipsoriatics, however, weekly dressing changes with a topical antipsoriatic under occlusion of a hydrocolloid dressing provide a new pharmacological principle with an intensified action of the topical antipsoriatic, increasing its bioavailability, decreasing its dose and providing optimal patient compliance (chapter 2.4).[7]

Topical corticosteroids

Topical corticosteroids (clobetasol (chapter 2.4) and budesonide[8]), on the whole, had a broad effect on all aspects of epidermal growth, differentiation and inflammation. Topical corticosteroids can therefore be regarded as a broad spectrum antipsoriatic treatment. In addition, the effects of topical corticosteroids proved to be rapid. Treatment by clobetasol-17-propionate lotion under hydrocolloid occlusion had even a faster clearing capacity and the same relapse characteristics compared to conventional twice daily application of clobetasol-17-propionate ointment without occlusion (chapter 2.4).

Phototherapy

Phototherapy (UVB) had a broad effect on all aspects of epidermal growth, differentiation and inflammation, except for PMN accumulation (chapter 2.3). Phototherapy therefore can be regarded as therapy that interferes with both epidermal processes and immune mechanisms.[9] Combination of dithranol and phototherapy (UVB) can be regarded as a synergistic treatment. The combination resulted in an improvement which was significantly more than the sum of the improvements of the individual treatments.

Conclusions

Based on the immunohistochemical observations it can be concluded that:

I. Topical corticosteroids and phototherapy (UVB) are both immunosuppressive treatments and also modulators of epidermal growth and differentiation.
II. Calcipotriol treatment is a more selective treatment with prominent effects on epidermal growth, differentiation and epidermal PMN accumulation, but with minimal effect on T-cell accumulation.
III. Dithranol is a selective antipsoriatic therapy with only minimal anti-inflammatory capacity. However, Micanol, dithranol micro-encapsulated in crystalline monoglycerides, proved to have a substantial anti-inflammatory effect.

5.3. IN VIVO MODELS FOR PSORIASIS

In vivo models for epidermal proliferation permit studies on the induction of the recruitment of cycling epidermal cells and the associated changes in the process of keratinocyte differentiation in vivo. The increased recruitment of cycling epidermal cells is the key-feature of psoriatic hyperproliferation of the epidermis.[10-12] In addition, in vivo models provide a 'standardised lesion' in contrast to the heterogeneity that characterises a genuine psoriatic lesion.

The effect of tape-stripping has often been used as a model to mimic certain aspects of the psoriatic lesion. This technique involves the removal of the stratum corneum by repeated applications of adhesive tape. The tape-stripping model is well-reproducible and non-invasive and results in a hyperproliferative response, slight cutaneous inflammation and a psoriasis-like epidermal differentiation pattern.[13] The model is adequate for studies on the in vivo effect of systemic drugs on epidermal growth and differentiation. A specific effect of systemic antipsoriatics could be shown by acitretin, in contrast to absence of such interference by cyclosporin.[14,15]

Previously, various experiments have been carried out to study the effect of topical antipsoriatic treatments on epidermal proliferation following tape-stripping. Calcipotriol proved to inhibit ornithine decarboxylase activity following tape-stripping.[16] Ornithine decarboxylase is the rate limiting enzyme of the polyamine synthesis which is pivotal in epidermal proliferation.[17] Other experiments showed that topical corticosteroids delay the wave of hyperproliferation following tape-stripping.[18,19] In these experiments, proliferation was assessed by measuring the percentage of cells in SG_2M phase using flowcytometry.

One could speculate that, from a pharmacological point of view, the tape-stripping model is less appropriate for topical drugs because of the vehicle effect. The vehicle will to some extent compensate for the removal of the stratum corneum (skin barrier) in tape-stripping and as such influence the process of regeneration. Another problem is that the removal of the skin barrier will provide a totally different bioavailability of topical drugs as compared to the unstripped skin. Therefore the search for alternative models to study the effect of topical treatments is indicated.

In the present thesis, a new in vivo skin model was developed (chapter 3.1). This model was obtained by irradiation of normal human skin with an intermediate dose of one MED (minimal erythema dose) UVB which resulted in a response pattern that is comparable with a psoriasis-like epidermal hyperproliferation and differentiation and did not result in a major inflammatory infiltrate or epidermal cell destruction. The UVB model was used to study the effect of topical antipsoriatic

treatments. A study was carried out on the interference of topical antipsoriatics with epidermal proliferation and differentiation using an UVB skin challenge (chapter 3.2). Clobetasol-17-propionate proved to inhibit recruitment of cycling epidermal cells, involucrin expression, transglutaminase expression and keratin 16 expression following UVB challenge. In contrast, calcipotriol did not modulate the induction of these aspects of proliferation and epidermal differentiation, apart from the inhibition of the number of transglutaminase positive cell layers. So far, no information is available on the effect of systemic treatments on UVB induced hyperproliferation.

From the interference of antipsoriatic treatments -topical and systemic- with trauma (tape-stripping) or UVB induced hyperproliferation, it can be concluded that acitretin and topical corticosteroids inhibit the recruitment of cycling cells and associated epidermal differentiation in vivo. Cyclosporin treatment has no effect on the induction of epidermal hyperproliferation in vivo in the tape-stripping model, in contrast to its growth inhibitory effect in vitro.[20-22] No information on the modulation of the induction of epidermal hyperproliferation is available so far for dithranol, photo(chemo)therapy and methotrexate.

Calcipotriol inhibits trauma induced hyperproliferation.[18,19] However, in UVB induced proliferation, calcipotriol was not effective in this respect. It is my own experience that unstable psoriasis, that is characterised by spreading lesions and pin-point papules, responds less to calcipotriol compared to chronic plaque psoriasis. This might explain the disability of calcipotriol to modulate the 'new lesion' induced by UVB radiation. An alternative explanation for the absence of an effect of calcipotriol an UVB inflammation is the relatively short application period of calcipotriol in the experimental model.

5.4. THE TOPICAL THERAPY OF PSORIASIS: AN UPDATE

5.4.1. Dithranol based therapies

Dithranol, introduced by Galewski and Unna more than 80 years ago, remains to be a very important treatment for psoriasis.[23] An important development is the short contact principle. Short contact applications enable a decreased penetration in the uninvolved skin of the psoriatic patient whilst having an adequate bioavailability in the psoriatic plaques.[24] The short contact treatment using dithranol in a cream that can easily be washed off, has increased the possibilities of the use of dithranol at home.

Dithranol micro-encapsulated in crystalline monoglycerides (Micanol) is a new development.[25] In a comparative study over eight weeks between Micanol monotherapy, Micanol combined with UVB phototherapy and UVB combined with the placebo of Micanol, a complete clearance of psoriasis was seen in respectively 29%, 54%, and 46% of the patients.[26]

The results of the dithranol short contact therapy depend on the intensity of the treatment and compliance of the patient. Table II shows that a complete clearance in 62% of the patients is reached by short contact applications of dithranol at the in-patient department. At the out-patient department, only 10% of the patients reached a complete clearance if the control visits were carried out at 1-3 weeks intervals.

Table II Clearance during short contact therapy with dithranol

Number of visits	Base	Literature	% of cleared patients
In-patient treatment	Emulsifying ointment	Paramsothy et al.[27]	62
Three times weekly	Micanol	Gerritsen et al.[26]	53
Three times weekly	Psoricreme® or petrolatum with salicylic acid	de Mare et al.[28]	50
Once weekly	Psoricreme® or petrolatum with salicylic acid	de Mare et al.[28]	33
Once weekly	Micanol	Gerritsen et al.[26]	29
Once per 1-3 weeks	Petrolatum with salicylic acid	Hindrycks et al.[29]	10

A new concept is the intensified out-patient treatment in which patients are treated and instructed by specially trained nurses with short contact dithranol cream applications in order to carry out this treatment at home.[30] One study showed that in intensified out-patient treatment, a 70%-reduction of the PASI-score takes more time compared to in-patient treatment with 24 hour applications of dithranol but takes significantly less time compared to UVB phototherapy.[30] At the moment an extensive, comparative, multi-centre study is being carried out on the cost-effectiveness and patient satisfaction of these three treatment possibilities.

In the present thesis, it was shown that the cream base (chapter 2.2) and the monoglyceride suspension (chapter 2.3) proved to be well-tolerated, proved to have a substantial antipsoriatic effect and could be washed off easily. The cream formulation, as monotherapy, approached the efficacy of classical in-patient treatment, although a prolonged treatment period was required to reach this result.[30] Dithranol in the monoglyceride formulation, however, proved to be more adequate as an adjunct treatment to UVB.[26]

5.4.2. Vitamin D_3 treatments

The last decade, vitamin D_3 analogues have been shown to have an important antipsoriatic effect. Recently, vitamin D_3 analogues were introduced as a routine treatment in dermatology. The mode of action of these derivatives is pluriform and comprises inhibition of cellular proliferation, enhancement of normal epidermal differentiation (formation of cornified envelopes, enhancement of transglutaminase activity and transcription of involucrin) and modulation of inflammatory events.[31]

Calcipotriol

Calcipotriol was introduced in the Netherlands in 1992. In comparison to calcitriol, the naturally occurring hormone (1,25-dihydroxy vitamin D_3), calcipotriol has 100-200 times less effect on the calcium metabolism after systemic administration in animals. Calcipotriol has become the therapy of first choice in mild to moderate psoriasis.

Calcipotriol ointment (50µg/g ointment), applied twice daily, has a marked effect in 80% of the patients. Irritation of the skin is seen in approximately 25% of the patients; 5% of the patients has to discontinue treatment because of skin irritation.[32-34] During a large long-term study, a clinically relevant increase of serum calcium and phosphate was not seen; the amount of ointment applied in these studies did not exceed the maximal dose of 100 grams per week.[34] From this study it can be concluded that the long-term treatment with calcipotriol is a safe and effective therapeutic principle.

Calcipotriol is a topical treatment with important possibilities for combination therapy and the combination of calcipotriol with other antipsoriatic therapies is a major step forward in the treatment of psoriasis. The effects of calcipotriol in vivo are mainly on epidermal proliferation, differentiation and accumulation of PMN. Cyclosporin, on the other hand, mainly has immunosuppressive effects. In a group of 69 patients, treated in a multi-centre study, with a low dose of cyclosporin (2 mg/kg/day) either or not combined with topical calcipotriol, an

improvement of the PASI-score of more than 90% was experienced in 50% of the patients treated with the combination of cyclosporin and topical calcipotriol ointment twice daily. Only 12% of the patients reached such an improvement using cyclosporin monotherapy without calcipotriol.[35] At the moment no comparable studies concerning methotrexate or acitretin are available.

The combination of calcipotriol and UVB phototherapy is more effective compared to calcipotriol monotherapy.[36-38] Calcipotriol enables a marked reduction of the UVA dose in PUVA therapy.[39] The therapeutic effect of the combination of calcipotriol and medium strength topical corticosteroids does not differ substantially from both monotherapies.[12,40] However the combination of calcipotriol and betamethasone dipropionate has been demonstrated to be more effective compared to each treatment as monotherapy.[41]

On the whole, 80% of the patients has a satisfactory response to calcipotriol.[32-34] Not only patients with psoriasis of limited extent benefit from calcipotriol. It was shown in chapter 4.2 that calcipotriol can also be effective in patients with extensive psoriasis, provided that optimal compliance is realised. In combination with cyclosporin, calcipotriol proved to be very effective as adjuvans in the management of erythrodermic psoriasis (chapter 4.1).

Tacalcitol

A limitation of the current calcipotriol therapy is irritancy which, in principal, excludes treatment of the face. Low irritancy was claimed by Japanese investigations for an alternative vitamin D_3 derivative: tacalcitol (1,24(R)-dihydroxy vitamin D_3). Tacalcitol (2 μg/g ointment; applied twice daily) has been reported to be effective in 80% of the patients resulting in a marked improvement.[42] In a left-right comparative study, it was established that tacalcitol is more effective compared to treatment with betamethason-17-valerate ointment and tacalcitol proved to have only a minor irritating potential in less than 1% of the patients.[43]

Recently, efficacy and safety of tacalcitol were investigated in Europe.[44] A dose-finding study on tacalcitol ointment was carried out, using once daily applications. This study revealed similar antipsoriatic effects of treatment with ointment containing 4 μg/g tacalcitol compared to ointment containing 16 μg/g tacalcitol. Ointment with less than 4 μg/g of the active substance demonstrated a substantially decreased therapeutic efficacy.[44]

In a multi-centre study, the therapeutic efficacy, safety and tolerability of tacalcitol ointment (4 μg/g) compared to placebo ointment were investigated. The ointment was applied once daily in Caucasian patients with psoriasis during eight weeks in a left-right comparative study.[40]

Hundred-twenty-two patients from different centres were included in the study. Already after a treatment period of two weeks, a highly significant difference in the clinical scores was observed between patients treated with tacalcitol ointment compared to patients treated with the vehicle. The clinical relevance was also apparent: 60% of the patients judged the treatment as good or very good. During the eight weeks of treatment, no changes were seen in the serum calcium concentration. Signs of skin irritation were seen in 12% of the patients. Only in one patient, the therapy was discontinued due to irritation. This considerable difference in irritating potential between the European and Japanese study can be explained by the difference in concentration of the tacalcitol ointment but also by the fact that far more emphasis is laid in Europe on the irritating potential of vitamin D_3 analogues. The European multi-centre study indicates that tacalcitol ointment in a concentration of 4 μg/g applied once daily is an effective therapy with only a relatively small irritation potential.[40]

In the present thesis, it was shown that tacalcitol was well-tolerated and safe, also during prolonged treatment (chapter 4.3). It is feasible that tacalcitol will broaden the vitamin D_3 principle to the treatment of the face and flexures.

5.4.3. Hydrocolloid dressings

Since the seventies, it has been known that occlusive dressings have an antipsoriatic potential.[45] Plastic foil as an occlusive dressing proved to have an irritating and macerating effect during prolonged applications. A hydrocolloid dressing, however, can be worn for a whole week without having such side-effects. In contrast to daily dressing changes or daily ointment or cream applications, an optimal compliance is guaranteed using occlusive therapy with once weekly dressing changes. Although monotherapy with hydrocolloid dressings has been proven to be beneficial in psoriasis, this effect is only minor.[7] The combination of a hydrocolloid dressing with a topical corticosteroid, on the other hand, is very effective, particularly in localised, recalcitrant psoriasis.[46,47] The hydrocolloid dressing represents an interesting pharmacological principle because of the prolonged effect of a single once weekly application of a topical corticosteroid. Calcipotriol under occlusion of a hydrocolloid dressing has also been proven to be more effective compared to calcipotriol monotherapy and as effective as clobetasol under occlusion although, in the latter study, calcipotriol was more irritating than the corticosteroid.[48,49] Further studies are needed to establish the relevance of this principle for other topical treatments for psoriasis.

The present thesis demonstrated that the relapse and the post-treatment remission characteristics following discontinuation of treatment with clobetasol-17-propionate in conjunction with a hydrocolloid dressing, were analogous to treatment with twice daily applications of clobetasol-17-propionate ointment without a hydrocolloid (chapter 2.4). There is no indication that a faster remission of the combination of a hydrocolloid in conjunction with a corticosteroid is accompanied by a shorter remission period compared to corticosteroid monotherapy. Also no differences in atrophogenicity between both treatment modalities could be observed (chapter 2.4).

5.5. GENERAL CONCLUSION AND SUMMARY

In chapter 1 of the present dissertation, a review is given about general aspects of the skin-disease psoriasis like: clinical features, histopathology, aetiology and current therapeutic possibilities. The subject of focal interest in the present thesis is the topical treatment of psoriasis. Lately progress has been made on this territory, mainly on dithranol based therapies, treatment with vitamin D_3 analogues and the principle of topical therapy under hydrocolloid occlusion.

The research that underlies this dissertation contains, besides an analysis of the clinical efficacy and safety of several topical antipsoriatics, an assessment of immunohistochemical effects of topical antipsoriatic therapies on psoriatic skin and also on a new model for a developing psoriatic lesion (UVB-model). Primarily, epidermal differentiation processes were studied with the following markers: involucrin, transglutaminase, filaggrin and cytokeratin 16. Markers for epidermal proliferation (Ki-67) and cutaneous inflammation (T-lymphocytes and polymorphonuclear granulocytes) were studied as well. At the end of chapter 1 the following questions were formulated:

1. What are the immunohistochemical effects of different topical antipsoriatics?
2. What are the effects of different topical antipsoriatics on a new in vivo model for a developing psoriatic lesion?
3. What is the clinical efficacy and safety of new (combinations of) topical antipsoriatics?

In chapter 2, a consistent pattern of in vivo effects of topical treatments on epidermal differentiation in psoriasis was found: a decrease of the percentage of involucrin and transglutaminase positive cell layers, a reduction of keratin 16 positive cells and an increase of the number of filaggrin positive cells. Early modulation of these markers for epidermal differentiation suggests that interference with keratinocyte differentiation is an important aspect of the topical treatment of psoriasis in general. The topical treatments that were studied also proved to inhibit the proliferation rate of epidermal keratinocytes.

In contrast to the in vivo situation, in vitro studies have been shown to have a more treatment-specific response pattern. The differences between the in vivo action of various topical antipsoriatic treatments were mainly caused by a selective interference pattern with cutaneous inflammation. Whereas phototherapy (UVB) and topical corticosteroids both inhibited T-lymphocyte accumulation, calcipotriol only caused a small effect on T-cell functioning. During treatment with dithranol, the vehicle seemed to be a determining factor for the cell-biological effect. Dithranol, in classical vehicles, left the mononuclear inflammatory infiltrate unaffected, during a prolonged time, despite evident clinical

improvement.[4] Dithranol in the Micanol formulation induced an obvious reduction of the percentage of T-lymphocytes in the inflammatory infiltrate.

The anti-inflammatory and immunomodulatory effect of phototherapy and corticosteroids is also expressed during the treatment of inflammatory conditions such as atopic dermatitis.[50] The effect of calcipotriol on PMN permits treatment of pustular psoriasis with this vitamin D_3 analogue. Dithranol, in fact, is a treatment which is specific for psoriasis, although some authors have shown efficacy in seborrhoeic dermatitis, a condition which shares histological and clinical features with psoriasis.[51,52]

In chapter 3, a new in vivo skin model was developed for an evolving psoriatic lesion. In vivo models for epidermal proliferation permit studies on the induction of the recruitment of cycling epidermal cells. The UVB-model proved to be a model suitable for interference studies with topical antipsoriatics. Clobetasol-17-propionate modulated many aspects of the effect of UVB on normal human skin in contrast to calcipotriol.

In chapter 4 and former chapters, the clinical efficacy of new and existing (combinations of) therapies was assessed and discussed. In a case-report, the additive value of calcipotriol in the cyclosporin treatment of erythrodermic psoriasis was shown. The efficacy of this combination has been demonstrated before in a large, clinical study in patients with mild to moderate psoriasis.[35]

In a left-right comparative study in patients with extensive psoriasis at the in-patient department, an analogues efficacy was found for the classical dithranol treatment and treatment with calcipotriol. From this study, it can be concluded that calcipotriol is a therapy that is also suitable for patients with extensive psoriasis, provided that patient-compliance is optimal.

The combination of therapies reduces side-effects of individual treatments due to a dose-reducing effect. On the other hand, combination treatments may also be more effective due to synergism. The efficacy of a therapy with mainly immunomodulating effects can be increased by a therapy with effects on epidermal proliferation and differentiation. The combination of phototherapy (UVB) and dithranol had synergistic effects on the cell-biological level. Clinical studies did also show a synergistic effect of the combination of cyclosporin as a selective immunomodulatory treatment with calcipotriol mainly interfering with epidermal growth and differentiation.[35]

Regarding safety aspects of the treatment of psoriasis, potent topical corticosteroids under hydrocolloid occlusion were found not to be more atrophogenic compared to corticosteroids without this occlusion and to have the same relapse characteristics after discontinuation of both treatments.

Further it was found that the vitamin D_3 analogue, tacalcitol, is an effective therapy with only a small irritative potential and without significant side-effects on calcium-, phosphate-, creatinin-concentration in the serum and 1-α-microglobuline in urine.

The assessment of the mode of action of antipsoriatic treatments is complex. It has been shown that in vitro effects may not always be relevant in the in vivo situation. Further studies are indicated to assess effects of treatment in vivo during treatment of psoriatic plaques and should aim for assessment of functional markers with respect to cutaneous inflammation and quantitative assessment of cell-biological changes.

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SAMENVATTING

In hoofdstuk 1 van dit proefschrift wordt een overzicht gegeven over algemene aspecten van de huidziekte psoriasis waaronder klinische kenmerken, histologie, aetiologie en huidige therapeutische mogelijkheden. Het aandachtsgebied in dit proefschrift is de lokale behandeling van psoriasis. De laatste tijd is er vooruitgang geboekt op dit terrein met name met betrekking tot de dithranol gebaseerde therapieën, therapie met vitamine D_3 analogen en het principe van een lokale therapie onder hydrocolloïde occlusie.

Bij het onderzoek dat ten grondslag ligt aan deze dissertatie is, naast de analyse van klinische effectiviteit en veiligheid van verschillende lokale therapeutica, met behulp van immunohistochemische technieken, het effect van lokale therapeutica op de psoriatische huid geïnventariseerd en is tevens het effect van deze therapieën geïnventariseerd in een nieuw model (UVB-model) voor een zich ontwikkelende psoriasis laesie. Primair is hierbij gekeken naar epidermale differentiatieprocessen met als markers: involucrine, transglutaminase, filaggrine en keratine 16 maar ook naar markers voor epidermale proliferatie (Ki-67) en cutane inflammatie (T-lymfocyten en polymorfkernige granulocyten). Aan het eind van hoofdstuk 1 werden drie vragen geformuleerd:

1. Wat zijn de immunohistochemische effecten van verschillende antipsoriatica?
2. Wat zijn de effecten van verschillende lokale antipsoriatica op een nieuw in vivo model voor een zich ontwikkelende psoriatische laesie?
3. Wat is de klinische effectiviteit en veiligheid van nieuwe (combinaties van) lokale antipsoriatica?

In hoofdstuk 2 werd een consistent patroon gevonden in de wijze waarop lokale antipsoriatica hun effect hebben op epidermale differentiatie processen in vivo in de psoriatische huid: een vermindering van het percentage involucrine en transglutaminase positieve cellagen, een reductie van het aantal keratine 16 positieve cellen in de epidermis en een toename van het aantal filaggrine positieve cellen. Het feit dat deze markers voor epidermale differentiatie al vroeg veranderden gedurende lokale psoriasis behandeling suggereert dat de differentiatie van keratinocyten belangrijk aspect is van de lokale behandeling van psoriasis in het algemeen. De bestudeerde lokale antipsoriatica remden tevens de proliferatie van de epidermale keratinocyten.

In tegenstelling tot de in vivo situatie worden met name bij in vitro studies meer therapie-specifieke effecten gevonden van de verschillende antipsoriatica. De verschillen tussen de lokale

antipsoriatica die in vivo gezien werden, lijken voornamelijk veroorzaakt te worden door een selectief patroon van interferentie met cutane inflammatie. Lichttherapie met UVB en lokale corticosteroïden remden beide de accumulatie van T-lymfocyten, in tegenstelling tot calcipotriol dat slechts een klein effect had op de T-cel functie. Bij de behandeling met dithranol bleek het vehiculum een bepalende factor te zijn voor het cel-biologisch effect. Tijdens behandeling met dithranol in klassieke vehicula persisteerde het T-celinfiltraat gedurende enkele weken ondanks sterke klinische verbetering. Dithranol in de Micanol crème-formulering bleek een duidelijke reductie van het percentage T-lymfocyten in het ontstekingsinfiltraat te induceren.

In hoofdstuk 3 is een nieuw model ontwikkeld voor een zich ontwikkelende psoriatische laesie. Door middel van in vivo modellen kan de inductie van rekrutering van epidermale proliferatie worden bestudeerd. Het UVB-model is gebleken een model te zijn dat geschikt is voor interventie studies met lokale antipsoriatica. Clobetasol-17-propionate moduleerde vele aspecten van het effect van UVB op normale menselijke huid, in tegenstelling tot calcipotriol.

In hoofdstuk 4 en eerdere hoofdstukken komt de klinische effectiviteit van nieuwe en bestaande (combinaties van) therapieën aan bod. In een case-report werd de toegevoegde waarde van calcipotriol bij de cyclosporine-behandeling van een erythrodermische psoriasis gedemonstreerd. De effectiviteit van deze combinatie was al beschreven in een grote studie bij milde tot matige psoriasis.

In een links-rechts vergelijkende studie bij patiënten met uitgebreide psoriasis in de klinische setting kon een vergelijkbare effectiviteit worden vastgesteld voor de klassieke behandeling met dithranol en behandeling met calcipotriol. Hieruit kan worden geconcludeerd dat ook calcipotriol een therapie kan zijn voor een uitgebreide psoriasis mits de therapietrouw optimaal is.

Combinaties van therapieën kunnen bijwerkingen reduceren door een dosis verminderend effect. Anderzijds kunnen gecombineerde behandelingen ook klinisch effectiever zijn ten gevolge van synergie. Zo kan de werking van een therapie met voornamelijk immunomodulerende effecten worden versterkt door een therapie met effecten op epidermale proliferatie en differentiatie. De combinatie van lichttherapie (UVB) met dithranol bleek synergistische effecten te hebben op het cel-biologische niveau.

Kijkend naar veiligheidsaspecten, werd gevonden dat potente lokale corticosteroïden onder hydrocolloïde occlusie niet meer atrofogeen zijn dan lokale corticosteroïden zonder deze occlusie

en tevens dat de relapse-karakteristieken na het stoppen van deze therapieën vergelijkbaar zijn. Verder werd gevonden dat het vitamine D_3 analoog, tacalcitol, een effectieve therapie is met een beperkt irritatief effect op de huid zonder significante bijwerkingen op calcium, fosfaat, kreatinine in serum en 1-α-microglobuline in urine.

In hoofdstuk 5 worden alle bevindingen bediscussieerd in het licht van de bestaande literatuur over deze onderwerpen.

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Curriculum vitae

Catharina Joanna Maria van der Vleuten werd geboren op 6 augustus 1967 te Oirschot. Aldaar bracht zij haar jeugd door. In 1984 behaalde zij het HAVO-diploma aan de Kempenhorst te Oirschot. In 1986 behaalde zij het VWO-diploma aan het Jacob-Roelandslyceum te Boxtel.

In 1986 werd zij inwoner van Nijmegen alwaar een aanvang werd gemaakt met de studie geneeskunde. Tijdens haar studie was zij actief betrokken bij studentenzaken en de vormgeving van het onderwijs. In 1988/89 was zij voorzitter van het Secretariaat ter Ondersteuning van Onderwijszaken voor Studenten, het officieel vertegenwoordigend orgaan van de faculteit ter behartiging van studentenbelangen. Als uitvloeisel van deze werkzaamheden was zij in 1988/89 lid van de faculteitsraad van de Faculteit der Medische Wetenschappen en in 1989-1991 was zij als student lid van het faculteitsbestuur. In 1991 behaalde zij het Doctoraal examen Geneeskunde.

In 1991 startte zij met de co-assistentschappen die zij in januari 1994 afrondde met haar artsexamen. Tijdens de co-assistentschappen werd de interesse voor de klinische èn experimentele dermatologie gewekt. In februari 1994 werd op de afdeling Dermatologie van het Academisch Ziekenhuis Nijmegen een aanvang gemaakt met het onderzoek waarvan U het resultaat in handen heeft. Sinds 1 juni 1996 is zij in opleiding tot dermatoloog (opleider: Prof. Dr. Dr. P.C.M. van de Kerkhof) op dezelfde afdeling.

Op de dag van haar promotie treedt zij in het huwelijk met Roland Verweij, advocaat te Tilburg.

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