

Flow-cytometric Investigation of Epidermal Cell Characteristics in Monogenic Disorders of Keratinization and Their Modulation by Topical Calcipotriol Treatment

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A flow-cytometric study was performed in monogenic disorders of keratinization, to assess DNA distribution as well as the expression of keratins and involucrin. In addition, the changes in expression of these markers under influence of calcipotriol treatment were investigated.

Proliferation, measured by the percentage of epidermal cells in SG2M-phase of the cell cycle, was increased in Darier's disease, lamellar ichthyosis, congenital bullous ichthyotic erythroderma of Brocq and the Comel–Netherton syndrome, whereas normal proliferation was found in autosomal dominant ichthyosis vulgaris, X-linked recessive ichthyosis, keratosis pilaris, ichthyosis bullosa of Siemens and the Sjögren–Larsson syndrome. Keratin 6 was enhanced in erythrodermic lamellar ichthyosis, congenital bullous ichthyotic erythroderma of Brocq and the Comel–Netherton syndrome, showing also reduction of keratin 10. Involucrin was only slightly reduced in erythrodermic lamellar ichthyosis, congenital bullous ichthyotic erythroderma of Brocq and the Comel–Netherton syndrome, compared to the pronounced reduction in all other skin disorders studied. Calcipotriol was found to enhance differentiation in Darier's disease, erythrodermic lamellar ichthyosis, and congenital bullous ichthyotic erythroderma of Brocq. Only Darier's disease did not show clinical improvement.

In conclusion, flow cytometry provides a useful method for quantification of epidermal cell characteristics in monogenic disorders of keratinization. Further studies need to be performed to establish its usefulness as a diagnostic and prognostic tool.

Key words: Darier's disease; ichthyosis; keratosis pilaris.

(Accepted August 28, 1995.)

Acta Derm Venereol (Stockh) 1996; 76: 97–101.

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One of the changes of normal, terminally differentiating keratinocytes is the specific expression of keratin intermediate filaments. Several methods for detection of these intermediate filament proteins are available. Two-dimensional polyacrylamide gel electrophoresis is most often used to characterize keratin proteins biochemically (1, 2). This method allows specific detection of the whole repertoire of keratins present in a tissue specimen, though the localization of the keratins is lost. Immunohistochemical methods using monoclonal antibodies recognizing single or sets of related keratins have the advantage that topographical information is maintained (3–5). A limitation of this approach is that only qualitative or semi-quantitative information can be obtained. Flow-cytometric analysis of intermediate filament proteins in single cell

suspensions of epidermal cells allows rapid simultaneous quantification of two or more characteristics per individual cell, with high reproducibility and statistical accuracy (6). Immunohistochemical and flow-cytometric approaches have been used to determine the epidermal cell characteristics in normal and psoriatic skin and to evaluate the effects of therapies on proliferation and differentiation in psoriasis (7–9). Little information is available on epidermal cell kinetics in monogenic disorders of keratinization. Some immunohistochemical studies have been performed with the monoclonal antibody Ki-67, recognizing a proliferation-associated nuclear antigen (7). However, no quantitative data are available on epidermal cell characteristics in monogenic disorders of keratinization, using a flow-cytometric approach. The vitamin D3 analogues calcipotriol and calcitriol (1 α ,25-dihydroxyvitamin D3) have been shown to inhibit cell proliferation (10–12) and induce terminal differentiation (10, 13–15) in cultured human keratinocytes. Immunohistochemical evaluation of treatment of psoriatic plaques with calcipotriol has revealed a rapid decrease of the number of proliferating cells, measured as Ki-67-positive nuclei, after 2 weeks, resulting in a total decrease of 48% after 12 weeks. Ks8.12-binding revealing keratin 16 expression has shown a tendency to diminish after 4–12 weeks of treatment (8). Flow-cytometric analysis has shown a decrease in DNA-content and a reduction of Ks8.12-binding in psoriasis, after 6 weeks of treatment with calcipotriol (9).

The aim of the present investigation was to assess, using a flow-cytometric approach, DNA distribution, as well as the expression of intermediate filament proteins and involucrin, markers for epidermal proliferation and differentiation, in order to characterize epidermal cell characteristics in Darier's disease, keratosis pilaris (KP), autosomal dominant ichthyosis vulgaris (ADIV), X-linked recessive ichthyosis (XRI), and congenital ichthyoses. Furthermore, the changes in expression of these keratins under influence of calcipotriol treatment were analyzed using flow cytometry.

MATERIALS AND METHODS

Subjects and skin sampling

Twenty-five patients (3 ADIV, 5 XRI, 5 Darier, 3 KP, 9 congenital ichthyosis) participated in the study. The group of congenital ichthyoses consisted of 1 erythrodermic lamellar ichthyosis (ELI), 2 non-erythrodermic lamellar ichthyosis (NELI), 1 ichthyosis bullosa of Siemens (Siemens), 2 congenital bullous ichthyotic erythroderma of Brocq (Brocq), 1 Comel–Netherton syndrome (CNS), and 2 patients with the Sjögren–Larsson syndrome (SLS). A superficial skin sample, about 0.2 mm thick and 3 mm in diameter, was removed from a representative area from each side with a razor blade in conjunction with a metal guard, from all patients before treatment. Similar samples were taken from 11 healthy volunteers. Eighteen patients (2 ADIV,

5 XRI, 3 Darier, 3 KP, 1 ELI, 2 NELI, 1 Siemens and 1 Brocq) completed a double-blind bilaterally paired comparative treatment period of 12 weeks with calcipotriol ointment 50 µg/g and the ointment vehicle only. At week 12, similar biopsies were taken from both sides. All 25 patients were recruited from the centre in Nijmegen. Twenty-four of them (except for one ADIV) constituted a subgroup, participating in a multicenter study from which the clinical results have been published before (16). For practical reasons only skin samples obtained from patients treated at the centre of dermatology in Nijmegen were analyzed using flow cytometry.

Monoclonal antibodies

- AF124 (National Cancer Institute, Bethesda, Maryland, USA) (17) to assess keratin 6, which is expressed in hyperproliferative epidermis.
- RKSE60 (Department of Pathology, University Hospital Maastricht, Netherlands) (18) to assess keratin 10 expression, present in differentiated epidermis.
- Anti-involucrin (Biomedical Technologies, Inc, Stoughton, USA) (19) to assess involucrin expression, a marker for terminal differentiation.
- 6B10 (Eurodiagnostics, Apeldoorn, Netherlands) (20) to assess keratin 4, which is expressed following treatment with retinoids.
- 1C7 (Eurodiagnostics, Apeldoorn, Netherlands) (20) to assess keratin 13, which is also expressed following retinoid treatment.

Analytical procedures

Cell suspensions were prepared using a trypsinization procedure in order to dissociate biopsy specimens into single cells. The biopsy specimen was washed in phosphate-buffered saline (PBS) and incubated with the dermal site downward in 1 ml of PBS containing 1% trypsin (DIFCO, Detroit, Mich.) and 0.3% dithioerythritol (Sigma, St. Louis, Mo.) at 37°C for 20 min. Subsequently, the specimen was fixed in 500 µl ice-cold 70% ethanol and dissociated by sonification (Sonifier B-12, Branson Sonic Power, Danbury, Conn.) for 5 s at 70 W. This resulted in a cell suspension containing only epidermal cells, for dermis and stratum corneum remained intact and floated on the surface.

Aliquots of these ethanol-fixed cell suspensions were immunostained with the monoclonal antibodies anti-involucrin, 6B10, 1C7, RKSE60 and AF124. As second antibody, goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (GAM-FITC; Tago, Burlingame, U.S.A.) was used for all stainings, except for detection of AF 124 which required a FITC-conjugated swine anti-rabbit IgG (SAR-FITC; Dakopatts, Copenhagen, Denmark). These antibodies were added in a 1:25 dilution for 30 min at room temperature. After the two incubation steps, the cells were washed twice and resuspended in 200 µl PBS, pH 7.8, containing propidium iodide (PI) 40 mg/l (Calbiochem, San Diego, U.S.A.). Afterwards the cell suspensions were incubated for 15 min with 50 µl RNase (1 mg/ml in PBS) (Sigma, St. Louis, U.S.A.) at room temperature and filtered in order to remove clumps; fluorescence was measured using an Ortho 50H flow cytometer (Ortho Instruments, Westwood, U.S.A.). Both FITC and PI were excited with a 5 W argon laser (164-05, Spectra Physics, Mountain View, U.S.A.) tuned at 488 nm, and emission was recorded at 515–530 nm (FITC) and >630 nm (PI). The data were stored and analyzed with a Digital PDP 11/34 computer (Digital Equipment, Galway, Republic of Ireland). Green fluorescence derived from FITC indicated staining with the primary antibodies. Red fluorescence derived from PI was used to establish the DNA content per cell, allowing a differentiation between the cells in the consecutive phases of the cell cycle. The cells with elevated DNA content reflected the cells in the SG2M phase of the cell cycle. Therefore, the percentage of cells in SG2M phase was measured as a marker of proliferation.

RESULTS

Epidermal cell characteristics in monogenic disorders of keratinization

Flow-cytometric measurements of cell suspensions obtained in the various monogenic disorders of keratinization are summarized in Table I.

The SG2M values have been measured for each of the monoclonal antibodies, resulting in a total amount of five SG2M values in each sample. In the tables, the mean values are shown $\pm$ S.E.M.

As most disorders are extremely rare, the number of patients in the various groups was too low to perform statistical analysis. To allow a comparison between the flow-cytometric data before calcipotriol treatment found in the various skin disorders, alterations more than one standard deviation from the mean values in the normal control group were taken into consideration.

The percentage of cells in the SG2M phase of the cell cycle was elevated in Darier, ELI, NELI, Brocq, and CNS. Values comparative to normal were observed in ADIV, XRI, KP, Siemens, and the SLS.

Keratin 6 revealed an increase in ELI, Brocq, and CNS.

A reduction of keratin 10 was observed in CNS. A reduction, less than one standard deviation from the normal controls, was noticed in Darier, ELI, NELI, Siemens, and SLS. Involucrin was diminished in all monogenic disorders of keratinization studied. ELI, Brocq, and CNS exhibited only a reduction of involucrin expression of more than one standard deviation, whereas a reduction of more than three standard deviations was observed in the other dermatoses.

The percentage of cells containing keratins 4 and 13 was low in all subgroups.

Modulation of epidermal cell characteristics by calcipotriol treatment

To allow a comparison between the flow-cytometric changes by calcipotriol treatment, alterations of the nett calcipotriol effect of more than one standard deviation from the mean pretreatment values were taken into consideration.

Changes in epidermal cell characteristics in the monogenic disorders of keratinization, after treatment with calcipotriol, compared to its vehicle, are summarized in Table II. An increase in involucrin expression in favour of the calcipotriol-treated side was observed in Darier, ELI, and Brocq.

Alteration of keratin 10 expression paralleled the changes observed in involucrin expression. Again, a unilateral increase in favour of the calcipotriol treated side was observed exclusively in Darier, ELI and Brocq.

Keratin 6 expression in ADIV, KP and Siemens was reduced by calcipotriol, while an elevation was noticed in Darier, ELI and Brocq.

Modulation of keratins 4 and 13 was rather inconsistent: keratins 4 and 13 were both upregulated by calcipotriol in ADIV and Darier. In KP both were downregulated. Contradictory modification was observed in ELI, NELI, and Siemens. In Brocq only keratin 4 was modified upward, whereas in XRI only keratin 13 was regulated downward.

Only moderate modulation was observed in the percentage of cells in SG2M phase. An increase was observed in ADIV,

Table I. Mean values ($\pm$ SEM) for percentage of cells positive for markers of proliferation and differentiation in normal skin and monogenic disorders of keratinization

Normal <i>n</i> = 11	ADIV <i>n</i> = 3	XRI <i>n</i> = 5	Darier <i>n</i> = 5	KP <i>n</i> = 3	ELI <i>n</i> = 1	NELI <i>n</i> = 2	Siemens <i>n</i> = 1	Brocq <i>n</i> = 2	CNS <i>n</i> = 1	SLS <i>n</i> = 2
Involucrin	45.2 $\pm$ 2.7	18.1*** $\pm$ 4.5	12.8*** $\pm$ 1.6	18.3*** $\pm$ 1.9	17.5*** $\pm$ 2.8	35.0* $\pm$ 0.1	4.0*** $\pm$ 1.3	29.4* $\pm$ 1.5	29.7* $\pm$ 0.1	9.5*** $\pm$ 1.6
Keratin 4	0.9 $\pm$ 0.2	2.7** $\pm$ 0.9	3.9*** $\pm$ 1.3	5.5*** $\pm$ 1.1	2.9*** $\pm$ 0.8	1.5 $\pm$ 0.0	1.7* $\pm$ 0.5	2.6** $\pm$ 0.4	2.1* $\pm$ 0.1	0.8 $\pm$ 0.0
Keratin 13	3.8 $\pm$ 0.5	2.2 $\pm$ 0.6	3.8 $\pm$ 1.2	5.7* $\pm$ 1.4	3.2 $\pm$ 0.8	1.6 $\pm$ 0.0	1.3 $\pm$ 0.2	2.0 $\pm$ 0.3	1.7 $\pm$ 0.3	0.6 $\pm$ 0.0
Keratin 10	55.4 $\pm$ 5.3	57.6 $\pm$ 4.0	56.1 $\pm$ 2.9	48.4 $\pm$ 4.1	59.7 $\pm$ 4.1	45.0 $\pm$ 1.2	51.9 $\pm$ 1.4	57.3 $\pm$ 4.6	36.5* $\pm$ 2.6	52.7 $\pm$ 4.1
Keratin 6	18.8 $\pm$ 2.9	9.9 $\pm$ 4.8	5.3 $\pm$ 1.0	16.0 $\pm$ 1.7	7.7 $\pm$ 0.9	54.2*** $\pm$ 1.6	19.5 $\pm$ 7.1	35.8* $\pm$ 4.9	47.1** $\pm$ 4.9	3.0 $\pm$ 0.5
SG2M	5.8 $\pm$ 0.8	6.5 $\pm$ 0.3	7.3 $\pm$ 0.4	10.7* $\pm$ 0.6	6.5 $\pm$ 0.2	11.1* $\pm$ 0.4	8.5* $\pm$ 0.4	10.6* $\pm$ 0.4	9.1* $\pm$ 0.6	5.8 $\pm$ 0.4

* > 1 SD from normal, ** 2 SD from normal, *** 3 SD from normal.

Table II. Changes ($\pm$ SEM) of epidermal cell characteristics in monogenic disorders of keratinization under influence of calcipotriol treatment

	ADIV (<i>n</i> = 2)	XRI (<i>n</i> = 5)	Darier (<i>n</i> = 3)	KP (<i>n</i> = 3)	ELI (<i>n</i> = 1)	NELI (<i>n</i> = 2)	Siemens (<i>n</i> = 1)	Brocq (<i>n</i> = 1)
Involucrin calcipotriol vehicle	7.1 $\pm$ 4.9	-0.2 $\pm$ 0.8	12.6 $\pm$ 4.4	12.0 $\pm$ 6.7	9.6	0.2 $\pm$ 0.0	6.8	25.6
difference	7.9 $\pm$ 5.7	2.1 $\pm$ 1.0	-0.9 $\pm$ 3.2	10.0 $\pm$ 5.8	-13.5	0.6 $\pm$ 0.7	15.4	7.9
Keratin 4 calcipotriol vehicle	-0.8 $\pm$ 0.8***	-2.3 $\pm$ 1.0	13.5 $\pm$ 7.5***	2.0 $\pm$ 10.3	23.1***	-0.4 $\pm$ 0.7	-8.6***	17.7***
difference	1.6 $\pm$ 0.4	-2.3 $\pm$ 1.7	7.2 $\pm$ 1.7	-0.3 $\pm$ 1.0	0.9	-0.2 $\pm$ 3.5	0.5	0.5
Keratin 13 calcipotriol vehicle	-1.4 $\pm$ 1.8	-1.3 $\pm$ 1.7	-0.4 $\pm$ 1.4	1.5 $\pm$ 2.3	0.7	-1.2 $\pm$ 0.6	2.6	-0.2
difference	3.0 $\pm$ 2.2**	-1.0 $\pm$ 2.9	7.6 $\pm$ 2.4***	-1.8 $\pm$ 3.2*	0.2***	1.0 $\pm$ 0.4*	-2.1***	0.7*
Keratin 10 calcipotriol vehicle	1.2 $\pm$ 0.1	-3.0 $\pm$ 1.7	5.5 $\pm$ 3.0	0.2 $\pm$ 0.5	1.2	-0.5 $\pm$ 0.1	0.3	0.4
difference	0.2 $\pm$ 0.6	0.3 $\pm$ 0.1	0.3 $\pm$ 0.9	1.8 $\pm$ 1.0	2.2	0.0 $\pm$ 0.0	-0.2	0.1
Keratin 6 calcipotriol vehicle	1.0 $\pm$ 0.5*	-3.3 $\pm$ 1.7*	5.2 $\pm$ 3.4*	-1.6 $\pm$ 1.4*	-1.0***	-0.5 $\pm$ 0.1*	0.5*	0.3
difference	-2.1 $\pm$ 4.8	-4.6 $\pm$ 2.0	3.1 $\pm$ 3.8	2.1 $\pm$ 2.2	-3.4	-1.1 $\pm$ 1.1	-2.2	20.4
Keratin 4 calcipotriol vehicle	0.6 $\pm$ 2.4	-4.8 $\pm$ 3.2	-7.5 $\pm$ 14.5	2.7 $\pm$ 5.2	-5.7	1.2 $\pm$ 0.1	5.4	7.0
difference	-2.7 $\pm$ 2.5	0.2 $\pm$ 2.4	10.6 $\pm$ 13.5*	-0.6 $\pm$ 3.3	2.3*	-2.3 $\pm$ 1.1*	-7.6*	13.4**
Keratin 13 calcipotriol vehicle	-6.0 $\pm$ 4.5	0.5 $\pm$ 1.8	10.6 $\pm$ 14.3	4.8 $\pm$ 4.0	18.1	-7.2 $\pm$ 5.1	0.0	28.9
difference	7.7 $\pm$ 5.4	-1.0 $\pm$ 2.8	5.3 $\pm$ 10.5	7.6 $\pm$ 2.2	9.2	-12.0 $\pm$ 4.1	11.8	6.9
SG2M calcipotriol vehicle	-13.7 $\pm$ 10.0*	1.5 $\pm$ 3.5	5.3 $\pm$ 4.3*	-2.8 $\pm$ 6.1*	8.9***	4.8 $\pm$ 1.0	-11.8***	22.0***
difference	0.6 $\pm$ 0.2	0.2 $\pm$ 0.4	-2.0 $\pm$ 0.9	0.3 $\pm$ 0.2	4.5 $\pm$ 1.1	1.5 $\pm$ 0.4	-4.3 $\pm$ 1.3	0.5 $\pm$ 2.4
SG2M calcipotriol vehicle	-0.1 $\pm$ 0.4	0.9 $\pm$ 0.5	-1.5 $\pm$ 0.2	0.7 $\pm$ 0.2	3.5 $\pm$ 1.9	-0.6 $\pm$ 0.3	2.3 $\pm$ 2.0	1.5 $\pm$ 1.0
difference	0.7 $\pm$ 0.0*	-0.7 $\pm$ 0.8	-0.5 $\pm$ 1.0	-0.4 $\pm$ 0.2*	1.0 $\pm$ 1.9**	2.1 $\pm$ 0.4***	-6.6 $\pm$ 2.1***	-1.0 $\pm$ 1.5*

The values indicated in the Table represent the changes in mean values of percentage of cells positive for the monoclonal antibodies from baseline to end of treatment. The changes are indicated for the calcipotriol- and verum-treated side. The nett calcipotriol effect is indicated by the difference.

* > 1 SD from normal, ** > 2 SD from normal, *** > 3 SD from normal.

ELI and NELI, whereas a decrease was found in KP, Siemens and Brocq.

DISCUSSION

In the present study, information on epidermal cell kinetics of monogenic disorders of keratinization was obtained, using a flow-cytometric approach. Furthermore, the influence of calcipotriol on proliferation and differentiation was analysed using flow cytometry. Proliferation, measured by the percentage of cells in SG2M phase of the cell cycle, revealed a hyperproliferative state of the epidermis in Darier, ELI, NELI, Brocq and CNS, whereas proliferation was comparative to normal in ADIV, XRI, KP, Siemens and SLS. In 1966, Frost et al. (21) established hyperproliferation to be a contributing factor in the pathogenesis of lamellar ichthyosis, epidermolytic hyperkeratosis and Darier's disease, but not in ADIV and XRI, using autoradiographic techniques with tritiated thymidine which is incorporated into cells in the period of DNA synthesis (22). Jagell & Liden demonstrated DNA synthesis of epidermal cells in SLS to be 2.7 times greater than normal, using the same technique (23). Our flow-cytometric data are in accordance with these previous observations, except for Siemens and SLS. This discrepancy may be explained by the fact that both Siemens and SLS are clinically characterized by prominent lichenification, implicating that relatively more superficial than basal epidermal cell layers may be obtained, using a 0.2 mm thick razor blade. Another marker for proliferation, AF124 staining keratin 6, was elevated in ELI, Brocq and CNS. Information on differentiation was obtained by the percentage of cells, positive for keratin 10, which is a marker for early differentiating keratinocytes. Keratin 10 was reduced more than one standard deviation compared to the normal controls only in CNS. However, all skin disorders characterized by hyperproliferation (as demonstrated with the SG2M values described previously) showed a reduced staining pattern for keratin 10, with the exception of Brocq, which is another ichthyosis subtype, clinically characterized by prominent lichenification. These data are in accordance with clonal analysis, growth kinetics, and cell cycle studies of normal human keratinocytes cultured in serum-free medium, which show that if keratinocyte proliferation is promoted, differentiation is inhibited (24).

Involucrin is one of the substrates for the formation of cornified envelopes and is synthesized in normal epidermis in the granular cell layer, implying that this protein is a marker of late-stage differentiation. However, in hyperproliferative skin disorders such as benign neoplasms, malignant tumours, psoriasis, and DLE, involucrin is synthesized much earlier (25, 26). Our study demonstrated diminished involucrin expression in all skin disorders studied. However, ELI, Brocq and CNS, all characterized by elevated keratin 6 expression and hyperproliferation, exhibited a more pronounced staining of anti-involucrin, compared to the other dermatoses studied.

Concerning the influence of calcipotriol on epidermal cell kinetics, we observed induction of differentiation, indicated by keratin 10 expression, as well as induction of terminal differentiation, indicated by involucrin expression, in Darier, ELI and Brocq, which is in accordance with previous studies on calcipotriol in psoriasis (27). Nevertheless, the expected reduction in proliferation, as was observed previously in psoriasis, was lacking in our study.

Keratins 4 and 13, which are expressed following treatment with retinoids (28), are normally found in fetal, but not in adult epidermis. Modulation after treatment with calcipotriol revealed no consistent findings.

Comparing the flow-cytometric data with the clinical data revealed a good correlation in KP and Siemens, which were unresponsive to calcipotriol, and in ELI and Brocq, which improved substantially. This is in contrast to NELI, XRI and ADIV, in which some clinical improvement was observed, whereas the flow cytometry remained unchanged. Finally, it is of interest that Darier was characterized by flow-cytometric changes, although clinical improvement was not observed. It is attractive to speculate that Darier's disease might be responsive to calcipotriol when used at a lower and less irritating concentration.

We conclude that flow cytometry provides a useful method for establishing epidermal cell kinetics of monogenic disorders of keratinization. Further studies are required to find out to what extent quantification of cell cycle kinetics and markers of keratinization is useful as a diagnostic and prognostic tool.

ACKNOWLEDGEMENTS

We wish to thank I. M. J. J. van Vlijmen-Willems and C. A. E. M. van Hooijdonk for their assistance, and Leo Pharmaceutical Products B.V. for providing the ointments and for financial support.

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