

CD 2394, a Novel Synthetic Retinoid, Initiates an Embryonic Type of Differentiation in Hyperproliferative Skin

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In human skin, there are 2 types of epidermal differentiation: normal differentiation, characterized by keratin 10 expression, and alternative differentiation. Alternative differentiation may be regeneration-associated differentiation (keratin 6 and 16) or re-induction of embryonic differentiation (expression of keratin 13, 15 and 19). The purpose of this study was to investigate the effect of the novel synthetic retinoid CD 2394 on hyperproliferative human skin, with respect to embryonic differentiation in particular. The effects of CD 2394 were compared with untreated and vehicle-treated skin 48 h after tape-stripping. In a multiparameter flow cytometric assay, parameters of proliferation, normal differentiation, embryonic differentiation and inflammation were assessed. With respect to proliferation, treatment with CD 2394 resulted in a decreased number of cells in the G₂M-phase. Normal differentiation was decreased in CD 2394 treated skin. Furthermore, most of the CD 2394 treated samples showed expression of keratin 13, which was not seen in the otherwise treated skin. A correlation between keratin 10 and keratin 13 expression could not be demonstrated. This study showed that CD 2394 is capable of inducing an embryonic pathway of differentiation, which is distinct from normal differentiation or regeneration-associated differentiation. Key words: keratins; proliferation; tape-stripping.

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Retinoids are used in skin disorders such as acne, psoriasis and several malignancies. In the present study, the new synthetic retinoid CD 2394 (2-hydroxy-4-[3-hydroxy-3-(3-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propynyl] benzoic acid; Galderma Research and Development, Sophia Antipolis, France), which binds selectively to the RAR- γ receptor subtype (the predominant receptor in epidermis) (1, 2), was investigated. The advantages of this new retinoid are its relatively low lipophilicity, resulting in rapid elimination from the body and less disturbance of lipid metabolism after systemic administration.

It is known that retinoids induce increased proliferation (3, 4) and decreased terminal differentiation (4–9). In addition, a characteristic effect of retinoids is the induction of keratins that are normally not present or are only marginally present in adult human skin (8–10), especially induction of keratin 13.

In human skin, there are 2 types of epidermal differentiation: normal differentiation, characterized by keratin 10 expression, and alternative differentiation. Alternative differentiation can be regeneration-associated differentiation

(expression of keratin 6 and 16) as well as the re-induction of an embryonic type of differentiation (expression of keratin 13, 15 and 19). This embryonic type of differentiation is also seen following treatment with retinoids.

Retinoids are known to induce the pathway of an embryonic type of differentiation: induction of keratin 13, 15 and 19 as well as cellular retinoic acid binding protein II (CRABP II) has been well documented (1, 8–10).

The aim of the present study was to investigate the effect of treatment with CD 2394 on hyperproliferative human skin in particular with respect to an embryonic type of differentiation. In order to induce hyperproliferation in normal skin, tape-stripping was performed. This method has been used as a standardized procedure to induce hyperproliferation in normal skin since 1951 (11), and can serve as an *in vivo* model for hyperproliferative skin disorders. After treatment with CD 2394 gel or vehicle, characteristics of normal differentiation (keratin 10-positive cells), an embryonic type of differentiation (keratin 13-positive cells), proliferation (cells in different phases of the cell cycle) and inflammation (vimentin-positive cells) were compared with untreated skin by using a multiparameter flow cytometric assay. Flow cytometry is a fast and accurate method for cell cycle analysis (12) and has proven to be able to detect differences in epidermal characteristics (13) (unpublished data). This method offers the opportunity to quantify epidermal effects of topical therapy as a valuable addition to clinical perceptible effects.

Using this quantitative approach, the following questions were answered: (i) Does CD 2394 affect epidermal proliferation, normal differentiation, and inflammation? (ii) Does CD 2394 induce an embryonic type of differentiation, i.e. expression of keratin 13? (iii) To what extent is the induction of an embryonic type of differentiation correlated with inhibition of normal differentiation?

MATERIALS AND METHODS

Subjects

Eight male patients, aged between 18 and 24 years, with stable mild to moderate acne vulgaris of the face participated. None of the patients had acne on the back or other skin diseases. No topical or systemic treatment had been used within the previous 2 and 4 weeks, respectively. Systemic retinoids had not been used within the previous 6 months.

Treatment, Sellotape-stripping and biopsy procedures

After obtaining the informed consent of the patient and permission from the ethics committee, 4 areas on the upper back (about 2 × 2 cm) were marked with a waterproof pencil. One zone of each subject was treated with 100 μ l 0.01% CD 2394 gel (CIRD Galderma, Sophia

Antipolis, France), 0.03% CD 2394 gel and vehicle of the gel, respectively. A fourth zone was left untreated. Assignment of the treatment modalities to the four zones was determined using a randomized latin square design. Gel was applied for 24 h and the treated zones were covered with Tegaderm (3M, Zoeterwoude, The Netherlands). After 24 h, a tape-stripping procedure was performed: Sellotape (Sellotape gb Ltd, Dunstable, UK) was applied with gentle pressure to the marked area, and then removed. This procedure was repeated until the skin glistened (14). Immediately after tape-stripping, the treatment modalities and Tegaderm were applied to the treated zones. After 48 h, the point of time at which proliferation was maximal (15–18), local anaesthesia was induced with xylocaine/adrenaline 1:100000 (Astra Pharmaceutica BV, Zoetermeer, The Netherlands) and 1 punch biopsy (3 mm) was taken from each tape-stripped area.

Cell isolation procedure

The tissue was preserved in phosphate-buffered saline (PBS) until cell isolation, for a maximum of 8 h, as described previously (15). In brief, the biopsies were kept in PBS (with calcium and magnesium) containing 0.5 mg/ml protease type x (Sigma, St Louis, MO, USA) at 4°C during 16–20 h. Subsequently, the dermis and epidermis were separated with 2 forceps, and the epidermis was incubated for 30 min at 37°C in PBS containing 0.025 mg/ml trypsin type III (Sigma) and 0.3 mg/ml dithioerythritol (Sigma). After 10% foetal calf serum was added, the epidermis was gently mixed on a vortex to detach the keratinocytes. After the stratum corneum was removed, the cells were pelleted, fixed in ice-cold ethanol (70% v/v) and stored at –20°C.

Flow cytometric analysis

A multi-labelling technique was used to obtain information about proliferation, differentiation and inflammation. The staining procedure was described previously (19). In brief, inflammation was assessed by using Vim3B4 in a dilution of 1:20 (Novocastra Laboratories, Newcastle upon Tyne, UK) a monoclonal antibody directed against vimentin. Differentiation was assessed using the monoclonal antibody RKSE60 (a gift from Professor F. C. S. Ramaekers, Department of Molecular Biology, University of Maastricht, The Netherlands) in a dilution of 1:10 directed against keratin 10. The fluorochrome TO-PRO-3 iodide (TP3) (Molecular Probes, Eugene, USA) was used for measuring DNA content per cell in the keratin 10-negative population. As secondary antibodies we used goat anti-mouse IgG2a conjugated with fluorescein-isothiocyanate (FITC) (Southern Biotechnology Associates, Birmingham, USA) and goat anti-mouse IgG1 conjugated with phycoerythrin (PE) (Southern Biotechnology Associates) binding to Vim3B4 and RKSE60, respectively.

After this triple-labelling was performed, all samples were additionally stained for keratin 13. In this procedure, the washed cells were incubated with 1C7 (a gift of J. Beck, Department of Pathology, University Hospital of Nijmegen, The Netherlands), an antibody against keratin 13 that was diluted 1:2. As secondary antibody, rabbit anti-mouse IgG conjugated with fluorescein-isothiocyanate (FITC) (DAKO, Copenhagen, Denmark) was used.

An EPICS[®] Elite flow cytometer (Coulter, Luton, UK) was used to measure 5000 gated cells. An argon ion laser (15 mW, 488 nm) excited FITC and PE, and a helium-neon laser (10 mW, 633 nm) excited TP3. Bandpass filters of 515–535 nm (green, FITC), 555–595 nm (orange, PE), and 665–685 nm (red, TP3) were used to measure emitted light. The area/peak ratio in the DNA-histogram was used to exclude doublets of diploid cells and gate the real single tetraploid cells for further analysis (20). The percentage of cells positive for vimentin, keratin 10 and keratin 13 were assessed by using gates. These gates offer the opportunity to determine proliferation characteristics for each of the subpopulations: the percentages of cells

in S- and G₂M-phase were determined with Multicycle[™] software (Phoenix Flow Systems, San Diego, CA, USA).

Because of a wrong biopsy, information about the vehicle-treated sample of subject 1 was lacking.

Statistical analysis

Data were statistically tested with a 1-way repeated ANOVA test. Correlation was tested with a multiple regression analysis. All percentages are represented as mean ± SEM. The mean value of the other vehicle-treated samples was used for the missing value for subject 1.

RESULTS

Proliferation characteristics as measured 48 h following tape-stripping in untreated, vehicle-treated and CD 2394 treated areas are summarized in Figs. 1A and 1B. Both concentrations of CD 2394 caused a reduction in cells in G₂M-phase compared with vehicle and untreated skin ($p < 0.05$). However, as shown in Fig. 1B, treatment with CD 2394 did not have a significant effect on the percentage of proliferative cells (cells in SG₂M-phase).

Differentiation parameters differed significantly between the 4 treatment modalities (Fig. 1C): most keratin 10-positive cells were present in the untreated group (66.1%), whereas the least differentiation was seen in the CD 2394 treated samples (38.1% for 0.01% CD 2394 and 46.4% for 0.03% CD 2394). The vehicle-treated samples were intermediate (55.7%) between the other groups. Significant differences were present between 0.01% CD 2394 and untreated and vehicle-treated skin ($p < 0.001$ and $p < 0.01$, respectively). The higher concentration of CD 2394 also differed significantly from that of untreated skin ($p < 0.01$).

Results with respect to keratin 13, as shown in Fig. 1D, show that the expression in the retinoid-treated samples was high (8.2% for 0.01% CD 2394 and 8.4% for 0.03% CD 2394), whereas in the untreated and vehicle-treated group almost no expression was seen (0.3% for untreated skin and 0.7% for vehicle-treated skin) ($p < 0.001$). Although the mean expression of keratin 13 in treated skin was high, 2 out of 8 samples showed nearly no expression (0.2 and 0.1%). Significant differences were seen between 0.01% and 0.03% CD 2394 on the one hand and the untreated and vehicle-treated samples on the other ($p < 0.01$). No correlations were found between keratin 13 expression and expression of keratin 10.

Differences with respect to inflammation parameters were minimal and not significant.

DISCUSSION

The present study showed that tape-stripped human skin treated with either 0.01% or 0.03% CD 2394 showed less cells in G₂M-phase than untreated or vehicle-treated stripped skin. A decrease in proliferation was seen in previous studies in which tape-stripped and psoriatic skin were treated with systemic retinoids (5, 21). In the present study, a borderline significant reduction in the number of cells in G₂M-phase revealed a modest indication of an antiproliferative effect *in vivo*. The fact that the differences that were seen in cells in G₂M-phase were not present in SG₂M-phase indicates that retinoids cause a delay in the cell cycle: Cells that were

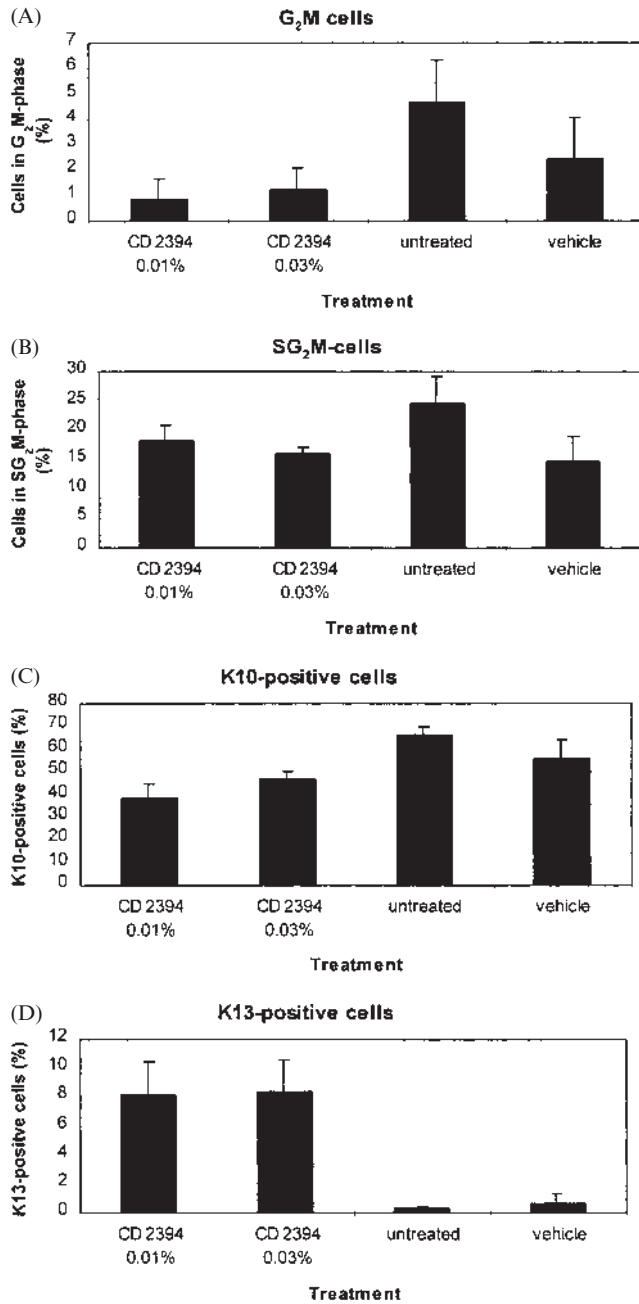


Fig. 1. Percentage of: (A) cells in G₂M-phase; (B) cells in SG₂M-phase; (C) keratin 10-positive cells; and (D) keratin 13-positive cells 48 h after tape-stripping after treatment with CD 2394, vehicle, or without treatment (mean \pm SEM, $n=8$)

triggered to enter the cell cycle are in the S-phase and have not reached the G₂M-phase 48 h after tape-stripping. This means that there is no supply of cells to the G₂M-phase, which leads to a decrease in cells in this phase.

Parameters with respect to differentiation showed that expression of keratin 10, the keratin that is expressed in normal differentiation, is significantly decreased after treatment with CD 2394. Most of the earlier studies showed the same result (1, 4–7) although some investigations revealed no change in keratin 10-positive cells (8, 9, 22). A possible explanation for the discrepancy between these findings could

be differences in dose and duration of treatment, cellular environment (age of patient, *in vivo* or *in vitro*, and presence of other growth factors in medium) (22), moment of sampling, and drug-specific effects.

Keratin 13 was expressed in most of the skin samples that were treated with the retinoid. Under normal conditions, keratin 13 expression is limited to non-keratinizing squamous epithelia and expression in skin is, together with expression of keratin 15 and 19, assumed to be a sign of re-induction of an embryonic type of differentiation (10). In 2 of the treated samples almost no expression of keratin 13 was seen. This finding was described earlier in a study by Rosenthal et al., in which only one-sixth of the skin samples with a weak histological response to retinoid treatment and half of the strong responding samples showed expression of keratin 13 (9). This can be an indication that the samples in the present study are weak responders, which could explain the almost absent keratin 13 expression. However, in the earlier mentioned study no significant correlation existed between keratin 13 expression and the magnitude of the histological response (9).

The tendency for low levels of keratin 10 expression to be accompanied by high percentages of keratin 13 pleads for the hypothesis that retinoids bring about differentiation via an alternative pathway. This means that, beside the normal differentiation (keratin 10 expression) and the regenerative pattern (keratin 6 and 16 expression), an embryonic route of differentiation (keratin 13, 15 and 19 expression) can be followed.

Although tape-stripping can serve as an *in vivo* model for hyperproliferative skin disorders, it should be noticed that these results were found in a model and cannot simply be extrapolated to other conditions. A clear-cut dose-response relationship was not seen in this study, which could be explained by the relatively small group. Further studies are required to complete data about CD 2394 in other conditions.

In the present study, it was confirmed that induction of keratin 13 is a retinoid-mediated event not positively correlated with epidermal proliferation (8, 9, 22): retinoid-treated skin contained less G₂M-cells, but more keratin 13 expressing cells than otherwise treated skin. Future experiments on the interrelation between keratin 10 and 13 expression should focus on coexpression of these keratins per cell, using double-labelling flow cytometry.

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