Vitamin D₃ Induces Caspase-14 Expression in Psoriatic Lesions and Enhances Caspase-14 Processing in Organotypic Skin Cultures

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Caspase-14 is a nonapoptotic caspase family member whose expression in the epidermis is confined to the suprabasal layers, which consist of differentiating keratinocytes. Proteolytic activation of this caspase is observed in the later stages of epidermal differentiation. In psoriatic skin, a dramatic decrease in caspase-14 expression in the parakeratotic plugs was observed. Topical treatment of psoriatic lesions with a vitamin D₃ analogue resulted in a decrease of the psoriatic phenotype and an increase in caspase-14 expression in the parakeratotic plugs. To investigate whether vitamin D₃ directly affects caspase-14 expression levels, we used keratinocyte cell cultures. 1 α ,25-Dihydroxycholecalciferol, the biologically active form of vitamin D₃, increased caspase-14 expression, whereas retinoic acid inhibited it. Moreover, retinoic acid repressed the vitamin D₃-induced caspase-14 expression level. In addition, the use of organotypic skin cultures demonstrated that 1 α ,25-dihydroxycholecalciferol enhanced epidermal differentiation and caspase-14 activation, whereas retinoic acid completely blocked caspase-14 processing. Our data indicate that caspase-14 plays an important role in terminal epidermal differentiation, and its absence may contribute to the psoriatic phenotype. (*Am J Pathol* 2004; 165:833–841)

The epidermis is the outermost layer of the skin and consists mainly of keratinocytes. The epidermal basal layer contains stem cells that continuously provide the epidermis with new cells.^{1,2} Once the keratinocytes leave the basal layer, they undergo a complex program of

differentiation involving morphological and biochemical changes as they proceed toward the surface of the skin.^{3–5} The different suprabasal layers distinguished in the epidermis are the spinous layer, the granular layer, and the cornified layer, which consists of terminally differentiated keratinocytes, also called corneocytes. The basal layer is composed of cuboidal cells that are anchored to the basal lamina through hemidesmosomes.⁶ In the spinous layer an increase in desmosomes is observed, and early differentiation is accompanied by the expression of typical markers for keratinocyte differentiation, such as keratins 1 and 10; involucrin; and transglutaminase types 1, 3, and 5.^{7–11} The keratinocytes of the granular layer are distinguished by the presence of keratohyalin granules, containing precursors of the cornified envelope, such as profilaggrin, loricrin, and cystatin- α .^{12,13} Major changes occur during the transition from the granular layer of living cells to the cornified layer, which is composed of dead cells. Massive enzymatic activity results in loss of the nucleus and other organelles, and the cornified envelope is formed through cross-linking by transglutaminase activity.^{5,14} Finally, only the cornified envelopes containing keratin macrofibrils remain. Eventually, the corneocytes are continuously shed into the environment through desquamation.

Based on sequence homology, caspase-14 was identified as one of the cysteine aspartic-specific proteases or caspases that are of absolute importance in apoptosis and inflammation.^{15–17} The proform of these proteases consists of an N-terminal prodomain, followed by the large p20 (20 kd) and the small p10 (10 kd) subunit, respectively. Within the unprocessed zymogen, the large prodomain caspases exert low enzymatic activity, whereas the proform of small prodomain caspases exerts none. In general, proteolytic maturation, which cleaves

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off the N-terminal prodomain and separates the two subunits, leads to formation of the fully active enzyme, a heterotetramer composed of two large and two small subunits.^{18,19} In contrast to many other caspases, caspase-14 is not ubiquitously expressed, and in the skin it is only present in the suprabasal layers of the epidermis and epidermal derivatives such as the hair follicle and sebaceous gland.^{20–22} Although other caspases are expressed in the epidermis,^{17,21,23,24} they are present as unprocessed inactive zymogens. However, caspase-14 is processed during keratinocyte terminal differentiation, and therefore may play a role in skin formation.^{21,22}

Psoriasis is a skin disease characterized by hyperproliferation of the basal layer and parakeratosis or abnormal formation of the cornified layer, because the cells retain the nucleus, which is absent from normal corneocytes.^{25,26} Compared to normal epidermis, an aberrant expression pattern of keratinocyte differentiation markers and death-related proteins is observed in psoriatic lesions.^{27–32} We previously reported inhibition of caspase-14 expression in the parakeratotic regions of psoriatic skin.²¹ Different compounds are known to regulate keratinocyte proliferation or differentiation. Among these are vitamin D₃ and vitamin A, which exert their effects mainly through binding of nuclear receptors that can act as ligand-activated transcription factors.^{33–35} The active form of vitamin D₃, 1,25-dihydroxyvitamin D₃, has a concentration-dependent inhibitory effect on proliferation, but enhances differentiation in keratinocyte cell cultures.^{36,37} In contrast, retinoic acid suppresses differentiation.^{38,39} Because of their ability to inhibit keratinocyte proliferation and induce differentiation, vitamin D₃ and analogues are widely used for the treatment of hyperproliferative skin diseases such as psoriasis.⁴⁰

To determine whether caspase-14 expression is influenced by vitamin D₃, we performed a clinical study in which psoriasis patients were treated with a balm containing a vitamin D₃ analogue. We also investigated the effects of vitamin D₃ and retinoic acid on caspase-14 expression levels in keratinocyte cultures. Moreover, we used skin equivalents as a model system to study the effects of vitamin D₃ and retinoic acid on caspase-14 processing.

Materials and Methods

Cells and Cell Culture

The immortalized keratinocyte HaCaT cell line was obtained from Dr. N. Fusenig (German Cancer Research Center, Heidelberg, Germany) and the cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Primary normal human epidermal keratinocytes were purchased from Clonetics and grown in keratinocyte growth medium (KGM) (Clonetics, San Diego, CA). Partial differentiation was induced by postconfluent growth of the cells, whereas complete differentiation was obtained in organotypic keratinocyte cultures. The generation of *in vitro*-reconstructed skin equivalents was based on a described method.⁴¹ Briefly, 3- μ m

pore culture insets (Becton Dickinson, Franklin Lakes, NJ) were placed in deep six-well trays (Becton Dickinson), on which was poured a suspension of 1.25×10^5 human fibroblasts in $1 \times$ Hanks' buffered saline solution (Invitrogen, Carlsbad, CA) adjusted to pH 7.4 with 1 mol/L NaOH and containing 2.4 mg/ml of bovine collagen type I (Nutacon, Leimuiden, The Netherlands) and 10% fetal calf serum. The collagen solution was allowed to gel for 2 hours at 37°C in a humidified atmosphere without CO₂. The gel was then equilibrated for 2 hours in 16 ml of prewarmed KGM (2 ml inside the inset, 14 ml outside the inset) at 37°C in a humidified CO₂ (5%) incubator. The KGM within the inset was then replaced with 2 ml of KGM containing 1.2×10^6 normal human epidermal keratinocytes and the cells were cultured submerged overnight. The next day the medium was replaced by 10 ml of supplemented keratinocyte differentiation medium (KGM supplemented with 1.3 mmol/L CaCl₂, 10 μ g/ml transferrin, 50 μ g/ml L-ascorbic acid, and 0.1% bovine serum albumin, but lacking bovine pituitary extract), with or without 10^{-7} mol/L 1 α ,25-dihydroxycholecalciferol (Sigma, St. Louis, MO) and/or 10^{-6} mol/L all-*trans* retinoic acid (Sigma). At this stage the keratinocytes were brought to the air-liquid interface. Supplemented keratinocyte differentiation medium was replaced every 2 days and samples are taken on days 4 and 8 after lifting the cultures to the air-liquid interface.

³H-Thymidine Incorporation Assay

Primary keratinocytes were seeded in 96-well plates at 10^4 cells per well and treated with 1 α ,25-dihydroxycholecalciferol (Sigma) concentrations ranging from 10^{-6} to 10^{-8} mol/L, 10^{-6} mol/L all-*trans* retinoic acid (Sigma), 10^3 U/ml human tumor necrosis factor- α (prepared in our laboratory), 100 nmol/L dexamethasone (Sigma) for 24 or 72 hours or with 10 μ g/ml mitomycin c (Sigma) for 2 hours. All treatments were performed in triplicate. The cells were pulsed for 8 hours with ³H-thymidine at 1 μ Ci per well. The cells were harvested and evaluated for ³H-radioactivity using a Topcount microplate scintillation counter (Packard Instrument Company, Meriden, CT).

Immunoblot Analysis

Partially differentiated keratinocytes or organotypic keratinocyte culture samples were lysed in a buffer containing 50 mmol/L Tris, pH 8.0, 300 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 15 mmol/L MgCl₂, 1% Nonidet P-40, 1 mmol/L phenylmethyl sulfonyl fluoride, 50 μ mol/L leupeptin, and 20 μ g/ml aprotinin. Protein concentration was determined and equal amounts of protein were used for polyacrylamide gel electrophoresis and immunoblotting. Blots were exposed, revealed by means of chemiluminescence (Perkin Elmer, MA) and signals were quantified by densitometric analysis and captured by a Lumi-Imager workstation (Roche Molecular Biochemicals, Basel, Switzerland). An anti-caspase-14 murine polyclonal antiserum, which cross-reacts with human caspase-14 in Western blots, was generated as described.²¹ The antibodies against β -actin (ICN Biomed-

Table 1. Vitamin D₃ Enhances Caspase-14 Expression Levels in Psoriatic Lesions

Patient	Parakeratotic plugs			Maximum epidermal thickness, (μm)			Caspase-14 expression		
	Normal skin	Psoriatic skin		Orthokeratotic skin	Psoriatic skin		Orthokeratotic stratum corneum	Parakeratotic plugs	
		Before	After		Before	After		Before	After
1	—	+	+	115	401	234	+	—	+
2	—	+	+	109	485	358	+	—	—
3	—	+	+	109	285	250	+	—	—
4	—	+	+	153	463	302	+	—	+
5	—	+	+	140	529	371	+	—	+
6	—	+	+	109	628	375	+	—	+
7	—	+	+	197	456	184	+	—	+

Seven psoriasis vulgaris patients participated in this clinical study. Prior to the study, selected psoriatic plaques were left untreated for 2 weeks, and biopsy punches from normal and psoriatic lesions were isolated. During the study, psoriatic plaques were topically treated with Daivonex twice daily, and biopsy punches were taken 3 weeks later. The samples were used for immunohistochemical examination. +, Present; —, absent; before, psoriatic skin before Daivonex treatment; after, psoriatic skin after Daivonex treatment.

cals, Irvine, CA), keratin 10 (Santa Cruz Biotechnology, Santa Cruz, CA), caspase-8 (BioSource, MA), and caspase-9 (Becton Dickinson) were purchased.

Vitamin D Treatment of Psoriatic Lesions

Seven patients with clinically diagnosed psoriasis vulgaris were selected, excluding patients receiving systemic treatments such as methotrexate, cyclosporine, or Psoralen Ultraviolet A (PUVA). The group consisted of males and females between 18 and 70 years of age. The selected plaques were left untreated for 2 weeks before the start of the study. During the study the plaques were treated topically with calcipotriol (Daivonex; Leo Pharmaceutical Products, Ballerup, Denmark) twice daily for 3 weeks. Full-thickness 3-mm punch biopsy specimens from psoriatic plaques were obtained for immunohistochemical examination before and after treatment. Samples were also obtained from anatomically normal-appearing uninvolved skin (at least 3 cm from the margin of a visible lesion) from the same patients. All patients gave informed consent for donation of biopsies, and the study was performed with the approval of the Ethical Committee of the Middelheim Hospital.

Histology and Immunohistochemistry

Samples of organotypic *in vitro* keratinocyte cultures or human skin were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin. Immunohistochemistry using a polyclonal anti-caspase-14 antiserum was performed as described.²¹ The anti-Ki-67 antibody was obtained from LabVision-Neomarkers (Fremont, CA) and used according to the manufacturer's instructions. Percentage of Ki-67-positive cells was scored by counting 200 cells of the basal layer in each sample.

Results

Vitamin D₃ Enhances Caspase-14 Expression Levels in Psoriatic Lesions

In normal skin, caspase-14 is detected in the cytoplasm and nuclei throughout the suprabasal epidermal layers. However, in the parakeratotic regions of psoriatic skin, which are characterized by the lack of nuclear destruction, caspase-14 expression is down-regulated.²¹ Vitamin D₃ and its analogues are typically used for psoriasis treatment, and so we evaluated the effect of the vitamin D₃ analogue calcipotriol on caspase-14 expression in psoriatic plaques. Seven patients were treated with Daivonex balm twice a day for 2 weeks, and 3-mm skin biopsies were taken from psoriatic plaques before and after treatment. Samples of normal skin from the same persons were also obtained. Parakeratosis was present in all samples of psoriatic skin before and after treatment, but not in normal skin (Table 1). In all patients, vitamin D₃ treatment resulted in a reduction of the psoriatic phenotype, and the decrease of epidermal thickness reflected a drop in keratinocyte hyperproliferation. In three patients the monolayered aspect of the basal layer was restored to normal (data not shown). Caspase-14 was not expressed in the parakeratotic plugs before vitamin D₃ treatment. In five patients, however, caspase-14 expression was clearly up-regulated in the parakeratotic plugs after vitamin D₃ treatment. Figure 1 demonstrates the morphology and caspase-14 expression levels in normal and psoriatic skin before and after treatment of one representative patient. Vitamin D₃ treatment resulted in increased caspase-14 expression levels both in the cytoplasm and the nuclei of cells in the parakeratotic layer.

Vitamin D₃ Enhances Caspase-14 Expression in Keratinocytes in a Dose-Dependent Manner

To investigate whether vitamin D₃ directly affects caspase-14 expression in keratinocytes, we treated ker-

Figure 1. Vitamin D₃ increases caspase-14 expression in the parakeratotic regions of psoriatic skin. Psoriatic skin was treated with a balm containing a vitamin D₃ analogue, and skin biopsies were used for staining with H&E (A, C, E), or caspase-14 immunodetection (B, D, F). A and B: Normal skin showing orthokeratosis. C and D: Psoriatic skin before treatment; the parakeratotic layer is indicated with **double asterisk**. E and F: Psoriatic skin after vitamin D₃ treatment; the parakeratotic region is indicated with an **asterisk**. Original magnifications, $\times 200$.

atinocyte cultures with a serial dilution of 1 α ,25-dihydroxycholecalciferol. It has been reported that in primary keratinocytes and HaCaT cells, caspase-14 was not expressed when the cells were grown subconfluently, but caspase-14 expression was induced when the cells were differentiated by postconfluent growth.²¹ HaCaT cells were treated with different concentrations of 1 α ,25-dihydroxycholecalciferol for 72 hours. Vitamin D₃ enhanced procaspase-14 expression in a dose-dependent manner, but it had no effect on keratin 10 expression (Figure 2A). The effect was strongest in the presence of 10⁻⁷ mol/L 1 α ,25-dihydroxycholecalciferol, a concentration generally used to inhibit keratinocyte proliferation and induce differentiation.^{37,42} In a time-kinetics experiment, HaCaT cells were treated with 10⁻⁷ mol/L vitamin D₃ for 4 days, and samples for Western blotting were prepared every 24 hours (Figure 2B). Subconfluently grown cells at 24 hours were not differentiated, as evidenced by the lack of keratin 10 expression. From 48 hours on the keratinocyte density steadily increased, leading to partial differentiation and expression of keratin 10 and procaspase-14. The enhancing effect of vitamin D₃ on procaspase-14 protein expression was clearly observed at 48 hours and

Figure 2. 1 α ,25-Dihydroxycholecalciferol enhances procaspase-14 protein expression. **A:** HaCaT cells were treated for 72 hours with different concentrations of 1 α ,25-dihydroxycholecalciferol as indicated. Cell lysates were used for Western blot analysis. **B:** HaCaT cells were treated with 10⁻⁷ mol/L 1 α ,25-dihydroxycholecalciferol for 24, 48, 72, and 96 hours. Protein extracts were analyzed for caspase-14, keratin 10, and caspase-3 expression.

later. The caspase-3 expression levels were not altered by vitamin D₃ treatment, indicating that vitamin D₃ did not affect caspase expression levels in general.

Retinoic Acid Inhibits the Vitamin D₃-Enhancing Effect on Procaspase-14 Expression in Keratinocytes

Retinoic acid inhibits keratinocyte differentiation and can antagonize the differentiation effects of vitamin D₃.^{38,42} Moreover, caspase-14 expression is suppressed by retinoic acid *in vitro*.⁴³ Therefore, we investigated the effect of retinoic acid on vitamin D₃-induced caspase-14 expression in keratinocytes (Figure 3). Treatment of differentiating primary keratinocytes and HaCaT cells with

Figure 3. Retinoic acid inhibits vitamin D₃-induced procaspase-14 expression. Western blot analysis of the expression of different caspases by HaCaT cells or primary normal human epidermal keratinocytes treated with different combinations of vitamin D₃, 9-*cis*-retinoic acid, or all-*trans* retinoic acid for 72 hours. 9cRA, 9-*cis*-retinoic acid; atRA, all-*trans* retinoic acid; vitD₃, 1 α ,25-dihydroxycholecalciferol.

Figure 4. Vitamin D₃-dependent caspase-14 up-regulation is not a mere consequence of growth arrest. Normal human epidermal keratinocytes were treated with different growth arrest-inducing agents as indicated. The cells were pulsed for 8 hours with ³H-thymidine, harvested, and ³H-radioactivity was measured (**A**, **C**, and **E**). Protein lysates of similarly treated cells were used for immunodetection of caspase-14 and β-actin (**B**, **D**, and **F**). Relative levels of caspase-14, normalized with β-actin levels, are indicated. atRA, all-*trans* retinoic acid; dex, dexamethasone; mito c, mitomycin c; vitD₃, 1α,25-dihydroxycholecalciferol.

10⁻⁷ mol/L 1α,25-dihydroxycholecalciferol enhanced procaspase-14 expression, whereas retinoic acid, at pharmacological concentrations,^{42,44,45} inhibited procaspase-14 expression induced by 10⁻⁷ mol/L vitamin D₃ or postconfluent growth. The levels of proapoptotic caspases such as caspase-8 and -9 were not influenced by vitamin D₃ or retinoic acid.

Vitamin D₃-Dependent Caspase-14 Up-Regulation Is Not a Mere Consequence of Growth Arrest

In keratinocytes vitamin D₃ treatment results in inhibition of proliferation^{37,42} and the induction of caspase-14 expression. To investigate whether caspase-14 up-regulation occurred as a mere consequence of any growth-inhibiting stimulus, we examined the effect of other growth arrest inducers on caspase-14 expression levels. Keratinocytes were treated for 24 or 72 hours with different concentrations of vitamin D₃ (10⁻⁶ mol/L to 10⁻⁸ mol/L) and pulsed with radiolabeled thymidine for 8 hours (Figure 4, A and C). Caspase-14 levels were evaluated by Western blotting and the ratios of induction were calculated (Figure 4, B and D). After 24 hours vitamin D₃ induced up-regulation of caspase-14 expression levels at

concentrations that did not affect the growth rate at this time point (5.4-fold induction at 10⁻⁷ mol/L), indicating that the vitamin D₃ effect on caspase-14 was not correlated with its growth inhibitory capacity. After 72 hours of vitamin D₃ treatment the inhibitory effect on proliferation reached ~90%, however the inducing effect on caspase-14 levels was less pronounced (3.6-fold induction). We also tested the effect of other known inducers of keratinocyte growth arrest such as tumor necrosis factor-α, dexamethasone, all-*trans*-retinoic acid and mitomycin c.⁴⁶ All compounds were found to suppress proliferation as assessed by ³H-thymidine incorporation (Figure 4E). In contrast, caspase-14 levels were not increased but rather somewhat suppressed in these conditions (Figure 4F), confirming that the vitamin D₃-dependent induction of caspase-14 in keratinocytes is not a direct result of growth arrest.

Vitamin D₃ Enhances and All-Trans Retinoic Acid Inhibits Caspase-14 Processing in Organotypic Keratinocyte Cultures

In vitro three-dimensional organotypic keratinocyte cultures allow complete epidermal differentiation when they are brought to the air-liquid interface. All layers present in normal skin epidermis can be distinguished, including the cornified layer consisting of terminally differentiated keratinocytes. In these skin equivalents, as in normal skin, caspase-14 is expressed and processed.²¹ We used organotypic keratinocyte cultures to study the effect of vitamin D₃ and retinoic acid on caspase-14 processing. 1α,25-Dihydroxycholecalciferol and/or all-*trans* retinoic acid was added to the medium when the keratinocytes were brought to the air-liquid interface. Samples were taken after 4 and 8 days of air-exposure and examined for morphology and caspase-14 processing (Figure 5). After 4 days the cornified layer showed only minor development and caspase-14 was only slightly processed [Figure 5A (a) and B]. Vitamin D₃ treatment of these skin equivalents enforced the program of terminal differentiation, resulting in a more rapid formation of cornified envelopes, fewer intermediate cell layers and earlier development of a cornified layer (Figure 5A, c). An increase in caspase-14 processing was also observed (Figure 5B). On day 8, nuclei were still present in some cells of the cornified layer of organotypic keratinocyte cultures treated with vitamin D₃ (Figure 5A, d). At this time-point, cornification and caspase-14 processing was already established and could not be further enhanced. In the samples treated with all-*trans* retinoic acid alone or in combination with vitamin D₃, the formation of cornified envelopes was impaired, and this was associated with a complete absence of caspase-14 processing [Figure 5, A (e to h) and B]. Proliferation in these samples was examined by Ki-67 staining (Figure 5C). In all samples Ki-67-positive cells were found and no marked changes in the number of proliferative cells was observed in the presence of vitamin D₃. The percentage of Ki-67-positive cells in the basal layer in untreated and vitamin D₃-

Figure 5. Vitamin D₃ induces and all-*trans* retinoic acid inhibits caspase-14 processing in organotypic keratinocyte cultures. Organotypic keratinocyte cultures were treated with 1 α ,25-dihydroxycholecalciferol and/or all-*trans* retinoic acid for 4 or 8 days. **A:** Morphological examination of paraffin-embedded sections of skin equivalents. **B:** Western blot detection of caspase-14 in cell lysates. **C:** Ki-67 immunodetection on paraffin-embedded sections of skin equivalents. **Arrows** indicate Ki-67-positive cells. The percentage of Ki-67-positive cells in the basal layer was: 25.5% in control cultures (4 days); 23% in vitamin D₃-treated cultures (4 days); 12.5% in control cultures (8 days); 14.5% in vitamin D₃-treated cultures (8 days). atRA, all-*trans* retinoic acid; vitD₃, 1 α ,25-dihydroxycholecalciferol. Original magnifications, $\times 200$ (**A**); $\times 400$ (**C**).

-treated samples was 25.5% and 23% after 4 days, respectively. After 8 days of organ culture 12.5% and 14.5% Ki-67-positive cells were scored in untreated and vitamin D₃-treated cultures, respectively.

Discussion

Although apoptotic caspases are expressed in the epidermis, they are not activated during normal epidermal differentiation, indicating that keratinocyte terminal differentiation and apoptosis occur through different signaling pathways.^{21,43} Activation of short prodomain caspases typically occurs by cleavage C-terminal to an Asp residue by caspases or granzyme B. However, caspase-14 processing during epidermal differentiation occurs between isoleucine152 and lysine153.⁴⁷ This cleavage probably amounts to activation of caspase-14, because it results in the formation of a heterotetrameric complex consisting of two p20 and two p10 subunits that contain all of the conserved caspase residues involved in substrate recognition and catalytic activity. The function of caspase-14 remains elusive, but the fact that it is induced in differentiating keratinocytes and processed during cornification suggests a role for this protease in normal epidermal differentiation.^{21,22} The events responsible for nuclear degradation occurring during the transition from the granular to the cornified layer of the epidermis are unknown, but probably require proteolytic activity. In normal skin, caspase-14 is found in the cytoplasm and in the nucleus, whereas in psoriatic skin it is absent in the parakeratotic region, which is characterized by a failure in nuclear degradation. The correlation between caspase-14 expression and normal nuclear destruction may suggest a role for caspase-14 in this event. Balms containing vitamin D₃ are used as a standard treatment for psoriatic lesions.⁴⁰ The treatment restores to normal the altered expression pattern of several proteins, such as Ki-67, keratin 14 and 16, involucrin, filaggrin, transglutaminase, and Bcl-X_L.^{31,48-51} We investigated the influence of a vitamin D₃ analogue on caspase-14 expression in psoriatic skin, and found that the loss of caspase-14 expression in the parakeratotic region was restored in five of seven patients. The increase of caspase-14 levels initiated by vitamin D₃ treatment *in vivo* matched our results obtained with keratinocyte cultures. 1 α ,25-Dihydroxycholecalciferol, the hormonally active metabolite of vitamin D₃, increased procaspase-14 levels in primary keratinocytes and HaCaT cells, but this effect was antagonized by retinoic acid at pharmacological concentrations. Meanwhile the expression levels of proapoptotic caspases remained unaltered. Other growth arrest-inducing agents such as tumor necrosis factor, dexamethasone, or retinoic acid did not enhance but rather suppressed caspase-14 expression. In addition, 24 hours of treatment of keratinocyte cultures with vitamin D₃ concentrations that do not affect proliferation were found to up-regulate the caspase-14 levels. These observations confirm previous findings showing that elevated Ca²⁺ levels, another growth arrest-inducing agent in keratinocytes, did not induce caspase-14 expression.²³ Taken together, these data indicate that vitamin D₃-induced caspase-14 expression is not a mere consequence of growth arrest. This implicates caspase-14 as a true keratinocyte differentiation marker. Moreover, scanning of the human caspase-14 promoter sequence in a transcription factor search program, MatInspector Professional,⁵² revealed the presence of putative

vitamin D and retinoic acid receptor binding sites (data not shown).

In vitro skin equivalents are valuable model systems for studying the different stages of the program leading to keratinocyte cornification, in which caspase-14 processing is observed.^{21,22,43} As previously described, addition of 1 α ,25-dihydroxycholecalciferol to the culture medium resulted in enhanced formation of corneocytes.⁵³ The stratum corneum is thickened, whereas the other suprabasal layers become more flattened. In the samples obtained after 4 days of air exposure, the increase in the formation of the cornified layer is correlated with an increase in caspase-14 maturation. It is unlikely that the increase in caspase-14 processing is because of an elevated expression of procaspase-14, because high concentrations of the latter do not result in autoprocesing.^{17,54} Whether increased processing of caspase-14 occurs in vitamin D₃-treated psoriatic lesions is currently not clear.

The vitamin D₃-mediated up-regulation of procaspase-14 expression observed in monolayer keratinocyte cultures was not reflected in organotypic keratinocyte cultures. This is because of the difference between the two *in vitro* model systems that were used in this study. In monolayer keratinocyte cultures, vitamin D₃ has an anti-proliferative effect and can act on expression levels of early markers such as keratin 1 and 10, involucrin, and transglutaminase 1.^{42,55,56} In three-dimensional organotypic keratinocyte cultures, vitamin D₃ stimulates the intermediate steps in keratinocyte differentiation because of an increased conversion rate of suprabasal cells to corneocytes.⁵³ These earlier observations are confirmed by the fact that we did not observe differences in the number of Ki-67-positive cells between untreated and vitamin D₃-treated skin equivalent cultures. This accelerated epidermal differentiation could be the reason for the incomplete removal of nuclei from the corneocyte layers in the day 8 skin equivalent samples. In contrast, addition of retinoic acid to skin equivalents results in inhibition of differentiation because no cornification takes place and differentiation markers such as keratin 1 and 10, loricrin, and transglutaminase 1 are down-regulated.^{44,57,58} We observed only a partial inhibitory effect of retinoic acid on procaspase-14 expression levels in organotypic keratinocyte cultures, compared to monolayer keratinocyte cultures. However, we found complete blockage of caspase-14 processing, even after 8 days of air exposure. This implies that the upstream caspase-14-processing factor is not expressed or activated in the presence of retinoic acid, or in the absence of cornification. Hence, caspase-14 processing was always correlated with stratum corneum formation, suggesting that its activity is required in this stage of keratinocyte terminal differentiation.

Although we observed opposite effects of vitamin D₃ and retinoids on *in vitro* epidermal differentiation and caspase-14 expression and processing, retinoids are also successfully used for treatment of psoriatic skin.^{59,60} Retinoic acid inhibits keratinocyte differentiation *in vitro*, it causes thickening of the granular layer and increased expression of differentiation markers such as involucrin,

transglutaminase, and filaggrin *in vivo*.^{44,61,62} The apparent paradox between the proliferation-inducing effects of retinoids on normal skin and its use as therapy for hyperproliferative diseases such as psoriasis has not been resolved so far, but it has been suggested that depending on the concentration, retinoids can act as vitamin D₃ agonists.^{42,63} Hence, it is possible that retinoic acid has a different effect on caspase-14 expression in psoriatic skin compared to its *in vitro* effects.

In summary, our results indicate that caspase-14 expression can be regulated as a keratinocyte differentiation marker. Moreover, its processing is strictly associated with epidermal cornification. In addition, the fact that clinical improvement of psoriatic lesions by treatment with vitamin D₃ correlates with an increased caspase-14 expression suggests that caspase-14 plays an important role in terminal epidermal differentiation, and its absence may contribute to the psoriatic phenotype.

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