1α,25-Dihydroxyvitamin D₃ Protects Human Keratinocytes from Apoptosis by the Formation of Sphingosine-1-Phosphate

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Owing to its ability to induce growth arrest and differentiation of keratinocytes, 1α,25-dihydroxyvitamin D₃ and its analogs are useful for the treatment of hyperproliferative skin diseases, such as psoriasis vulgaris. It has been implicated that the 1α,25-dihydroxyvitamin D₃-induced differentiation of keratinocytes is mediated, at least in part, by the formation of ceramides; however, ceramides have also been identified to induce apoptosis in many cells, including keratinocytes. Therefore, it was of interest to investigate the influence of 1α,25-dihydroxyvitamin D₃ on apoptosis in keratinocytes. Most interestingly, physiological concentrations of 1α,25-dihydroxyvitamin D₃ did not induce apoptosis in keratinocytes, despite the formation of ceramides. Moreover, 1α,25-dihydroxyvitamin D₃ appeared cytoprotective and made keratinocytes resistant to apoptosis induced by ceramides, ultraviolet irradiation, or tumor necrosis factor-α. The cytoprotective effect was accompanied by the formation of the sphingolipid breakdown product sphingosine-1-phosphate, which prevented apoptosis in analogy to 1α,25-dihydroxyvitamin D₃. The effect of 1α,25-dihydroxyvitamin D₃ was specific as the almost inactive precursor cholecalciferol neither induced sphingosine-1-phosphate formation nor prevented cells from apoptosis. Besides this, the cytoprotective aptitude of 1α,25-dihydroxyvitamin D₃ was completely abolished by the sphingosine kinase inhibitor N,N-dimethylsphingosine, which blocked sphingosine-1-phosphate formation. Moreover, sphingosine-1-phosphate was able to restore the cytoprotective effect of 1α,25-dihydroxyvitamin D₃ in the presence of N,N-dimethylsphingosine. Taken together, here we report for the first time that 1α,25-dihydroxyvitamin D₃ protects keratinocytes from apoptosis and additionally this cytoprotection is mediated via the formation of sphingosine-1-phosphate. *Key words: Bcl-2 family/cytoprotection/programmed cell death/sphingolipids/vitamin D₃. J Invest Dermatol 117:1241–1249, 2001*

Besides its long recognized role in calcium homeostasis, 1α,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) is known to inhibit proliferation and to promote differentiation in a variety of cell types, including breast and colon carcinoma cells as well as leukemic and epidermal cells (Bouillon *et al*, 1995; Kobayashi *et al*, 1998; Nolan *et al*, 1998). Therefore, the therapeutic potential of 1,25-(OH)₂D₃ and its derivatives (e.g., calcipotriol) has been investigated for the treatment of cancer, acute myeloid leukemia, and psoriasis. 1,25-(OH)₂D₃ exerts its effects by binding to the vitamin D₃ receptor, which belongs to the nuclear hormone-receptor gene family (Baker *et al*, 1988). The hormone-receptor complex heterodimerizes with the retinoid X receptor and binds to cognate response elements in the promoters of target genes involved in the regulation of cell growth and differentiation (Minghetti and Norman, 1988; Kuroki

et al, 1995). Recent studies have also indicated that 1,25-(OH)₂D₃ induces several rapid, apparently nongenomic biologic effects influencing a number of signal transduction pathways, including phosphoinositide signaling, intracellular calcium increase, and protein kinase C activation (Wali *et al*, 1990).

1,25-(OH)₂D₃ was the first compound identified as an inducer of neutral Mg^{2+}-dependent sphingomyelinase leading to the hydrolysis of the membrane lipid sphingomyelin and a concomitant increase of intracellular ceramide levels. Cell-permeable ceramide analogs or bacterial sphingomyelinase mimic the effects of 1,25-(OH)₂D₃ on cell differentiation (Bielawska *et al*, 1992b) indicating an important role for ceramides in the actions of 1,25-(OH)₂D₃. Moreover, ceramide has been identified as a crucial component in the induction of apoptosis. A variety of proapoptotic stimuli, including tumor necrosis factor (TNF)-α, Fas-ligand, growth factor withdrawal, anticancer drugs, oxidative stress, heat shock, ionizing radiation as well as ultraviolet (UV) light have been found to stimulate sphingomyelinase activity leading to an enhanced cellular level of ceramide (Hannun and Obeid, 1995; Hannun, 1996; Jarvis *et al*, 1996; Spiegel *et al*, 1996; Kolesnick and Kronke, 1998). Indeed, in a number of cancer cells 1,25-(OH)₂D₃ has also been recognized to induce apoptosis (Diaz *et al*, 2000; van den Bemd *et al*, 2000; Wang *et al*, 2000). In contrast 1,25-(OH)₂D₃ fails to induce programmed cell death in HL-60 cells as well as in human

thyrocytes despite ceramide formation (Studzinski *et al*, 1986; Xu *et al*, 1992, 1993; Wang and Studzinski, 1997). In fact, prolonged incubation of HL-60 cells and thyrocytes with $1,25\text{-}(OH)_2D_3$ prevents the appearance of apoptotic cell death induced by calcium ionophores, anticancer drugs, and even cell-permeable ceramide analogs. Anti-sense inhibition of vitamin D_3 receptor expression revealed that this protective effect is mediated via $1,25\text{-}(OH)_2D_3$ binding to its nuclear receptor (Hewison *et al*, 1996).

Recently, we demonstrated that $1,25\text{-}(OH)_2D_3$ enhances sphingosine kinase activity in HL-60 cells leading to a concomitant increase of sphingosine-1-phosphate (SPP), which prevents ceramide-induced apoptosis (Kleuser *et al*, 1998).

Hydrolysis of sphingomyelin after stimulation with $1,25\text{-}(OH)_2D_3$ or calcipotriol has also been documented in keratinocytes. Here, $1,25\text{-}(OH)_2D_3$ increases expression of TNF-α, which induces ceramide formation via an autocrine mechanism (Geilen *et al*, 1996, 1997). As TNF-α is in analogy to ceramides in a classical inductor of apoptosis, it was of interest to investigate the effect of $1,25\text{-}(OH)_2D_3$ on keratinocyte survival. We found that $1,25\text{-}(OH)_2D_3$ in physiological concentrations did not enhance apoptosis in human keratinocytes despite the expression of TNF-α and the subsequent formation of ceramide. Moreover, our studies demonstrate for the first time that $1,25\text{-}(OH)_2D_3$ even protects keratinocytes from apoptosis and this resistance is a consequence of SPP formation.

MATERIALS AND METHODS

Materials $1,25\text{-}(OH)_2D_3$ and calcipotriol were kindly donated by Dr. Lise Binderup (Leo-Pharmaceutical Products, Ballerup, Denmark). [*methyl*-^{3}H]thymidine (35 Ci per mmol), [^{3}H]putrescine (80 Ci per mmol), and [γ-^{32}P]adenosine triphosphate (4500 Ci per mmol) were purchased from ICN Biomedicals (Costa Mesa, CA). Cardiolipin and standard phospholipids were from Avanti Polar Lipids (Birmingham, AL). SPP, *N,N*-dimethylsphingosine (DMS), sphingosine, and *N*-acetylsphingosine (C2-cer) were purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Annexin V–fluorescein isothiocyanate (Annexin V-FITC) was obtained from Bender (Vienna, Austria). Dimethylcasein, putrescine, propidium iodide (PI), ceramides (bovine brain, type III), leupeptin, aprotinin, dithiothreitol, phenylmethylsulfonylfluoride, o-phthaldialdehyde, cholecalciferol, sodium orthovanadate, deoxypyridoxine, bovine serum albumin, HEPES, Triton X-100, actinomycin D, and Dulbecco's modified Eagle's medium were purchased from Sigma (St. Louis, MO). TNF-α was from Seromed Biochrom (Berlin, Germany). *Escherichia coli* diacylglycerol kinase and octyl-β-D-glycopyranosides were obtained from Calbiochem (La Jolla, CA). Keratinocyte basal medium, epidermal growth factor, insulin, hydrocortisone, bovine pituitary extract, gentamicin sulfate, and amphotericin B were purchased from Clonetics (San Diego, CA). All high-performance liquid chromatography solvents were obtained from Merck (Darmstadt, Germany).

Cell culture To isolate human keratinocytes juvenile foreskin from surgery was incubated at $4°C$ in a solution of 0.25% trypsin and 0.2% ethylenediamine tetraacetic acid (EDTA) for 20 h. Trypsinization was terminated by the addition of ice-cold Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were washed with phosphate-buffered saline (PBS) and centrifuged at $250 \times g$ for 5 min. The pellet was resuspended in keratinocyte growth medium that was prepared from keratinocyte basal medium by the addition of 0.1 ng per ml recombinant epidermal growth factor, 5.0 μg insulin per ml, 0.5 μg hydrocortisone per ml, 0.15 mM Ca^{2+}, 30 μg bovine pituitary extract per ml, 50 μg gentamicin sulfate per ml, 50 μg amphotericin B per ml.

Keratinocytes were pooled from several donors and cultured at $37°C$ in 5% CO_2. For all experiments only cells of the second or third passage were used.

DNA synthesis Keratinocytes (4×10^4 cells per well) were grown in 24-well plates for 24 h. Then medium was replaced by fresh keratinocyte growth medium and cells were incubated with $1,25\text{-}(OH)_2D_3$ for 72 h. Keratinocytes were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine per well and incubated for 23 h. The medium was removed and cells were washed twice each with PBS and ice-cold trichloroacetic acid (5%). The precipitated material was dissolved in 0.3 M NaOH solution and

incorporated [*methyl*-^{3}H]thymidine was determined in a scintillation counter (MicroBeta Plus, Wallac Oy, Turku, Finland).

Sphingosine kinase activity Human keratinocytes grown in 100 mm dishes until a confluence of approximately 60% were treated with $1,25(OH)_2D_3$ or the indicated agents for various incubation periods. Cells were then washed twice with ice-cold PBS and suspended in 200 μl of kinase buffer [20 mM Tris buffer (pH 7.4) containing 20% (vol/vol) glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 10 μg per ml leupeptin and aprotinin, 1 mM phenylmethylsulfonylfluoride, and 0.5 mM 4-deoxypyridoxine] as described previously (Olivera and Spiegel, 1993). Cells were disrupted by freeze-thawing, and the cytosolic fraction was prepared by centrifugation at $13,000 \times g$ for 30 min at $4°C$. The cytosolic fraction (120 μl) was incubated with 10 μl of sphingosine (1 mM), delivered as a sphingosine–bovine serum albumin complex (4 mg bovine serum albumin per ml). Kinase buffer was added to a final volume of 190 μl, and reactions were started by adding 10 μl of [γ-^{32}P]adenosine triphosphate (1–2 μCi, 20 mM) containing 100 mM $MgCl_2$. Samples were incubated for 30 min at $37°C$, followed by the addition of 20 μl of 1 M HCl. Lipids were extracted by the addition of 0.8 ml chloroform/methanol/concentrated HCl (100:200:1, vol/vol/vol). After vigorous vortexing, 240 μl of chloroform and 240 μl of 2 M KCl were added for phase separation. The samples were vortexed and centrifuged. Labeled lipids in the organic phase were separated by thin-layer chromatography on silica gel G60 using 1-butanol/methanol/acetic acid/water (80:20:10:20, vol/vol/vol/vol) as the solvent. The radioactive spots corresponding to authentic SPP were visualized by autoradiography, scraped from the plates, and counted in a scintillation counter.

Mass measurement of SPP SPP was determined as recently described (Ruwisch *et al*, 2001). Briefly, keratinocytes were washed twice with PBS and scraped in 1 ml of methanol containing 2.5 μl concentrated HCl. Lipids were extracted by addition of 1 ml chloroform and 1 ml 1 M NaCl. For alkalization, 100 μl of a 3 M NaOH solution were added. After centrifugation ($300 \times g$, 5 min), the alkaline aqueous phase containing SPP was transferred into a siliconized glass tube, the organic phase was re-extracted with 0.5 ml methanol, 0.5 ml 1 M NaCl and 50 μl 3 M NaOH. The aqueous phases were acidified with 100 μl concentrated HCl and extracted twice with 1.5 ml chloroform. The combined organic phases were evaporated using a vacuum system (Savant, Bethesda, MD). The dried lipids were resolved in 275 μl methanol/0.07 M K_2HPO_4 (9:1) by rigorous vortexing and sonication on ice for 5 min.

A derivatization mixture of 10 mg of o-phthaldialdehyde, 200 μl of ethanol, 10 μl of β-mercaptoethanol, and 10 ml of a 3% boric acid solution adjusted to pH 10.5 with potassium hydroxide was prepared. 25 μl of this derivatization mixture were added to the resolved lipids for 15 min at room temperature. The derivatives were analyzed by a Merck Hitachi LaChrom high-performance liquid chromatography system (Merck Hitachi, Darmstadt, Germany). Fluorescence was measured at an emission wavelength of 455 nm and an excitation wavelength of 340 nm after separation on a RP 18 Kromasil column (Chromatographie Service, Langerwehe, Germany) kept at $35°C$. The flow rate was adjusted to 1.3 ml per min, methanol and 0.07 M K_2HPO_4 were used as eluents. Resulting profiles were evaluated using the Merck system manager software.

Mass measurement of ceramide Keratinocytes, cultured in 100 mm dishes, were treated with vehicle or 100 nM of $1,25\text{-}(OH)_2D_3$ in the presence or absence of 5 μM DMS. Then lipids were extracted by the addition of 3 ml of methanol/chloroform/water (1:1:1, vol/vol/vol) and an aliquot of 200 μl of the chloroform phase was dried under a nitrogen stream. The lipids or standard bovine brain type III ceramides were suspended in 40 μl of 7.5% (wt/vol) octyl-β-D-glycopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid, 10 mM imidazole (pH 6.6) and then solubilized by freeze-thawing followed by sonication. The enzymatic reaction was started by the addition of 20 μl of dithiothreitol (20 mM), 20 μl of *Escherichia coli* diacylglycerol kinase (0.88 units per ml), 20 μl of [γ-^{32}P]adenosine triphosphate (10 mM, 1 μCi per nmol), and 100 μl of reaction buffer [100 mM imidazole (pH 6.6), 100 mM NaCl, 25 mM $MgCl_2$, and 2 mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid]. Lipids were incubated for 1 h at room temperature and then extracted by the addition of 1 nl of chloroform/methanol/concentrated HCl (100:200:1, vol/vol/vol) and 170 μl of PBS containing 10 mM EDTA. An aliquot of 50 μl of the organic phase was analyzed by thin-layer chromatography (Silica Gel G60) with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1,

vol/vol/vol/vol/vol) as the solvent. Radioactive spots corresponding to ceramide-1-phosphate (R_f = 0.23 ± 0.08) were counted.

Annexin V binding and PI dye exclusion by flow cytometry Keratinocytes (1.7×10^5 cells per well) were cultured in keratinocyte basal medium containing 5.0 µg insulin per ml, 0.5 µg hydrocortisone per ml, 50 µg gentamicin sulfate per ml, 50 ng amphotericin B per ml and incubated with the indicated agents for 24 h. Then cells were trypsinized and washed twice with binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Apoptosis was determined by flow cytometric detection of phosphatidylserine translocation using fluorescein-labeled Annexin V (Vermes *et al*, 1997). To discriminate between early apoptotic cells (Annexin V⁺/PI⁻) as well as late apoptotic and necrotic cells (Annexin V⁺/PI⁺), dye exclusion of the nonvital dye PI was simultaneously measured. Therefore, cells were resuspended in binding buffer followed by the addition of Annexin V-FITC (final concentration 0.5 µg per ml). The mixture was incubated for 10 min in the dark at room temperature, washed, and resuspended in binding buffer. Then PI was added (1 µg per ml) and samples were analyzed by bivariate flow cytometry.

TUNEL staining Keratinocytes were directly stained on chamber slides by the *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany), as recommended by the manufacturer. Cell layers were fixed with a 4% buffered paraformaldehyde solution and then blocked with 3% H₂O₂ in methanol before permeabilization with 0.1% Triton X-100 in 0.1% sodium citrate. Cells were incubated with fluorescein-labeled nucleotides (fluorescein-deoxyuridine triphosphate) and terminal deoxynucleotidyl transferase at 37°C for 1 h. Cells were washed with PBS and incorporated fluorescein at the damaged sites of the DNA was detected by an anti-fluorescein antibody conjugated horseradish peroxidase. After substrate reaction using diaminobenzidine, approximately 100 cells were evaluated in randomly selected high-power fields by light microscopy. Negative control was obtained by replacing the primary incubation with a nucleotide mixture without terminal deoxynucleotidyl transferase.

Transglutaminase assay Transglutaminase activity was determined by the method described by Wakita *et al* (1994). Cells were cultured in keratinocyte growth medium and incubated with the test substances for 96 h. Keratinocytes were collected with a rubber policeman in 20 mM Tris–HCl buffer containing 2 mM EDTA (pH 8.0) and homogenized by freeze-thawing. After centrifugation at $600 \times g$ for 10 min, 100 µl of the supernatant were mixed with 600 µl 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM CaCl₂, 5 mM dithiothreitol, 540 µg dimethylcasein, 1 mM putrescine, and 2.5 µCi [³H]putrescine (80 Ci per mmol). The mixture was incubated for 1 h at 37°C and the enzymatic reaction was stopped by the addition of 600 µl ice-cold trichloroacetic acid (10%). The protein precipitate was washed three times with ice-cold trichloroacetic acid (5%) containing 10 mM putrescine and once with ethanol (95%). The pellet was solubilized in 200 µl 1 M NaOH solution and radioactivity was determined in the scintillation counter.

Protein kinase C activity After treatment of keratinocytes with the indicated agents for 24 h, cells were washed twice with PBS, scraped from dishes, and suspended in kinase buffer [0.1 M Tris–HCl (pH 7.4)

containing 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 10 mg leupeptin per ml and aprotinin each, 1 mM phenylmethylsulfonylfluoride, and 0.5 mM 4–deoxypyridoxine]. Cells were lyzed by freeze-thawing and centrifuged at $140,000 \times g$ for 30 min. The supernatant, containing the cytosolic fraction, was collected, whereas the pellet was resuspended by passing through a 27 gauge needle 10 times in kinase buffer containing 0.1% Triton X-100. After centrifugation at $140,000 \times g$ for 30 min, supernatant was saved and designated as the membrane fraction. Protein concentrations were determined and equal amounts from cytosolic and membrane fractions were assayed for protein kinase C (PKC) activity, using a commercial assay kit (Upstate Biotechnology, Inc, Lake Placid, NY). The procedure was performed as described in the manufacturer's instructions.

Immunoblot analysis for Bcl-2 The expression of Bcl-2 was determined by western blot analysis using a mouse monoclonal antibody to human Bcl-2 (PharMingen, San Diego, CA). Lysates of keratinocytes were prepared by scraping cells from plates and suspended in PBS. The cells were collected by centrifugation and the resulting pellets were suspended in ice-cold lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM ethyleneglycol-bis-(β-aminoethylether)–*N,N,N′,N′*-tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate and 1 µg leupeptin per ml). After incubation on ice for 30 min, samples were centrifuged at $13,000 \times g$ for 20 min. The Triton-soluble fraction was collected, and 15 µg of protein were subjected to a 12.5% polyacrylamide gel and transferred to nitrocellulose membranes. The blots were blocked in Tris-buffered saline/Tween 20 (0.1%) with 5% nonfat dry milk for 1 h, incubated with the primary antibody for 3 h at 37°C and a horseradish peroxidase-conjugated second antibody (New England Biolabs, Beverly, MA) for 1 h at room temperature. Immune complexes were detected with an enhanced chemoluminescence detection method (Santa Cruz Biotechnology, CA).

Statistical analysis Data are the mean from triplicate assays and are expressed as mean ± SD. All experiments were repeated at least three times independently. Statistics were performed using Student's t test, with $p \leq 0.05$ considered significant.

RESULTS

Influence of 1,25-(OH)₂D₃ on cell growth, apoptosis, and necrosis 1,25-(OH)₂D₃ has been well established to inhibit cell growth of human keratinocytes. In agreement with many previous studies a slight but significant anti-proliferative effect was visible with 1 nM of 1,25-(OH)₂D₃, whereas a concentration of 100 nM of 1,25-(OH)₂D₃ reduced [*methyl*-³H]thymidine incorporation by more than 60% (**Table I**). To prove whether the anti-proliferative effect in this concentration range is accompanied by the induction of apoptosis, we used TUNEL staining and additionally measured the translocation of phosphatidylserine, an early event in the apoptotic process, by flow cytometry using Annexin V-FITC. To distinguish between early apoptotic as well as late apoptotic and

Table I. Effect of 1,25-(OH)₂D₃ on proliferation, apoptosis and necrosis in human keratinocytes[a]

1,25-(OH)₂D₃	Proliferation		Apoptosis/necrosis		
	[*methyl*-³H] thymidine incorporation		TUNEL-positive cells (%)	Annexin V⁺/PI⁻ cells (%)	Annexin V⁺/PI⁺ cells (%)
	(cpm × 10³)	(% of control)			
Control	53.3 ± 5.5	100	14.7 ± 2.5	3.4 ± 0.7	7.5 ± 2.1
0.1 nM	51.7 ± 6.2	96	16.3 ± 3.1	4.2 ± 1.4	6.9 ± 0.6
1 nM	41.1 ± 4.9[b]	77	12.0 ± 4.4	3.6 ± 1.1	8.7 ± 0.4
10 nM	29.0 ± 6.5[b]	55	10.3 ± 3.2	3.1 ± 0.3	5.9 ± 2.1
100 nM	21.7 ± 5.7[b]	40	6.7 ± 1.5[b]	3.4 ± 1.2	8.4 ± 1.4
1 µM	22.5 ± 4.3[b]	42	29.7 ± 8.0[b]	7.5 ± 1.6[b]	64.5 ± 8.9[b]
10 µM	19.5 ± 4.1[b]	37	84.3 ± 6.0[b]	8.7 ± 3.4[b]	85.6 ± 12.3[b]

[a]Keratinocytes were treated with the indicated concentrations of 1,25-(OH)₂D₃ for 3 d. Proliferation was measured by [*methyl*-³H]thymidine incorporation as described in *Materials and Methods*. Annexin V⁺/PI⁻ and Annexin V⁺/PI⁺ cells were determined by flow cytometric detection of phosphatidylserine translocation and PI uptake. Additionally, TUNEL staining was performed. Data are mean ± SD of triplicate determinations.
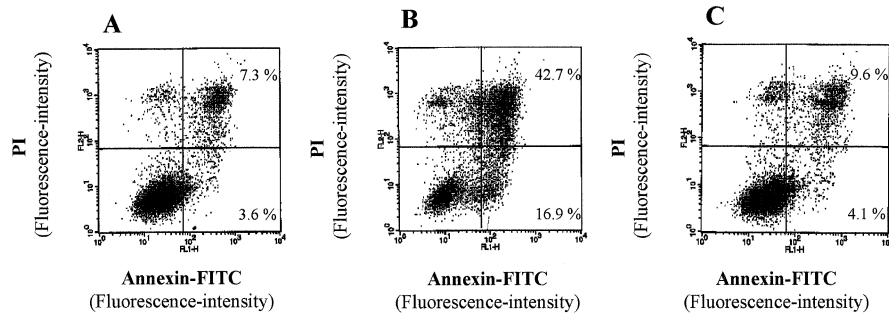[b]$p \leq 0.05$ considered significant.

Figure 1. Flow cytometric analysis indicating a protective effect of 1,25-(OH)$_2$D$_3$ against ceramide-induced apoptosis in human keratinocytes. Keratinocytes were preincubated with either vehicle (*A*, *B*) or 100 nM of 1,25-(OH)$_2$D$_3$ (*C*) for 24 h. Then C2-cer (25 μM) was added (*B*, *C*) for 3 h. Cells were harvested and double staining with Annexin V-FITC and PI was performed as described in *Materials and Methods*.
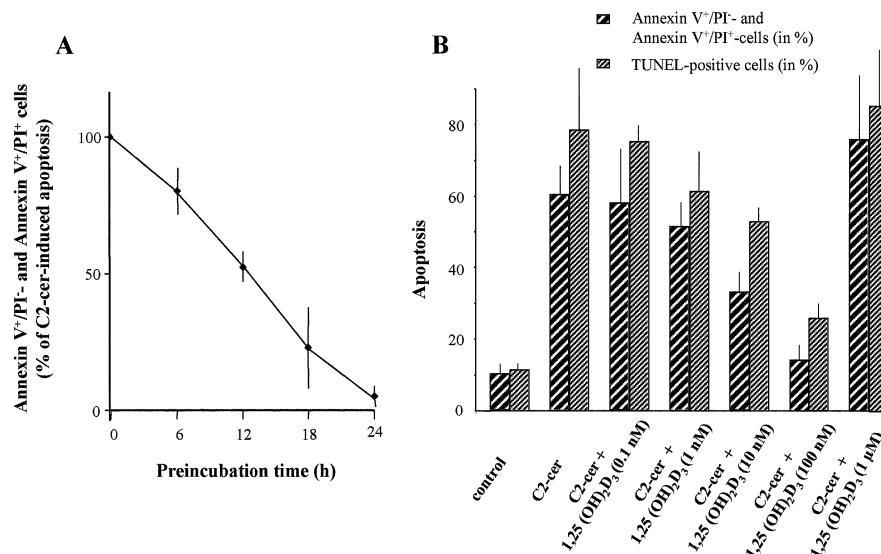

Figure 2. Effect of the 1,25-(OH)$_2$D$_3$-preincubation time and the 1,25-(OH)$_2$D$_3$-dose on prevention of ceramide-induced apoptosis. Human keratinocytes were pretreated with 100 nM of 1,25-(OH)$_2$D$_3$ for the indicated time periods (*A*) or with the indicated concentrations of 1,25-(OH)$_2$D$_3$ for 24 h (*B*). Apoptosis was induced by addition of 25 μM C2-cer for 3 h. Then double staining with Annexin V-FITC and PI as well as TUNEL staining were performed as described in *Materials and Methods*. Data are the mean ± SD of triplicate assays.

necrotic cells, dye exclusion of the nonvital dye PI was simultaneously measured. It is of interest that 1–100 nM of 1,25-(OH)$_2$D$_3$ neither increased Annexin V binding nor PI uptake indicating that 1,25-(OH)$_2$D$_3$ did not possess apoptotic or necrotic actions itself in this concentration range (**Table I**). But it should be noted that 1,25-(OH)$_2$D$_3$ concentrations exceeding 1 μM, however, resulted in an increased detachment of the cells from the dishes. Indeed, measurement of apoptosis/necrosis demonstrated an increase of Annexin V$^+$/PI$^+$-cells suggesting a cytotoxic effect of 1,25-(OH)$_2$D$_3$ at concentrations above 1 μM (**Table I**). This result was confirmed by TUNEL staining and is in agreement with other studies (Benassi *et al*, 1997; Bektas *et al*, 2000)

Protective effect of 1,25-(OH)$_2$D$_3$ on cell survival in ceramide-, UV-, and TNF-α-induced apoptosis It has been shown that treatment with 1,25-(OH)$_2$D$_3$ protects HL-60 cells and thyrocytes against ceramide-induced apoptosis. To investigate an anti-apoptotic role of 1,25-(OH)$_2$D$_3$ in keratinocytes, cells were treated with the cell permeable ceramide analog C2-cer. Treatment with C2-cer resulted in an increase in Annexin V$^+$/PI$^-$ and more dramatically of Annexin V$^+$/PI$^+$ cells, which is visible by a right shift of the cell populations presented in **Fig 1B**. The ability of C2-cer to induce apoptosis became obvious at a concentration of 25 μM; however, when keratinocytes were preincubated with 100 nM of 1,25-(OH)$_2$D$_3$ Annexin V binding and PI uptake was almost completely abolished indicating a protective role for 1,25-(OH)$_2$D$_3$ against C2-cer induced apoptosis (**Fig 1C**). The cytoprotective effect of 1,25-(OH)$_2$D$_3$ was verified using the TUNEL technique (**Fig 2B**). The capacity to prevent apoptosis was dependent on the preincubation time with 1,25-

(OH)$_2$D$_3$. A significant resistance against ceramide-induced apoptosis was first visible after a preincubation period of 12 h with 1,25-(OH)$_2$D$_3$, whereas a maximal reduction of the number of Annexin V$^+$/PI$^-$ and Annexin V$^+$/PI$^+$ cells was evident after 24 h (**Fig 2A**). The effect was also concentration dependent showing a maximal cytoprotection at 100 nM of 1,25-(OH)$_2$D$_3$. Concentrations exceeding 1 μM did not prevent keratinocytes from apoptosis, which is consistent with the cytotoxic effect of 1,25-(OH)$_2$D$_3$ (**Fig 2B**). As UV and TNF-α have been found to increase ceramide levels, we investigated the effect of 1,25-(OH)$_2$D$_3$ on Annexin V binding and PI uptake induced by these stimuli. Indeed, 1,25-(OH)$_2$D$_3$ inhibited the expression of the apoptotic traits induced by UV and TNF-α demonstrating a protective effect of 1,25-(OH)$_2$D$_3$ independent of the apoptosis-triggering stimulus (**Fig 3**).

To investigate the specificity of 1,25-(OH)$_2$D$_3$ on cell survival, keratinocytes were also preincubated with calcipotriol and cholecalciferol. In analogy to the results with 1,25-(OH)$_2$D$_3$, a resistance against apoptosis was also detected with 100 nM of calcipotriol whereas pretreatment of keratinocytes with 100 nM of cholecalciferol, the almost inactive precursor of 1,25-(OH)$_2$D$_3$, neither diminished Annexin V binding nor PI uptake induced by ceramide.

Protective effect of SPP on cell survival in ceramide-induced apoptosis Recently, we have identified SPP as a regulatory component in the prevention of apoptosis in HL-60 cells after treatment with 1,25-(OH)$_2$D$_3$. Therefore, we examined the role of SPP on apoptosis in human keratinocytes. Cells were pretreated with SPP followed by the incubation with C2-cer. Exposure of keratinocytes to SPP prevented ceramide-induced
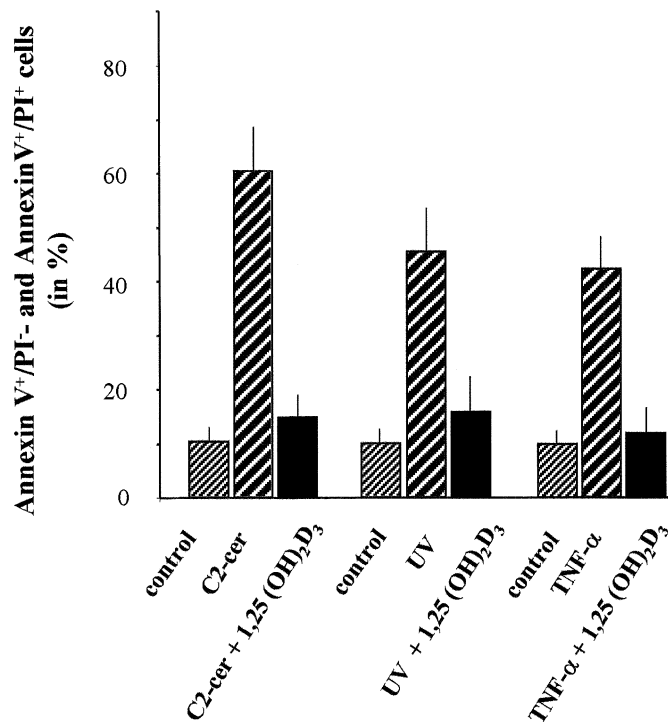
Figure 3. Cytoprotective effect of 1,25-(OH)₂D₃ against ceramide-, UV-, and TNF-α-induced apoptosis in human keratinocytes. Human keratinocytes were treated with vehicle or 1,25-$(OH)_2D_3$ for 24 h. Then apoptosis was induced by addition of 25 μM C2-cer for 3 h, UVB irradiation (11.76 mJ per cm^2) or 10 ng TNF-α per ml in the presence of 1 μg actinomycin per ml. After 24 h double staining with Annexin V-FITC and PI was performed as described in *Materials and Methods*. Data are the mean ± SD of triplicate assays.
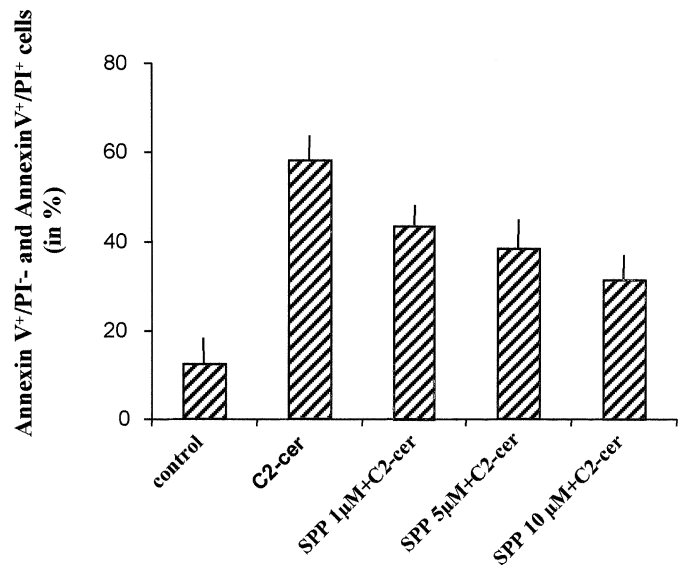

Figure 4. Cytoprotective effect of SPP against ceramide-induced apoptosis in human keratinocytes. Human keratinocytes were treated with vehicle or the indicated concentrations of SPP added as a bovine serum albumin complex 10 min prior to the addition of 25 μM C2-cer. After 3 h double staining with Annexin V-FITC and PI was performed. Values are the mean ± SD of triplicate assays. Results were repeated in at least three independent experiments.

apoptosis corroborating other studies with HL-60 and U937 cells. SPP suppressed C2-cer-induced Annexin V binding and PI uptake by more than 55%. The resistance against apoptosis was concentration dependent showing an optimal concentration at 10 μM SPP (**Fig 4**).

Involvement of SPP in the cytoprotective effect of 1,25-(OH)₂D₃ We next investigated whether the protective effect of 1,25-$(OH)_2D_3$ is mediated by the formation of SPP. For this reason the activity of sphingosine kinase, the critical enzyme in the formation of SPP, was determined after exposure to 1,25-$(OH)_2D_3$. Indeed, 1,25-$(OH)_2D_3$ increased sphingosine kinase activity (**Fig 5A**). A significant enhancement was first detected after 8 h and highest levels were observed after 24 h of exposure, which correlates with the cytoprotective effect of 1,25-$(OH)_2D_3$. The stimulation of sphingosine kinase was also concentration dependent (**Fig 5B**). A slight increase occurred already at 1 nM of 1,25-$(OH)_2D_3$, whereas highest enzyme activities leading to an 1.7-fold increase were detected at 100 nM of 1,25-$(OH)_2D_3$. During the time period of 24 h no phenotypic changes of differentiation were detected as measured by transglutaminase activity. Once more for the investigation of specificity, the influence of calcipotriol and cholecalciferol on sphingosine kinase activity was investigated. Calcipotriol (100 nM), which has been shown to possess a cytoprotective effect, increased sphingosine kinase activity in analogy to 1,25-$(OH)_2D_3$, whereas 100 nM of cholecalciferol did not affect the enzyme activity.

To prove whether stimulation of sphingosine kinase activity is associated with alterations of SPP mass levels, intracellular levels were examined after exposure to 1,25-$(OH)_2D_3$. SPP formation increased after treatment with 1,25-$(OH)_2D_3$ in accordance with the stimulation of sphingosine kinase activity and the cytoprotec-

tive effect of 1,25-$(OH)_2D_3$. The levels of SPP started to rise after 12 h and reached highest levels after 24 h of exposure to 100 nM of 1,25-$(OH)_2D_3$. At 24 h there was a significant increase of mass levels of SPP by almost 40% (**Table II**).

Effect of DMS on sphingosine kinase activity, ceramide levels, and cytoprotection in the presence of 1,25-(OH)₂D₃ To substantiate further the crucial role of SPP in the cytoprotective effect of 1,25-$(OH)_2D_3$ in keratinocytes, we utilized DMS, a well known inhibitor of sphingosine kinase. As there are controversial reports indicating that DMS also inhibits PKC activity, we measured the influence of DMS on PKC and sphingosine kinase activity after exposure to 1,25-$(OH)_2D_3$. The addition of 5 μM DMS did not reduce membrane-associated PKC activity in 1,25-$(OH)_2D_3$-treated keratinocytes (**Fig 6A**). In contrast, enhancement of sphingosine kinase activity was completely inhibited indicating that DMS is a specific inhibitor of this enzyme in keratinocytes only (**Fig 6B**).

Treatment of keratinocytes with DMS augmented the level of intracellular ceramides from 111.5 ± 4.4 pmol per 10^5 cells to 128.2 ± 3.1 pmol per 10^5 cells. In agreement Annexin V binding and PI uptake were increased confirming the crucial part played by SPP in the prevention of apoptosis. Moreover, when keratinocytes were pretreated with 1,25-$(OH)_2D_3$ in the presence of DMS the intracellular ceramide level was further enhanced to 149.2 ± 6.0 pmol per 10^5 cells suggesting that ceramide is the source of formation for SPP. In addition, the cytoprotective effect of 1,25-$(OH)_2D_3$ was completely blocked and the rate of Annexin V$^+$/PI$^-$ and Annexin V$^+$/PI$^+$ cells was enhanced. Most importantly, exogenous SPP overcame this inhibition, which proves the crucial part played by SPP in the cytoprotective effect of 1,25-$(OH)_2D_3$ (**Fig 7**).

Increase of Bcl-2 gene products by 1,25-(OH)₂D₃ and SPP As 1,25-$(OH)_2D_3$ has been identified to regulate Bcl-2 levels in thyrocytes, we investigated whether the anti-apoptotic action of 1,25-$(OH)_2D_3$ in keratinocytes is accompanied by an increase in Bcl-2 protein. In immunoblot analysis of Bcl-2, protein expression was assessed at 48 h after treatment with 1,25-$(OH)_2D_3$
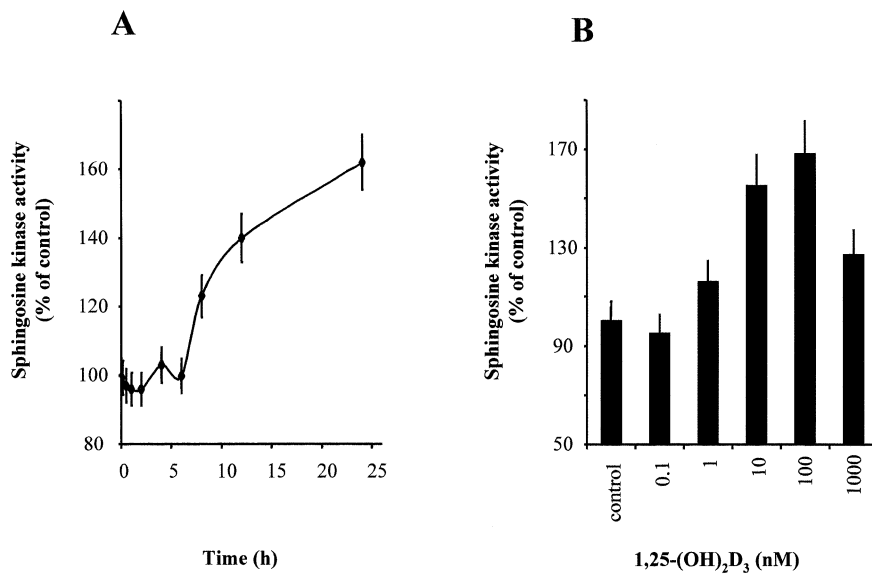
A   **B**

Figure 5. Influence of 1,25-(OH)$_2$D$_3$ on sphingosine kinase activity. Human keratinocytes were incubated with 100 nM of 1,25-(OH)$_2$D$_3$ up to 24 h (*A*) or treated with the indicated concentrations of 1,25-(OH)$_2$D$_3$ for 24 h (*B*). Sphingosine kinase activity was measured as described in *Materials and Methods*. Data are the mean ± SD of triplicate assays expressed as percent of control. The experiment was three times independently repeated obtaining similar results.

Table II. Effect of 1,25-(OH)$_2$D$_3$ on intracellular SPP levels and cytoprotection after ceramide–induced apoptosis in human keratinocytes[a]

Incubation time (h)	SPP levels after treatment with 1,25-(OH)$_2$D$_3$		Cytoprotection of 1,25-(OH)$_2$D$_3$ against C2-cer induced apoptosis	
	SPP level (pmol per 10^6 cells)	% of control	Annexin V$^+$/PI$^-$ and Annexin V$^+$/PI$^+$ cells (%)	Protection in %
0	7.1 ± 0.7	100	61.5 ± 7.7	0
6	6.1 ± 1.9	86	53.2 ± 8.4	17
12	8.0 ± 0.7	113	37.1 ± 5.3[b]	49
18	8.6 ± 1.0	122	19.3 ± 14.2[b]	85
24	9.8 ± 0.9[b]	138	14.3 ± 3.9[b]	95

[a]Keratinocytes were treated with 100 nM of 1,25-(OH)$_2$D$_3$ for the indicated time periods and SPP levels were subsequently measured as described in *Materials and Methods*. For measurement of the cytoprotective effect keratinocytes were pretreated for the indicated time periods with 100 nM of 1,25-(OH)$_2$D$_3$ followed by the addition of 25 µM of C2-cer for 3 h. Then Annexin V binding and PI uptake were measured as described. Control cells without C2-cer showed 11.5 ± 3.3 Annexin V$^+$/PI$^-$ and Annexin V$^+$/PI$^+$ cells. Data are mean ± SD of triplicate determinations.
[b]p ≤ 0.05 considered significant.

and SPP (**Fig 8**). Indeed, 1–100 nM of 1,25-(OH)$_2$D$_3$ increased Bcl-2 protein levels in a concentration-dependent manner, which is similar to the effective dose for its cytoprotective actions. Moreover, when cells were treated with 1 µM of 1,25-(OH)$_2$D$_3$ Bcl-2 protein levels were drastically diminished indicating a cytotoxic effect of 1,25-(OH)$_2$D$_3$ starting at this concentration. In agreement SPP also enhanced Bcl-2 protein levels in a concentration-dependent manner obtaining highest levels at a concentration of 10 µM.

DISCUSSION

The effects of 1,25-(OH)$_2$D$_3$ on cell growth arrest and induction of differentiation in keratinocytes have been well characterized, but the mechanism of its anti-proliferative and differentiating actions has not been fully clarified. 1,25-(OH)$_2$D$_3$ was the first compound identified as an inducer of sphingomyelin hydrolysis leading to enhanced ceramide formation (Okazaki *et al*, 1989). In keratinocytes 1,25-(OH)$_2$D$_3$ and calcipotriol increase ceramide levels via an autocrine mechanism by upregulation of TNF-α secretion (Geilen *et al*, 1997). The anti-proliferative effect in keratinocytes is mimicked by naturally occurring ceramides and the partial synthetic derivative C2-cer suggesting that these sphingolipids are important for cell growth arrest induced by 1,25-(OH)$_2$D$_3$ (Bielawska *et al*,

1992a). Well known inductors of programmed cell death, e.g., TNF-α or Fas-ligand and several lines of evidence suggest that ceramide plays a regulatory part in this process and that the formation of this sphingolipid does not appear to arise as a consequence of activation of the cell death machinery (Hannun, 1996; Zhang *et al*, 1996; Dbaibo *et al*, 1997). Enigmatically, 1,25-(OH)$_2$D$_3$ has been shown to act as both an inducer of apoptosis as well as a protective compound of cell survival indicating that apoptosis is not a universal response to 1,25-(OH)$_2$D$_3$ treatment. In breast cancer cell lines pretreatment with 10 nM 1,25-(OH)$_2$D$_3$ potentiates the effects of anti-cancer drugs (Wang *et al*, 2000), whereas in HL-60 cells and thyrocytes identical 1,25-(OH)$_2$D$_3$ concentrations make cells resistant to chemotherapeutic agents (Xu *et al*, 1993; Wang *et al*, 1999). Previously, it has been reported that 1,25-(OH)$_2$D$_3$ induces apoptosis in human keratinocytes as well as in HaCaT cells (Benassi *et al*, 1997; Bektas *et al*, 2000); however, it should be mentioned that this induction of apoptosis became first visible after 3 d with concentrations higher than 1 µM of 1,25-(OH)$_2$D$_3$. This is not contradictory to our results as we have shown an increase of Annexin V binding and PI uptake induced by 1,25-(OH)$_2$D$_3$ starting at this concentration as well. Most interestingly, here we report for the first time that treatment of keratinocytes with lower 1,25-(OH)$_2$D$_3$ concentrations (1–100 nM), which are

A

B

Figure 6. DMS inhibits sphingosine kinase activity but not PKC activity in keratinocytes. Keratinocytes were incubated in the absence or presence of DMS (5 μM) for 3 h and then treated with 1,25-(OH)₂D₃ 100 mN for 24 h. Subsequently, membrane-associated PKC activity (*A*) or sphingosine kinase activity (*B*) was measured as described in *Materials and Methods*. Values are the mean ± SD of triplicate assays. The results are from one representative experiment of three.
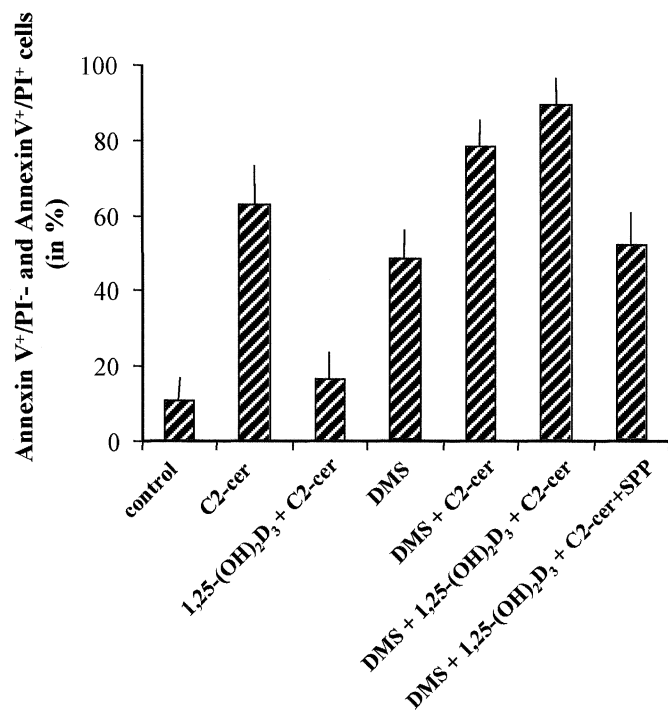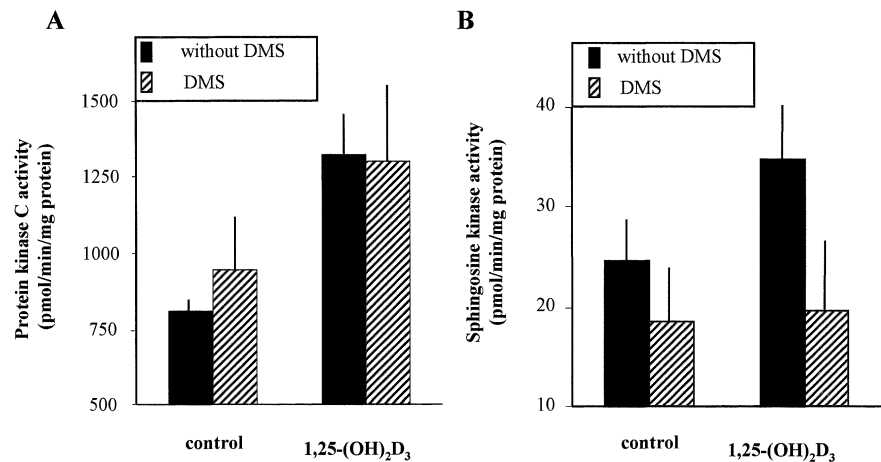

Figure 7. DMS inhibits the cytoprotective effect of 1,25-(OH)₂D₃. Keratinocytes were treated for 24 h with or without 100 nM of 1,25-(OH)₂D₃, 5 μM of DMS, and 10 μM of SPP as indicated. Then double staining with Annexin V-FITC and PI was performed. *Data points,* mean percentage Annexin V⁺/PI⁻ and Annexin V⁺/PI⁺ cells remains; *bars,* SD. Similar results were obtained in three additional experiments.
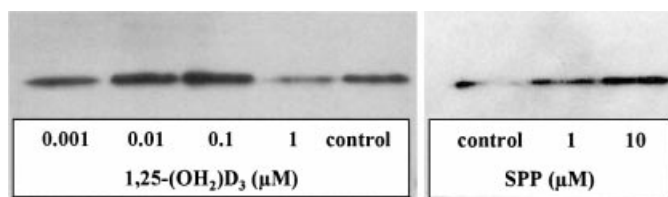

Figure 8. Influence of 1,25-(OH)₂D₃ and SPP on Bcl-2 protein expression. Total proteins were obtained from human keratinocytes after 48 h of stimulation with 1,25-(OH₂)D₃ or SPP with the indicated concentrations. Fifteen micrograms of protein per lane were loaded on a 12.5% polyacrylamide gel and western blot analysis were performed as described in *Materials and Methods*. Individual bands represent protein expression of Bcl-2 at 26 kDa.

effective to induce cell growth arrest and differentiation, do not enhance apoptosis despite the formation of ceramide. Even more, 1,25-(OH)₂D₃ makes keratinocytes resistant to ceramide-induced cell death.

Data presented here demonstrate that SPP mediates the survival effect of 1,25-(OH)₂D₃. The anti-apoptotic effect of 1,25-(OH)₂D₃ correlates with the formation of SPP, which is able to rescue keratinocytes from ceramide-mediated cell death. Moreover, the cytoprotective effect of 1,25-(OH)₂D₃ is completely blocked by the addition of DMS, which has been identified as a competitive inhibitor of sphingosine kinase and, therefore, is used to investigate the biologic role of SPP (Edsall *et al*, 1998); however, DMS has also been shown to inhibit PKC activity *in vitro* (Igarashi and Hakomori, 1989; Felding-Habermann *et al*, 1990; Khan *et al*, 1990). In keratinocytes, DMS effectively inhibits 1,25-(OH)₂D₃-stimulated sphingosine kinase activity without affecting PKC activity significantly. This is in agreement with studies in U937 monoblastic leukemia cells, Swiss 3T3 fibroblasts, and PC12 pheochromocytoma cells (Edsall *et al*, 1998).

Currently, it is less well understood, how 1,25-(OH)₂D₃ and SPP mediate their survival strategy. In HL-60 cells it has been clearly demonstrated that a variety of prominent regulatory components of apoptosis such as c-myc and p53 are not involved in the protective effect of 1,25-(OH)₂D₃ (Wolf and Rotter, 1985; Solary *et al*, 1993; Wang and Studzinski, 1997).

An important target of 1,25-(OH)₂D₃ and SPP may be alterations in the levels of Bcl-2 family members. Apoptotic response is often dependent on the ratio of apoptosis inducing (Bax, Bcl-Xs) to apoptosis-protective members (Bcl-2, Bcl-xl, Mcl-1) (McDonnell *et al*, 1996). Indeed, treatment of HL-60 cells with 1,25-(OH)₂D₃ results in a progressive increase of mitochondrial Mcl-1 protein and a transient increase in Al protein level. As in these investigations Raf-1 protein has also been detected in the mitochondrial fractions, a recruitment of activated Raf-1 to the mitochondria membrane takes place, which increases Mcl-1-mediated resistance to apoptosis (Wang and Studzinski, 1997). In this context it is of interest that SPP has been recognized as a positive regulator of Raf-1 protein and a suppressor of Bax-protein (Wu *et al*, 1995; Goetzl *et al*, 1999). Besides this, overexpression of Bcl-2 protein has been shown to protect cells from C2-cer induced apoptosis (Zhang *et al*, 1996). In human thyrocytes 1,25-(OH)₂D₃ increases Bcl-2 messenger RNA and protein levels, elevates the Bcl-2/Bax ratio, and protects thyrocytes from apoptosis (Wang *et al*, 1999), whereas in breast cancer cells 1,25-(OH)₂D₃ decreases Bcl-2 expression and induces apoptosis (James *et al*, 1996).

In keratinocytes an increase of Bcl-2 has been shown to mediate anti-apoptotic actions as well (Pincelli *et al*, 1997), whereas a decrease induced by C2-cer is connected to an enhanced apoptotic DNA fragmentation (Di Nardo *et al*, 2000). Nerve growth factor, which is synthesized and released by keratinocytes, acts as a survival factor through its high-affinity receptor by altering the Bcl-2/Bax

ratio and has been identified as a very potent activator of sphingosine kinase (Rius *et al*, 1997). Indeed, here we show an anti-apoptotic action of 1,25-$(OH)_2D_3$ as well as SPP, which is associated with an acceleration of Bcl-2 protein expression.

In analogy to the Bcl-2/Bax rheostat, a variety of investigations implicate that the ratio of intracellular ceramide and SPP levels are a determining factor in the fate of the cell (Cuvillier *et al*, 1996; Kleuser *et al*, 1998). Several targets of SPP besides Raf-1 protein activation have been elucidated. It has been suggested that the stress-activated protein kinase (SAPK/JNK) pathway is involved in ceramide-mediated apoptosis and SPP prevents the activation of these kinase cascades. Moreover, SPP stimulates extracellular signal–regulated kinases Erk1 and Erk2 suggesting that specific activation of different family members of mitogen-activated protein kinases are responsible for the opposing effects of ceramide and SPP on apoptosis (Cuvillier *et al*, 1996). Additionally, SPP counteracts activation of caspases, a family of aspartate-specific cysteine proteases involved in the induction of apoptosis (Cuvillier *et al*, 1998).

The clinical implications of our findings are complex. The main pathophysiologic features of psoriasis include an increase in keratinocyte proliferation and abnormal cell differentiation as well as the presence of a dermal inflammatory cell filtrate (Lowe *et al*, 1995). 1,25-$(OH)_2D_3$ and calcipotriol possess the capacity to induce cell growth arrest and differentiation.

In conclusion, the present study suggests that the inhibition of cell growth is not a consequence of apoptosis as 1,25-$(OH)_2D_3$ has a cytoprotective effect in keratinocytes. These findings are important for the optimal dose of 1,25-$(OH)_2D_3$ in the treatment of hyperproliferative skin diseases. Moreover, the formation of SPP is crucial for the anti-apoptotic effect of 1,25-$(OH)_2D_3$. An exciting goal for future research will be to elucidate further the part played by SPP in epidermal cells.

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