Report

1,25-dihydroxyvitamin D₃ protects human keratinocytes against UV-B-induced damage

In vitro analysis of cell viability/proliferation, DNA-damage and -repair

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The skin is the only organ that has the capacity to photo-synthesize the biological active vitamin D metabolite 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] from 7-dehydrocholesterol (7-DHC), following exposure to ultraviolet (UV)-B irradiation. The aim of the present work was to investigate the capacity of 1,25(OH)₂D₃ to protect human keratinocytes (HaCaT) and squamous cell carcinoma cell lines (SCL-1) against the hazardous effects of UV-B irradiation. Human keratinocytes (HaCaT) and squamous cell carcinoma cell lines (SCL-1) were pretreated with 1,25(OH)₂D₃ over 48 hours and then irradiated once with UVB-radiation. We evaluated the results of several assays (colony-forming-unit-culture assay, WST-1-assay and crystal violet assay), comparing viability/proliferation in 1,25(OH)₂D₃-pretreated cells with controls that were pretreated with the carrier substance ethanol alone. Additionally, we analyzed the effects of 1,25(OH)₂D₃ on UV-induced DNA damage in HaCaT-keratinocytes by detection of cyclobutane pyrimidine dimers (CPDs) via dot blot analysis. We prove that 1,25(OH)₂D₃, in a concentration of 10⁻⁷ M, protects human keratinocytes (HaCaT) as well as squamous cell carcinoma cell lines (SCL-1) against the hazardous effects of UV-B-radiation (100 J/cm²–1,000 J/cm²) in vitro. Moreover, we demonstrate that the number of CPDs induced in HaCaT-keratinocytes after irradiation with UV-B (100 J/cm²–1,000 J/cm²) was decreased after pretreatment with 1,25(OH)₂D₃, as compared to carrier-treated controls. Analysis of the time course revealed that the elimination of UV-B-induced DNA-damage in HaCaT-keratinocytes occurs quicker when cells are pretreated with 1,25(OH)₂D₃ (as compared to controls). To put it in a nutshell, our data support the hypothesis that 1,25(OH)₂D₃ protects cultured

human keratinocytes against the hazardous effects of UV-B radiation.

Introduction

Increasing evidence indicates that the UV-B-mediated cutaneous photosynthesis of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the active form of vitamin D, represents an evolutionary highly-conserved endocrine system that protects the skin against environmental hazards, including ultraviolet radiation.¹ Since vitamin D₃ is formed in the skin by the action of ultraviolet-B radiation from 7-dehydrocholesterol (7-DHC), the major source of vitamin D is casual exposure to sunlight.² Endogenously synthesized and dietary vitamin D₃ is transported in the blood to the liver, where it is hydroxylated at C-25 position to form the circulating pro-hormone, 25-hydroxyvitamin D₃. The active hormone, 1,25-dihydroxyvitamin D₃ is then synthesized in the kidney by hydroxylation of 25-hydroxyvitamin D₃ at C-1 position.³ In recent years, it has been demonstrated that keratinocytes and numerous other cell types possess the enzymatic machinery (CYP27B1) to synthesize 1,25(OH)₂D₃.^{4,5} Although their function in skin is not fully understood, it is well accepted that the normal differentiation of keratinocytes is partly regulated by 1,25(OH)₂D₃.⁶ In this study, we analyzed the effects of 1,25(OH)₂D₃ (10⁻⁷ M) on proliferation and viability in UV-B-irradiated human keratinocytes (HaCaT) and squamous cell carcinoma cell lines (SCL-1) using colony-forming-unit-culture assay, WST-1-assay and crystal violet assay. On one hand, human skin is well known as a source of vitamin D and 1,25(OH)₂D₃ production. On the other hand, it is one of the major target organs of 1,25(OH)₂D₃. We now investigated the question whether 1,25(OH)₂D₃ protects the skin against the environmental hazard of UV-B radiation.

The hazardous effects of solar UV radiation, in particular of UV-B with a wavelength range between 290 and 320 nm, are well recognized as the most important etiological factor in the development of non-melanoma skin cancer.⁷ UVB induces photochemical changes in the skin that may lead to acute effects such as sunburn and immune suppression or chronic effects like premature skin aging and skin cancer.⁷ Approximately 70–80% of DNA damage

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induced by UV-B irradiation are cyclobutane pyrimidine dimers (CPDs), mostly formed between adjacent thymidine nucleotides on the same DNA strand.⁸ Due to the fact that UV-B radiation plays a significant role in the genesis of DNA damage but also in the production of vitamin D in the skin, the aim of this study was to investigate the capacity of 1,25(OH)₂D₃, produced in the skin after exposure to UV-B-light, to protect human keratinocytes (HaCaT) and squamous cell carcinoma cell lines (SCL-1) against the hazardous effects of ultraviolet B irradiation. Additionally, we investigated the ability of 1,25(OH)₂D₃ to influence UV-B-induced DNA damage in HaCaT-keratinocytes by detection of cyclobutane pyrimidine dimers (CPDs) via dot blot analysis.

Results

Pharmacological doses of 1,25(OH)₂D₃ inhibit the growth of squamous cell carcinoma cell lines (SCL-1) and, depending on culture conditions, inhibit or increase the growth of HaCaT-keratinocytes. We demonstrate that human keratinocytes and squamous cell carcinoma cell lines represent target cells for 1,25(OH)₂D₃. Incubation of HaCaT-keratinocytes and SCL-1 cells with 1,25(OH)₂D₃ in a concentration of 10⁻⁷ M resulted in a significant suppression of cell proliferation in SCL-1 and a significant increase of cell proliferation in HaCaT-keratinocytes. As detected using colony-forming-unit-culture assay (with a total period of growth of 10 days), pretreatment with 1,25(OH)₂D₃ increases colony-forming of HaCaT-keratinocytes up to 137.4% and suppresses colony-forming of squamous cell carcinoma cell lines (SCL-1) down to 42.3%, as compared to controls that were treated with the carrier substance ethanol alone (Fig. 1A and B). In contrast, crystal violet and WST-1 assays reveal antiproliferative effects both on HaCaT-keratinocytes and SCL-1 cells, that were most pronounced after 48 h (Figs. 2 and 3).

UV-B radiation exerts antiproliferative, cytotoxic effects on HaCaT-keratinocytes and squamous cell carcinoma cell lines (SCL-1). Treatment of HaCaT-keratinocytes and squamous cell carcinoma cell lines (SCL-1) with UV-B radiation (500 J/cm², 800 J/cm², 1,000 J/cm²) resulted in a dose-dependent and significant inhibition of cell proliferation, as assessed by WST-1 and CV assays. The effect on HaCaT-keratinocytes is significant at 12 h (WST) and 24 h (CV) after irradiation with 500 J/cm² (data not shown) and most pronounced after 48 h (Fig. 2A and B). In our experiments with SCL-1 (WST and CV), dose-dependent cytotoxic effects of UV-B radiation are found 24 h after irradiation with 500 J/cm², but are significant only 48 h after irradiation with 1,000 J/cm² (Fig. 3A and B). The results of the CFUc-assay demonstrate a reduction of the number of colonies down to 62.3% (in medium-treated cells) and 56.8% (in ethanol-controls) for HaCaT-keratinocytes, 10 days after irradiation with 100 J/cm² UV-B. The numbers of SCL-1 colonies in CFUc-assay are reduced to 57.91% (in medium-treated cells) and 56.75% (in ethanol-controls) 10 days after irradiation with 100 J/cm² UV-B, compared to the unirradiated controls. Irradiation of HaCaT-keratinocytes and SCL-1 cells with UV-B radiation in a dose of 500 J/cm² and more (800 J/cm², 1,000 J/cm²) has such a cytotoxic effect on the cells, that all colonies are dead 10 days after irradiation (Fig. 1A and B).

Figure 1. Pharmacological doses of 1,25(OH)₂D₃ increase the growth of HaCaT-keratinocytes (A) and inhibit the growth of squamous cell carcinoma cell lines (SCL-1, B), as assessed by colony-forming-unit-culture assay, and protect the cells against the cytotoxic effects of UV-B radiation. HaCaT-keratinocytes and SCL-1 cells were pretreated over 48 h with 1,25(OH)₂D₃ (10⁻⁷M), vehicle (ethanol) or medium alone and then irradiated once with UV-B light (0–1000 J/cm²). After irradiation, cells were provided with fresh medium. The colonies were stained with crystal violet after a growth time period of 7 days (post-irradiation) and then counted under an enlargement-lamp. The results show that incubation of HaCaT-keratinocytes with 1,25(OH)₂D₃ in a concentration of 10⁻⁷ M results in a significant increase of cell proliferation (A). On the other hand, incubation of SCL-1 cells with 1,25(OH)₂D₃ in a concentration of 10⁻⁷ M results in a significant inhibition of cell proliferation (B). After irradiation with 100 J/cm², 1,25(OH)₂D₃ protects the cells against the hazardous effects of UV-B radiation (A and B). After irradiation with UV-B doses of 500 J/cm² and more (data not shown), the cytotoxic effects of UV-B light overbalance the protective effects of 1,25(OH)₂D₃ and no HaCaT- or SCL-1-colony survives (A and B). *p < 0.05; **p < 0.01; #p > 0.05.

1,25(OH)₂D₃ protects human keratinocytes (HaCaT) and squamous cell carcinoma cell lines (SCL-1) against the hazardous effects of UV-B radiation. Incubation of HaCaT-keratinocytes and SCL-1 cells with 1,25(OH)₂D₃ (10⁻⁷ M) protects both cell lines against the hazardous effects of UV-B radiation, as assessed by CFUc-assay. Comparison of the 1,25(OH)₂D₃-pretreated HaCaT-keratinocytes with controls show that the number of colonies counted after a growth period of 10 days is significantly reduced to 62.3% (medium-control) and 56.8% (ethanol-control), whereas the number of colonies grown with a supplement of 1,25(OH)₂D₃ (10⁻⁷ M) is only reduced to 90.36% (Fig. 1A). In SCL-1, the number of colonies counted after 10 days is reduced

Figure 2. Pharmacological doses of 1,25(OH)₂D₃ inhibit the growth of HaCaT-keratinocytes as assessed by WST-1 (A) and crystal violet (B) assay, and protect the cells against the cytotoxic effects of UV-B radiation. HaCaT-keratinocytes were pretreated over 48 h with 1,25(OH)₂D₃ (10⁻⁷M), vehicle (ethanol) or medium alone and then irradiated once with UV-B light (0–1000 J/cm²). After irradiation, cells were provided with fresh medium. 0 h, 6 h, 12 h, 24 h and 48 h after irradiation, the absorption of the samples was measured in an ELISA-reader. Data shown in (A) and (B) were measured after 48 h. The results show that incubation of HaCaT-keratinocytes with 1,25(OH)₂D₃ in a concentration of 10⁻⁷ M results in a significant inhibition of cell proliferation. After irradiation with 100–1000 J/cm², 1,25(OH)₂D₃ protects the cells against the hazardous effects of UV-B radiation. After irradiation with UV-B doses of 500 J/cm² and more, we observe a dose-dependent reduction of the NADH contents (WST-1) resp. of the crystal violet contents (crystal violet assay) of the cells with increasing UV-B doses. *p < 0.05; **p < 0.01; #p > 0.05.

to 57.91% (medium-control) and 56.75% (ethanol-control), whereas the number of colonies grown with a supplement of 1,25(OH)₂D₃ (10⁻⁷ M) increases up to 128.18% from the initial value (Fig. 1B). In WST-1 and CV assays, the viability of SCL-1 and HaCaT-keratinocytes diminishes with increasing doses of UV-B irradiation. Nevertheless, cells treated with 1,25(OH)₂D₃ (10⁻⁷ M) show significantly higher viability/proliferation, as compared to ethanol-controls (Figs. 2 and 3).

Treatment with UV-B radiation (100 J/cm²–1,000 J/cm²) increases the number of cyclobutane pyrimidine dimers (CPDs) in HaCaT-keratinocytes. Analyzing the presence of cyclobutane pyrimidine dimers via dot blot analysis, we demonstrate that the number of CPDs increases with rising doses of UV-B radiation

Figure 3. Pharmacological doses of 1,25(OH)₂D₃ inhibit the growth of squamous cell carcinoma cell lines (SCL-1), as assessed by WST-1 (A) and crystal violet (B) assay, and protect the cells against the cytotoxic effects of UV-B radiation. SCL-1 cells were pretreated over 48 h with 1,25(OH)₂D₃ (10⁻⁷ M), vehicle (ethanol) or medium alone and then irradiated once with UV-B light (0–1000 J/cm²). After irradiation, cells were provided with fresh medium. 0 h, 6 h, 12 h, 24 h and 48 h after irradiation, the absorption of the samples was measured in an ELISA-reader. Results after 48 h are shown in this figure. The results show that incubation of SCL-1 cells with 1,25(OH)₂D₃ in a concentration of 10⁻⁷ M results in a significant inhibition of cell proliferation. After irradiation with 100–1000 J/cm², 1,25(OH)₂D₃ protects the cells against the hazardous effects of UV-B radiation. After irradiation with UV-B doses of 500 J/cm² and more, we observe a dose-dependent reduction of the NADH contents (WST-1) resp. of the crystal violet contents (crystal violet assay) of the cells with increasing UV-B doses. *p < 0.05; **p < 0.01; #p > 0.05.

(0 J/cm², 100 J/cm², 1,000 J/cm²). Compared to the initial number, the amount of CPDs increases in HaCaT keratinocytes up to 1605% in medium-controls and up to 2821% in ethanol-controls after irradiation with 1,000 J/cm² UV-B (Fig. 4).

The number of cyclobutane pyrimidine dimers in HaCaT-keratinocytes after irradiation with UV-B (100–1,000 J/cm² is decreased when cells are pretreated with 1,25(OH)₂D₃ (10⁻⁷ M). We found no significant difference between the number of CPDs in unirradiated HaCaT-keratinocytes pretreated with 1,25(OH)₂D₃ (10⁻⁷ M) as compared to vehicle-treated controls (ethanol-controls). After irradiation with 100 J/cm² and 1,000 J/cm², the number of CPDs in cells pretreated with 1,25(OH)₂D₃ (10⁻⁷ M) is lower than the number of CPDs in ethanol-controls (Fig. 5A–C).

Figure 4. Treatment with UV-B radiation (100–1000 J/cm²) increases the number of cyclobutane pyrimidine dimers (CPDs) in HaCaT-keratinocytes (dot blot analysis). HaCaT-keratinocytes were pretreated over 48 h with vehicle (ethanol) or medium alone and then irradiated once with UV-B light (0–1000 J/cm²). After irradiation, cells were provided with fresh medium. 0 h, 1 h, 3 h and 24 h after irradiation, DNA was isolated from the keratinocytes. The number of formed CPDs was quantified via dot blot analysis and enhanced chemiluminescence. This diagram shows the data established directly after irradiation (0 h). The results demonstrate an augmentation of the number of CPDs with increasing UV-B doses. (*p < 0.05/**p < 0.01/#p > 0.05)

Faster elimination of UV-B-induced DNA-damage when HaCaT-keratinocytes are pretreated with 1,25(OH)₂D₃ (10⁻⁷ M). Twenty-four hours after irradiation with UV-B (100 J/cm², 1,000 J/cm²), the number of CPDs in HaCaT keratinocytes is decreased, as compared to the amount of CPDs counted immediately after irradiation. In 1,25(OH)₂D₃-pretreated keratinocytes, the pixel density (attributed to the number of CPDs in the dot blot) diminishes on average from 20090.51 to 6901.13 pixel after irradiation with 100 J/cm², which signifies a reduction of 65.65% compared to the initial value. In ethanol-controls, the pixel density is reduced from 52493.29 to 22263.02 pixel, which means that the reduction comes up to merely 57.59% of the initial value. After irradiation with 1000 J/cm², the pixel density diminishes on average from 81712.44 to 37623.27 pixel in 1,25(OH)₂D₃-pretreated keratinocytes (reduction of 53.96% of the initial value) and from 85350.11 to 72341.62 pixel in ethanol-treated controls (reduction of 15.24% of the initial value). Table 1 and Figure 5 clarify and illustrate these results.

Discussion

In conclusion, our in vitro results identify 1,25(OH)₂D₃ as a protective agent against the hazardous effects of UV-B radiation in human keratinocytes. Three well-known biological effects are at least in part responsible for cytotoxic effects of UV-B radiation: the formation of CPDs and other DNA-photoproducts, the induction of apoptosis and the production of interleukin-6.^{9,10} Due to the fact that UV-B radiation is well recognized as the most important etiological factor in the development of non-melanoma skin cancer,⁷ intensive research has already been done to characterize these hazardous effects on human keratinocytes. The aim of the current study was to describe the actions of UV-B

Figure 5. Pharmacological doses of 1,25(OH)₂D₃ decrease the number of cyclobutane pyrimidine dimers (CPDs) in HaCaT-keratinocytes after irradiation with UV-B light (dot blot analysis). HaCaT-keratinocytes were pretreated over 48 h with 1,25(OH)₂D₃ (10⁻⁷ M) or vehicle (ethanol) and then irradiated once with UV-B light (0–1000 J/cm²). After irradiation, cells were provided with fresh medium. 0 h, 1 h, 3 h and 24 h after irradiation, DNA was isolated from the keratinocytes. The number of formed CPDs was quantified via dot blot analysis and enhanced chemiluminescence. (A) shows the data established directly after irradiation (0 h). (B) and (C) illustrate the time course. The results demonstrate a reduction of the number of CPDs after pretreatment with 1,25(OH)₂D₃. *p < 0.05; **p < 0.01; #p > 0.05.

radiation on viability and proliferation of human keratinocytes (HaCaT) and squamous cell carcinoma cell lines (SCL-1). We demonstrate that UV-B radiation exerts dose-dependent cytotoxic, antiproliferative effects on HaCaT-keratinocytes and SCL-1 cells. In CFUc, the cell colonies survived a growth time period of 10 days only up to a UV-B-dose of 500 J/cm². In WST-1 and CV assays, viability of the cells began to diminish 12 h (HaCaT)—24 h (SCL-1) after irradiation with 500 J/cm² and was most reduced after 48 h and following irradiation with higher UVB-doses (800 J/cm², 10,000 J/cm²). All in all, squamous

cell carcinoma cell lines (SCL-1) appear to be less sensitive to UV-B radiation, as compared to HaCaT-keratinocytes.

Differential effects of 1,25(OH)₂D₃ on cell proliferation and differentiation, that may depend on concentration and/or cell culture conditions, have previously been shown for keratinocytes.¹¹⁻¹⁵ Upgrading the results of Garach-Jehoshua et al.¹⁴ and Lyakhovich et al.¹⁵ our findings demonstrate an enhancing effect of 1,25(OH)₂D₃ (10⁻⁷ M) on proliferation of human keratinocytes (HaCaT), when cells are cultivated subconfluent on RPMI-medium + 10% FCS + 1% BSA over a time period of 10 days (CFUc-assay). As we could show in WST-1- and CV-assay under distinct cell culture conditions, 1,25(OH)₂D₃ has an antiproliferative effect on HaCaT-keratinocytes at earlier time points (48 h). On the other hand, proliferation of squamous cell carcinoma cell lines was clearly inhibited by 1,25(OH)₂D₃ (10⁻⁷ M) in all experiments of this study (CFUc, WST-1, CV). Hager et al. showed in 2001 that 1,25(OH)₂D₃ suppresses the growth of squamous cell carcinoma cell lines in larynx and tongue carcinoma by p21- and p27-regulated cell cycle arrest in G₀/G₁.¹⁶ In agree with these findings, we here demonstrate that 1,25(OH)₂D₃ in a concentration of 10⁻⁷ M also inhibits proliferation and reduces viability in human skin squamous cell carcinoma cell lines.

Vitamin D₃ is synthesized in human skin after exposure to UV-B and then, mediated by 25-hydroxylase (CYP27A1) and 1 α -hydroxylase (CYP27B1) in keratinocytes and other cell types, it is converted in the active form 1,25(OH)₂D₃. This leads to the question whether 1,25(OH)₂D₃ acts as a natural protector against UV-B radiation. In several recent studies it could be demonstrated that 1,25(OH)₂D₃ protects human keratinocytes against UV-B-induced cell-damage.^{9,10,17,18} Mechanisms are multiple and mostly associated to UV-B-induced suppression of apoptosis.⁹ Significant cytoprotective effects were shown after incubation with 1,25(OH)₂D₃ in a concentration of 10⁻⁸ M for at least 8 hours.⁹ The aim of the present work was not only to analyze protective effects of 1,25(OH)₂D₃ against UV-B radiation in human keratinocytes. We also dedicated our experiments to investigate photoprotective effects in squamous cell carcinoma cell lines (SCL-1). Thus, our results confirm significant photoprotective effects after pretreatment of HaCaT-keratinocytes as well as squamous cell carcinoma cell lines (SCL-1) with 1,25(OH)₂D₃ in a concentration of 10⁻⁷ M over a time period of 48 hours. Cytotoxic effects of UV-B radiation were reduced up to an irradiation with 1,000 J/cm² (WST-1 and CV assay).

Another finding of relevance is the reduction of UV-B-induced DNA-damage by pretreatment with 1,25(OH)₂D₃ in a concentration of 10⁻⁷ M 48 hours before irradiation. CPDs were detected via dot blot analysis to quantify the DNA-damage after

Table 1 Pharmacological doses of 1,25(OH)₂D₃ decrease the number of cyclobutane pyrimidine dimers (CPDs) in HaCaT-keratinocytes after irradiation with UV-B light (dot blot analysis)

a)		100 J/cm ²		reduction of:	
CPDs (mean value)		0h	24h	CPD	%
1,25(OH) ₂ D ₃		20090,51	6901,13	13189,38	65,65
ethanol		52493,29	22263,02	50230,27	57,59
b)		1000 J/cm ²		reduction of:	
CPDs (mean value)		0h	24h	CPD	%
1,25(OH) ₂ D ₃		81712,44	37623,27	44089,17	53,96
ethanol		85350,11	72341,62	13008,49	15,24

Table 1. Pharmacological doses of 1,25(OH)₂D₃ decrease the number of cyclobutane pyrimidine dimers (CPDs) in HaCaT-keratinocytes after irradiation with UV-B light (dot blot analysis). HaCaT-keratinocytes were pretreated over 48 h with 1,25(OH)₂D₃ (10⁻⁷ M) or vehicle (ethanol) and then irradiated once with UV-B light (100 J/cm² and 1000 J/cm²). After irradiation, cells were provided with fresh medium. 0 h, 1 h, 3 h and 24 h after irradiation, DNA was isolated from the keratinocytes. The number of formed CPDs was quantified via dot blot analysis and enhanced chemiluminescence. This table shows the data established directly after irradiation (0 h) and those established 24 h later to illustrate the reduction of the number of CPDs in the first 24 h after irradiation. The results demonstrate a faster elimination of UV-B-induced DNA-damage when HaCaT-keratinocytes are pretreated with 1,25(OH)₂D₃ (10⁻⁷ M).

irradiation with UV-B (0 J/cm²–1,000 J/cm²). As shown in former studies,¹⁹ we attested a dose-dependent rise in the number of CPDs with increasing UV-B-doses. At the same time, our experiments demonstrate that the number of CPDs after irradiation of cells pretreated with 1,25(OH)₂D₃ is lower than the number of CPDs in ethanol-controls, which speaks in favour of a photoprotective effect of 1,25(OH)₂D₃. Moreover, observation of the time course revealed that the number of CPDs decreases within the first 24 h after irradiation. This finding suggests the beginning of DNA-repair in this period of time. To complete the data of previous investigations,^{18,20} the present work proves the fact that the elimination of UVB-induced DNA-damage in HaCaT-keratinocytes occurs faster when cells are pretreated with 1,25(OH)₂D₃ (10⁻⁷ M).

To put it in a nutshell, our data support the hypothesis that 1,25-dihydroxyvitamin D₃ protects cultured human keratinocytes against the hazardous effects of ultraviolet B radiation.

Materials and Methods

Cell culture. Normal human keratinocytes (HaCaT) and squamous cell carcinoma cell lines (SCL-1) were maintained in RPMI 1640 medium (PAA Laboratories) supplemented with 1% L-glutamine and 10% foetal calf serum (Biochrom). They were grown in a humidified atmosphere of 5% CO₂ at 37°C. Cell culture medium was changed every two days.

1,25-dihydroxyvitamin D₃-treatment. When treating cells with 1,25-dihydroxyvitamin D₃, 1% of BSA was added to the medium. Moreover, we supplemented a mix of 1% penicillin/streptomycin. The rest of the cell culture conditions were identical to those described above. Vitamin D-treatment was accomplished by addition of 1,25-dihydroxyvitamin D₃ in a concentration of 10⁻⁷ M (5 μ l 1,25-dihydroxyvitamin D₃ per 5 ml medium). Due to the fact that the utilized 1,25-dihydroxyvitamin D₃ was solved in ethanol to a concentration of 10⁻⁴ M, we compared

the 1,25-dihydroxyvitamin D₃-treated cells with controls that we treated only with pure ethanol (5 µl ethanol per 5 ml medium). Another control was effectuated with medium only. Cells were pretreated with 1,25-dihydroxyvitamin D₃ 48 h before irradiation with UV-B. After irradiation, cell culture medium was changed every 2 days with supplementation of 5 µl of 1,25-dihydroxyvitamin D₃ resp. ethanol or medium.

UV-B irradiation. Before irradiation with the UV-B-Lamp (Waldmann), cells were washed with phosphate-buffered saline (PBS) and then irradiated through a thin film of PBS. Depending on the experiments, cells were irradiated with UV-B-doses between 100 J/cm² and 1,000 J/cm², as measured with a dosimeter (Waldmann). After irradiation, cells were provided with fresh medium.

Colony-forming-unit culture assay. HaCaT-keratinocytes were seeded in 10 cm culture plates, 2,000 cells in 5 ml of medium per plate. Slower proliferating SCL-1 cells were seeded denser with a number of 5,000 cells in 5 ml of medium per 10 cm culture plate. Cells were pretreated with 1,25-dihydroxyvitamin D₃ over 48 h and then irradiated with UV-B (0 J/cm², 100 J/cm², 500 J/cm², 800 J/cm² and 1,000 J/cm²). For the purpose of control, each 1,25-dihydroxyvitamin D₃-pretreated culture plate comes along with one medium- and one ethanol-control. After irradiation, cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Cell culture medium was changed every two days. After a growth time period of seven days, the colonies were stained with crystal violet and then counted under an enlargement-lamp.

Crystal violet staining. Medium was removed from the 10 cm culture plates and cells were washed two times with 3 ml of cold PBS and then incubated with 5 ml of 70% ethanol at 4°C for at least 30 min. After removing the ethanol, cells were incubated with 5 ml of a 0.1% crystal violet solution for 30 min at room temperature. Crystal violet was then removed and the cells were washed with water. Cell culture plates were dried overnight and the colonies were counted the following day.

WST-1 assay. HaCaT-keratinocytes and SCL-1 cells were seeded in 96-well culture plates (3 × 10³ cells in 100 µl RPMI + 10% FCS-medium/well) and incubated in a humidified atmosphere of 5% CO₂ at 37°C for 24 h. The wells were filled as follows:

In 6-wells the cells were pretreated with 1,25-dihydroxyvitamin D₃ 48 hours before irradiation. As independent controls, cells were treated in another 6-wells with ethanol resp. medium.

The remaining wells were filled with PBS. Each well contained 100 µl. Vitamin D-treatment was effectuated as described above. Cells were then irradiated with UVB: 0 J/cm², 100 J/cm², 500 J/cm², 800 J/cm² or 1,000 J/cm². 0 h, 6 h, 12 h, 24 h and 48 h after irradiation, the absorption of the samples was measured in an ELISA-reader as follows: 10 µl of WST-1-reagent were added to each well and the absorption of the samples was measured 0 h, 1 h, 2 h, 3 h and 4 h after adding the reagent at 450 nm using the ELISA-reader.

Crystal violet assay. Seeding, treatment and irradiation of HaCaT-keratinocytes and SCL-1 cells was effectuated as previously described for the WST-1 assay. Afterwards, medium was removed from the 96-well culture plates and cells were washed two times with 200 µl of cold PBS/well and then incubated with 150 µl of 70% ethanol at 4°C for at least 30 min. After removing the ethanol, cells were incubated with 100 µl of 0, 1% crystal violet solution/well for 30 min at room temperature. Crystal violet was then removed and the cells were washed with water. Cell culture plates were dried overnight. The next day, wells were incubated with 200 µl of 70% ethanol for at least 30 min at room temperature to dissolve the crystal violet stain before measuring its absorption at 550 nm in a Titertek Multiskan Plus-Reader.

Dot blot analysis. HaCaT-keratinocytes were pretreated with 1,25-dihydroxyvitamin D₃, ethanol and medium as described above. DNA was isolated 0 h, 1 h, 3 h and 24 h after irradiation of the cells with 0 J/cm², 100 J/cm² or 1,000 J/cm². DNA isolation was carried out with High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's manual. DNA-concentration in each sample was determined by spectroscopy with an Ultrospec 1000 photometer. The volume corresponding to 2 µg of DNA was filled up with autoclaved water to a terminal volume of 10 µl. 30 µl of denaturation buffer were added to each sample before the mix was applied to a nitrocellulose membrane on the dot blot apparatus (Bio-Dot SF microfiltration apparatus, Bio Rad). A vacuum pump permitted adsorption of the DNA to the nitrocellulose membrane. 100 µl of neutralization buffer were then applied to the membrane and also adsorbed using the vacuum pump. The membrane was removed from the dot blot apparatus and dried for 15 min at 80°C in an oven (Heraeus). Non-specific sites were blocked by soaking the membrane for 1 h in 2% blocking reagent in TBST. The membrane was then incubated overnight with 10 µl of primary antibody (thymidine-dimer-antibody, Kamiya Biomedical Company) in 5 ml of 2% blocking reagent in TBST (1:500 dilution). Membrane was washed three times with TBST (3 × 5 min) and then incubated for 1 h with secondary antibody (goat-anti-mouse IgG, Sigma) in a 1:1,000 dilution in 2% blocking reagent in TBST. Membrane was rewashed with TBST (3 × 5 min) and then incubated in plastic wrap with ECL western blotting detection reagent 1 (Perbio Science, Germany) for 5 min. Afterwards, the membrane was exposed to an X-ray film (lumi-film chemiluminescent detection film, Roche) for 30 s, 1 min, 5 min and 30 min in the dark room and the results were quantified with the Image J analysis software 1.36.

Statistical analysis. All data are represented as a mean for at least four experiments. Statistical significance was calculated by a two-tailed Student's t-test for unpaired samples, using the

Microsoft EXCEL software. Mean differences were considered to be significant when $p < 0.05$.

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