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1,25-Dihydroxyvitamin D_3 Inhibits Ultraviolet B-Induced Apoptosis, Jun Kinase Activation, and Interleukin-6 Production in Primary Human Keratinocytes

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Abstract We investigated the capacity of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] to protect human keratinocytes against the hazardous effects of ultraviolet B (UVB)-irradiation, recognized as the most important etiological factor in the development of skin cancer. Cytoprotective effects of 1,25$(OH)_2D_3$ on UVB-irradiated keratinocytes were seen morphologically and quantified using a colorimetric survival assay. Moreover, 1,25$(OH)_2D_3$ suppressed UVB-induced apoptotic cell death. An ELISA, detecting DNA-fragmentation, demonstrated that pretreatment of keratinocytes with 1,25$(OH)_2D_3$ 1 μM for 24 h reduced UVB-stimulated apoptosis by 55–70%. This suppression required pharmacological concentrations 1,25$(OH)_2D_3$ and a preincubation period of several hours. In addition, 1,25$(OH)_2D_3$ also inhibited mitochondrial cytochrome c release (90%), a hallmark event of UVB-induced apoptosis. Furthermore, we demonstrated that 1,25$(OH)_2D_3$ reduced two important mediators of the UV-response, namely, c-Jun-NH_2-terminal kinase (JNK) activation and interleukin-6 (IL-6) production. As shown by Western blotting, pretreatment of keratinocytes with 1,25$(OH)_2D_3$ 1 μM diminished UVB-stimulated JNK activation with more than 30%. 1,25$(OH)_2D_3$ treatment (1 μM) reduced UVB-induced IL-6 mRNA expression and secretion with 75–90%. Taken together, these findings suggest the existence of a photoprotective effect of active vitamin D_3 and create new perspectives for the pharmacological use of active vitamin D compounds in the prevention of UVB-induced skin damage and carcinogenesis. J. Cell. Biochem. 89: 663–673, 2003. © 2003 Wiley-Liss, Inc.

Key words: 1,25-dihydroxyvitamin D_3; ultraviolet B; keratinocytes; apoptosis; c-Jun NH_2-terminal kinase; interleukin-6

Ultraviolet-irradiation represents a major source of environmental stress, causing hazardous effects on health, such as cutaneous aging [Fisher et al., 1997], exacerbation of infections [Norval et al., 1999], and induction of skin cancer [de Gruijl et al., 2001]. The carcinogenic effect of solar irradiation has been mainly attributed to its UVB (290–320 nm) fraction, since the potentially dangerous UVC (100–290 nm) rays are absorbed by the ozone layers and therefore lose physiological significance [de Gruijl et al., 1993].

Apart from its hazardous effects, UVB is also essential for the cutaneous production of vitamin D_3. When UVB photons strike the epidermis, their energy can be used for the photolysis of 7-dehydrocholesterol (pro-vitamin D_3) to pre-vitamin D_3, which can be further converted to vitamin D_3 by thermal isomerization [Holick, 1994]. In the past few years it has been demonstrated that keratinocytes not only generate vitamin D_3 but also convert it to 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the hormonally active form of vitamin D_3 [Lehmann et al., 2001]. Since epidermal keratinocytes also contain the vitamin D receptor [Segaert et al., 1998] and respond to 1,25$(OH)_2D_3$ with growth

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arrest [Segaert et al., 1997] and differentiation [Bikle and Pillai, 1993; Bikle et al., 2003] these data suggest the existence of a unique vitamin D endocrine system within the epidermis.

In view of the close interplay between UVB-exposure and the generation of vitamin D_3 in the epidermis, one might suspect a mutual influence. Indeed, it has already been described that UVB suppresses VDR gene expression and $1,25(OH)_2D_3$ responsiveness in cultured keratinocytes [Courtois et al., 1998]. Conversely, active vitamin D_3 compounds may be involved in modulating UVB effects and act as photoprotective agents.

A hallmark event of UVB exposure is the induction of apoptosis in keratinocytes. Execution of this apoptotic program is a complex process in which nuclear and cell membrane effects are independently involved [Kulms et al., 1999; Kulms and Schwarz, 2000]. It has recently been demonstrated in fibroblasts that c-Jun NH_2-terminal kinase (JNK), a member of the mitogen-activated protein kinases (MAPK), is required for UV-induced apoptosis via the induction of cytochrome c release [Tournier et al., 2000]. In addition, some evidence suggests that JNK activation is involved in the development of skin cancer [Chen et al., 2001].

The most common response to solar UVB-irradiation is the sunburn reaction. This reaction is partly mediated by the release of interleukin-6 (IL-6) into the circulation from epidermal cells [Urbanski et al., 1990]. IL-6 is also known as a mediator of UVB-dependent immunosuppression [Nishimura et al., 1999] and has recently been implicated in the tumorigenesis of basal cell carcinoma, a frequent, mostly UVB-induced cutaneous neoplasm [Jee et al., 2001].

To explore a possible photoprotective effect of active vitamin D_3 compounds, we investigated the influence of $1,25(OH)_2D_3$ treatment on apoptotic cell death, JNK activation, and IL-6 production in UVB-irradiated primary human keratinocytes.

MATERIALS AND METHODS

Cell Culture and UVB-Irradiation

Normal human keratinocytes were isolated from foreskins of young donors (less then 6 years) as described [Kitano and Okada, 1983]. Keratinocytes were grown in Keratinocyte Serum Free Medium (Life Technologies, Paisley, Scotland) supplemented with bovine pituitary extract (50 μg/ml) and human epidermal growth factor (5 ng/ml). Third- or fourth-passage cells were used. $1,25(OH)_2D_3$ (gift from Dr. J.P. van de Velde, Solvay, Weesp, The Netherlands) was used from a stock in absolute ethanol. Final ethanol concentrations never exceeded 0.05% and did not affect experiments. Treatment with $1,25(OH)_2D_3$ started 24 h before irradiation (unless otherwise mentioned). Three Philips TL20W12 tubes with a peak output around 310 nm, were used as a UVB source. Before irradiation, cells were washed with PBS and then irradiated through a thin film of PBS. Cells were irradiated with physiologically relevant UVB doses (8–64 mJ/cm^2) as measured with an IL 700 A Research Radiometer (International Light, Newburyport, MA). After irradiation, cells were provided fresh medium.

Light and Fluorescence Microscopy

Morphological assessment of cell death after UVB-irradiation, with or without $1,25(OH)_2D_3$ pretreatment, was performed with a Zeiss light microscope. Typical apoptotic nuclear morphology was visualized by fluorescence microscopy after staining with Hoechst 33342 (Sigma Chemical Co., St. Louis, MO). Twenty-four hours after UVB-irradiation cells were incubated with 5 μg/ml Hoechst dye for 30 min at 37°C in an incubator. Subsequently nuclei were visualized under a Zeiss fluorescence microscope with appropriate filter combination.

MTT Assay

Cell survival assays were performed as described [Mosmann, 1983] using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake (Sigma Chemical Co.). Briefly, 8×10^3 cells were seeded in 96-well plates in 100 μl of growth medium/well and treated with increasing $1,25(OH)_2D_3$ concentrations. During UVB-irradiation one half of the plate was covered with Whatman-paper (= sham irradiation). Twenty-four hours after irradiation, MTT (50 μl of a 5 mg/ml solution in PBS) was added and incubated at 37°C for 2 h. The MTT-containing medium was removed from the wells, and the purple formazan product was solubilized in 200 μl DMSO for 5–10 min at room temperature. The plate was read at 570 nm. Eight replicate wells were used to

obtain all data points, and all the experiments were performed at least twice.

Northern Blot Analysis

Total RNA was isolated using the RNeasy kit from Qiagen (Qiagen, Inc., Valencia, CA). Northern blot analysis was performed as described [Segaert et al., 1997] on 15 μg of total RNA. A TNFα probe (American Type Culture Collection, Manassas, VA) was labeled with α^{32}P-dCTP by the random priming method (oligolabelling kit; Amersham Biosciences Corp., Piscataway, NJ). The IL-6 probe was amplified by PCR using respectively 5′-TTCCA-AAGATGTAGCCGCC-3′ and 5′-AATGAGATG-AGTTGTCATGTCC-3′ as forward and reverse primers on pT7-7(IL-6) as template (IL-6 was obtained from American Type Culture). The amplified product was cloned in pGEM-T (Promega Corp., Madison, MI) and subsequently sequenced. To detect IL-6 mRNA, α^{32}P-radiolabeled PCR fragments were generated using the same reverse primer as mentioned above. For loading control we used a probe for glyceraldehyde-3-phosphate dehydrogenase, labeled with the oligolabelling kit of Amersham Biosciences.

Western Blot Analysis

Protein extraction was performed as described [Segaert et al., 1997], except for phosphorylated proteins (25 mM HEPES pH 7.5, 0.3 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 0.5 mM dithiothreitol, 20 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml antipain) and for cytoplasmic cytochrome c (20 mM HEPES pH 7.5, 10 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 0.01% digitonin). Protein concentration was determined using the BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL). Samples (50–100 μg of protein) from cell lysates were separated by SDS–PAGE, followed by wet electrotransfer onto Hybond-C super membrane (Amersham Biosciences Corp.). The membrane was subsequently blocked and probed with a commercially obtained antibody. Immunoreactive proteins were visualized using a chemiluminescence detection system (NEN; Amersham Biosciences Corp.). The antibodies used were: monoclonal anti-human PARP and monoclonal anti-cytochrome c (BD Pharmingen, San Diego, CA); rabbit anti-phospho-p38 MAP kinase (Thr180/Tyr182) and rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) which specifically recognizes the phosphorylated forms of the kinases (New England Biolabs, Beverly, MA). Peroxidase conjugated goat antirabbit and rabbit antimouse (Dako A/S, Glostrup, Denmark) were used as secondary antibodies.

ELISA

Hundred μl medium per condition was used in IL-6 ELISA (Pelikine Compact™ human IL-6 ELISA kit; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and TNFα ELISA (OptEIAHuman TNFα set; BD Pharmingen). Experiments were performed according to manufacturer's protocol. Cells were analyzed for apoptosis by Cell Death Detection ELISA (Roche Molecular Biochemicals, Indianapolis, IN). The enrichment of mono- and oligonucleosomes released into the cytoplasm of cell lysates was detected by biotinylated anti-histone- and peroxidase-coupled anti-DNA-Ab and is calculated by using the formula: absorbance of sample cells/absorbance of control cells. This enrichment factor was used as a parameter of apoptosis.

Statistics

Data were analyzed by a two-tailed Student's *t*-test for unpaired samples, using a statistical software program (NCSS, Kaysville, UT). $P < 0.05$ was accepted as significant.

RESULTS

$1,25(OH)_2D_3$ Pretreatment Enhances Survival of Human Keratinocytes Upon UVB-Irradiation

By lightmicroscopy we compared the morphology of UVB-irradiated human keratinocytes with or without $1,25(OH)_2D_3$ pretreatment. Irradiation with 32 mJ/cm^2 UVB resulted in extensive cell death with small, shrunken and partly fragmented cells (Fig. 1A, a). These are characteristic morphological features of cells undergoing apoptosis [Kulms and Schwarz, 2000]. However, when keratinocytes were treated with $1,25(OH)_2D_3$ 1 μM 24 h prior to irradiation, a clear reduction of cell death was seen (Fig. 1A, b).

To further quantify this protective effect of active vitamin D_3 on UVB-induced cell death, we used an MTT-assay. $1,25(OH)_2D_3$ treatment, without UVB-irradiation, resulted in a dose-dependent reduction of cell number (Fig. 1B),

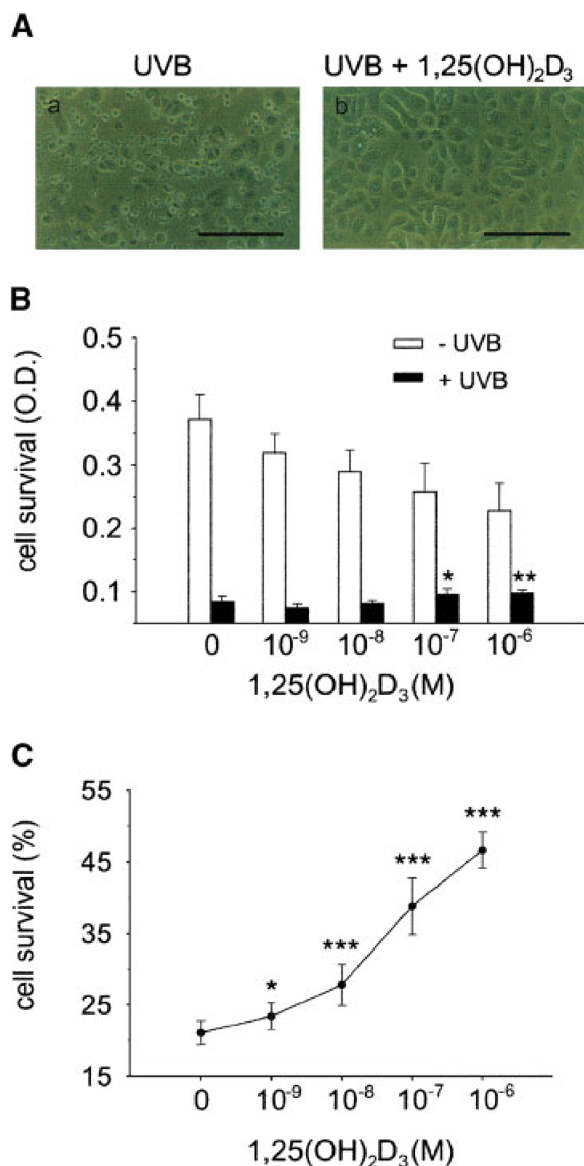

Fig. 1. 1,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] protects human keratinocytes against UVB-induced cell death. **A**: Light microscopy analysis of UVB-irradiated (32 mJ/cm^2) human keratinocytes in the absence (**a**) or presence (**b**) of $1,25(OH)_2D_3$ 1 μM pretreatment (24 h). Photographs were taken 24 h after irradiation. Scale bar: 100 μM. **B**, **C**: Cell survival assays, using MTT. Keratinocytes were incubated with variable concentrations $1,25(OH)_2D_3$ and irradiated with UVB 32 mJ/cm^2. Data are expressed as optical density (O.D.), reflecting the amount of viable cells (B) or as percentage cell survival, corrected for the antiproliferative effect of $1,25(OH)_2D_3$ (C). Data represent the mean of eight replicates ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ when comparing $1,25(OH)_2D_3$ pretreated irradiated cells with untreated irradiated cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

which was maximal with $1,25(OH)_2D_3$ concentrations of 0.1 and 1 μM. This reduction in cell number probably reflects the antiproliferative effect of $1,25(OH)_2D_3$ [Bikle and Pillai, 1993; Segaert et al., 1997]. Twenty-four hours after irradiation with 32 mJ/cm^2 only 20% of the cells remained viable (Fig. 1B). Based on our microscopical data, these extensive cell losses can be mainly ascribed to UVB-induced cell death. Pretreatment with $1,25(OH)_2D_3$ 0.1 and 1 μM reduced the UVB-induced cell losses with 13% ($P < 0.05$) and 15% ($P < 0.01$), respectively (Fig. 1B). However, the reduced cell numbers induced by pharmacological doses of active vitamin D_3 may lead to underestimation of its effect on UVB-induced cell death. Therefore we corrected the MTT-values of the vitamin D_3-pretreated and UVB-irradiated cells for the antiproliferative effect of $1,25(OH)_2D_3$ (Fig. 1C). After this correction, active vitamin D_3 protected keratinocytes significantly against UVB-caused cell death when preincubated for 24 h at higher, pharmacological doses. Treatment with $1,25(OH)_2D_3$ 1 μM reduced UVB-induced cell death with more than 25% ($P < 0.001$). This protection could be confirmed for other UVB doses (16 and 64 mJ/cm^2) (results not shown).

$1,25(OH)_2D_3$ Protects Human Keratinocytes Against UVB-Induced Apoptosis

To further confirm this suggested photoprotective effect of $1,25(OH)_2D_3$, we investigated its influence on UVB-induced apoptosis. There are a number of studies revealing that UVB-irradiation is proficient in inducing apoptosis as mode of cell death in primary human keratinocytes in culture [Kulms and Schwarz, 2000]. We initially used fluorescent microscopic analysis with Hoechst 33342. Untreated cells were uniformly stained indicating unaltered nuclei (Fig. 2A, a). Calcitriol-treated cells exhibited the same homogeneously stained nuclei, although less numerous, probably due to the previously mentioned vitamin D_3-induced growth arrest (Fig. 2A, b). Exposure to UVB 32 mJ/cm^2 resulted in nuclear condensation and fragmentation, important features of apoptotic cell death (Fig. 2A, c). Pretreatment of the keratinocytes during 24 h with $1,25(OH)_2D_3$ 1 μM however, clearly diminished the number of apoptotic nuclei (Fig. 2A, d).

Analyzing the cleavage of poly (ADP-ribose) polymerase (PARP) further corroborated these observations. Activation of a cascade of cystein

proteases, called caspases, appears to be crucial for executing apoptosis induced by UVB. The final effector protease, caspase-3, causes cleavage of several substrates, including PARP, which ultimately results in apoptosis [Kulms et al., 1999]. The cleavage of PARP is therefore one of the hallmarks of apoptosis. As shown in Figure 2B, PARP-cleavage was clearly induced by UVB-irradiation at later time points. Pretreatment with $1,25(OH)_2D_3$ 1 μM efficiently, but not completely, inhibited this UVB-induced PARP-cleavage.

To quantify the anti-apoptotic effect of $1,25(OH)_2D_3$, we used an ELISA, detecting the DNA-fragmentation seen during apoptosis (Cell Death Detection ELISA; Roche). Pretreatment of keratinocytes with $1,25(OH)_2D_3$ 1 μM for 24 h reduced UVB-stimulated apoptosis with 55% ($P < 0.01$) to 70% ($P < 0.001$) (Figs. 2C and 3). This protective effect was confirmed for different UVB-doses (Fig. 2C) and was dose-dependent, already significant at 10 nM (Fig. 3A) and time-dependent, with a minimum incubation period of 8 h (Fig. 3B).

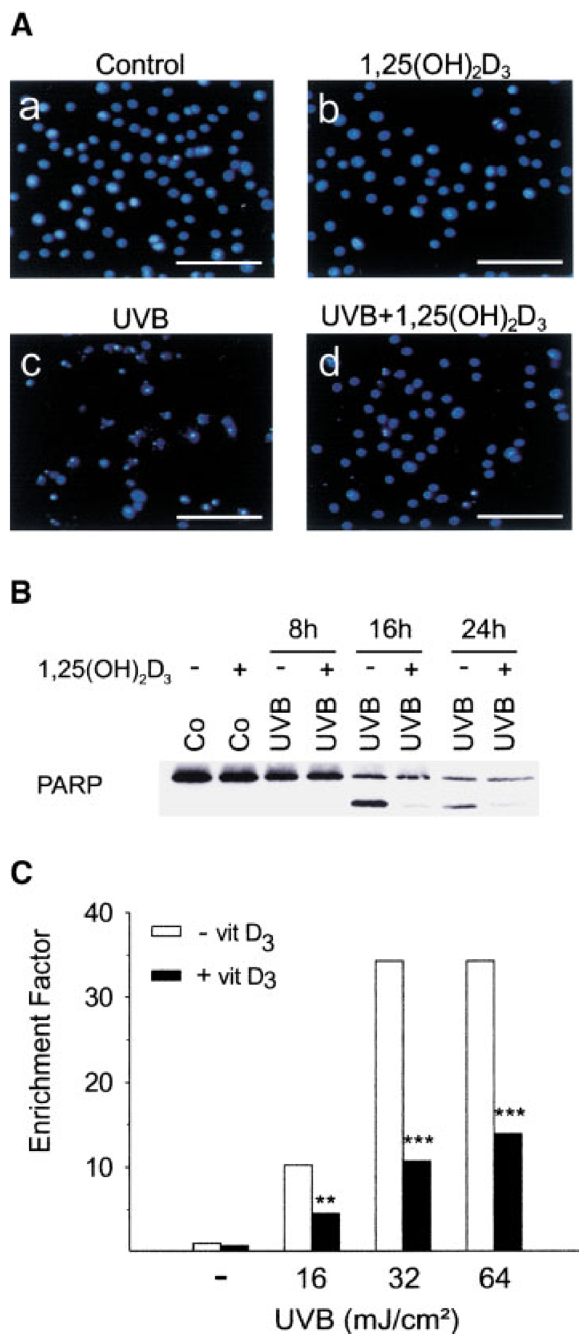

Fig. 2. $1,25(OH)_2D_3$ protects human keratinocytes against UVB-induced apoptosis. **A:** Fluorescence microscopy analysis of untreated keratinocytes (**a**), keratinocytes treated with $1,25(OH)_2D_3$ 1 μM (**b**), keratinocytes irradiated with UVB 32 mJ/cm^2 (**c**), and keratinocytes pretreated with $1,25(OH)_2D_3$ 1 μM prior to irradiation (**d**). Twenty-four hours after irradiation cells were incubated with Hoechst dye for 30 min and subsequently visualized under a Zeiss fluorescence microscope. Scale bar: 50 μM. **B:** Keratinocytes were irradiated with UVB 32 mJ/cm^2 in the absence or presence of $1,25(OH)_2D_3$ 1 μM pretreatment. Lysates were prepared at different time points after irradiation for the analysis of PARP-cleavage by Western blot. **C:** Quantification of apoptosis induced by different UVB-doses in untreated or in $1,25(OH)_2D_3$ (vit D_3) 1 μM pretreated cells. Apoptosis was evaluated 24 h after irradiation with a cell death detection ELISA. Rate of apoptosis is reflected by the enrichment of nucleosomes in the cytoplasm shown on the y axis (mean ± SD of triplicate samples). The shown data represent one of three independently performed experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ when comparing $1,25(OH)_2D_3$ pretreated irradiated cells with untreated irradiated cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

$1,25(OH)_2D_3$ Inhibits UVB-Induced JNK Activation and Cytochrome c Release in Human Keratinocytes

We next examined the effect of $1,25(OH)_2D_3$ on JNK activation, a well known participant of the UV response that has been implied in the induction of apoptosis [Davis, 2000]. In agreement with previous data [Assefa et al., 1997], we demonstrated a profound and rapid activation of JNK upon UVB-irradiation of normal human keratinocytes. Pretreatment of the keratinocytes with $1,25(OH)_2D_3$ 1 μM during 24 h caused a reduction of 30–50% in the UVB-induced phosphorylation of JNK (Fig. 4A). The UVB-induced activity of both JNK isoforms was equally affected by $1,25(OH)_2D_3$ treatment. In contrast, $1,25(OH)_2D_3$ treatment had no apparent effect on UVB-activated p38 MAPK (Fig. 4B), another member of the MAPK-group

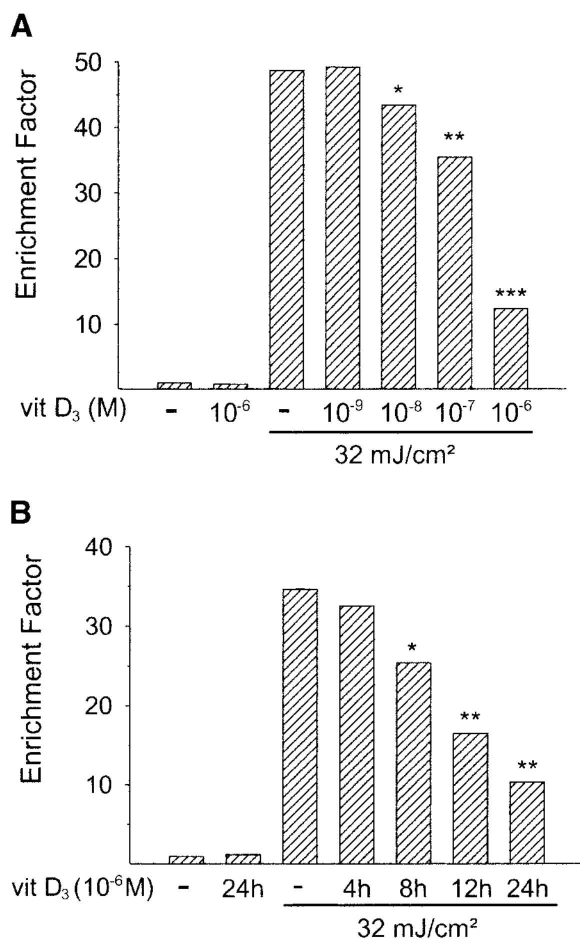

Fig. 3. Protection of $1{,}25(OH)_2D_3$ against UVB-induced apoptosis in human keratinocytes requires pharmacological concentrations and an incubation period of several hours. Keratinocytes were irradiated with UVB 32 mJ/cm^2 with or without $1{,}25(OH)_2D_3$ pretreatment. **A**: Pretreatment with variable concentrations $1{,}25(OH)_2D_3$ (vit D_3) for 24 h. **B**: Variable duration of $1{,}25(OH)_2D_3$ (vit D_3) 1 μM pretreatment before irradiation. Apoptosis was evaluated 24 h after irradiation with a cell death detection ELISA. Enrichment factor was used as a parameter of apoptosis and is given as the mean ± SD of triplicate samples. Data are from one representative of three independently performed experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ when comparing $1{,}25(OH)_2D_3$ pretreated irradiated cells with untreated irradiated cells.

that has been related to UVB-induced apoptosis [Assefa et al., 2000].

It is hypothesized that JNK-dependent apoptosis is mediated through mitochondrial cytochrome c release [Tournier et al., 2000]. Cytochrome c release has also been observed as an early event in UV-mediated apoptosis in HaCaT cells [Assefa et al., 2000] and HeLa cells [Goldstein et al., 2000]. Therefore we investigated the release of cytochrome c in UVB-irradiated normal human keratinocytes and the influence of $1{,}25(OH)_2D_3$ hereupon. Indeed,

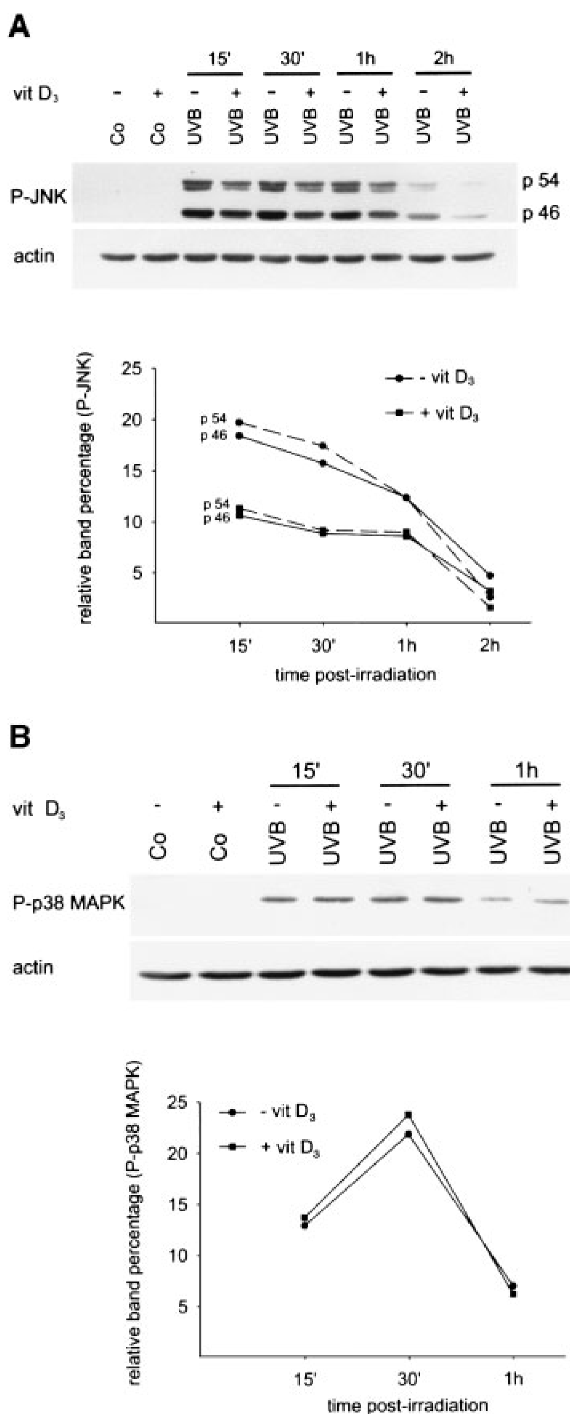

Fig. 4. $1{,}25(OH)_2D_3$ reduces UVB-induced JNK activation in human keratinocytes without affecting p38 MAPK activation. Keratinocytes were irradiated with UVB 32 mJ/cm^2 in the absence or presence of $1{,}25(OH)_2D_3$ (vit D_3) 1 μM. Lysates of the cells were harvested at the indicated time points after irradiation and subjected to Western blot analysis using phospho-JNK (P-JNK) antibody (**A**) and phospho-p38 MAPK (P-p38 MAPK) (**B**). Densitometric data normalized for β-actin were plotted.

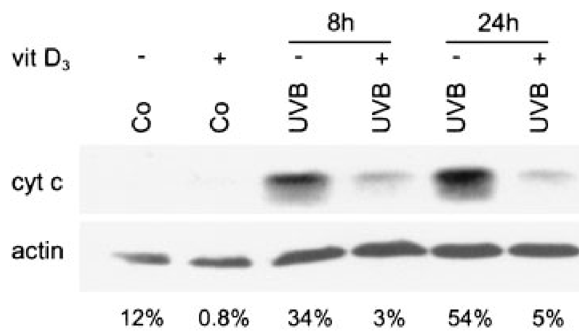

Fig. 5. $1,25(OH)_2D_3$ inhibits UVB-induced cytochrome c release in human keratinocytes. Keratinocytes were irradiated with UVB 32 mJ/cm^2 in the absence or presence of $1,25(OH)_2D_3$ (vit D_3) 1 μM pretreatment. Lysates of the cells were harvested at different time points after irradiation and subjected to Western blot analysis using the cytochrome c (cyt c) antibody. The indicated values were obtained by densitometric scanning and normalized for β-actin.

UVB-irradiation clearly induced cytochrome c release in the cytosol and $1,25(OH)_2D_3$ suppressed this release with more than 90% (Fig. 5).

$1,25(OH)_2D_3$ Suppresses UVB-Induced IL-6 Production in Human Keratinocytes

Another important feature of UVB-irradiated keratinocytes is the induction of various cytokines [Takashima and Bergstresser, 1996], such as IL-6 [Petit-Frère et al., 1998]. We confirmed that UVB-irradiation strongly induces accumulation of IL-6 mRNA and release of IL-6 protein by human keratinocytes in a dose- and time-dependent manner (Fig. 6). $1,25(OH)_2D_3$ by itself did not influence the IL-6 production significantly, whereas pretreatment of the keratinocytes with $1,25(OH)_2D_3$ 1 μM, 24 h prior to irradiation, resulted in a marked suppression of IL-6 mRNA expression (75–89%) (Fig. 6A) and IL-6 secretion (65%, $P < 0.05$ to 90%, $P < 0.001$) (Fig. 6B,C). As for the anti-apoptotic effect of $1,25(OH)_2D_3$, the inhibition of UVB-stimulated IL-6 production was shown to be dose-dependent and required pharmacological doses of $1,25(OH)_2D_3$ (Fig. 7).

In addition, we examined the effect of active vitamin D_3 on the production of TNFα, another pro-inflammatory UVB-inducible cytokine [Kock et al., 1990]. Basal TNFα expression levels were very low in unstimulated keratinocytes, but could be upregulated by both $1,25(OH)_2D_3$ treatment and UVB-stimulation separately (Fig. 8). Incubation of the keratinocytes with $1,25(OH)_2D_3$ (1 μM) followed by UVB-irradiation resulted in a synergistic superinduction of TNFα mRNA expression (Fig. 8A)

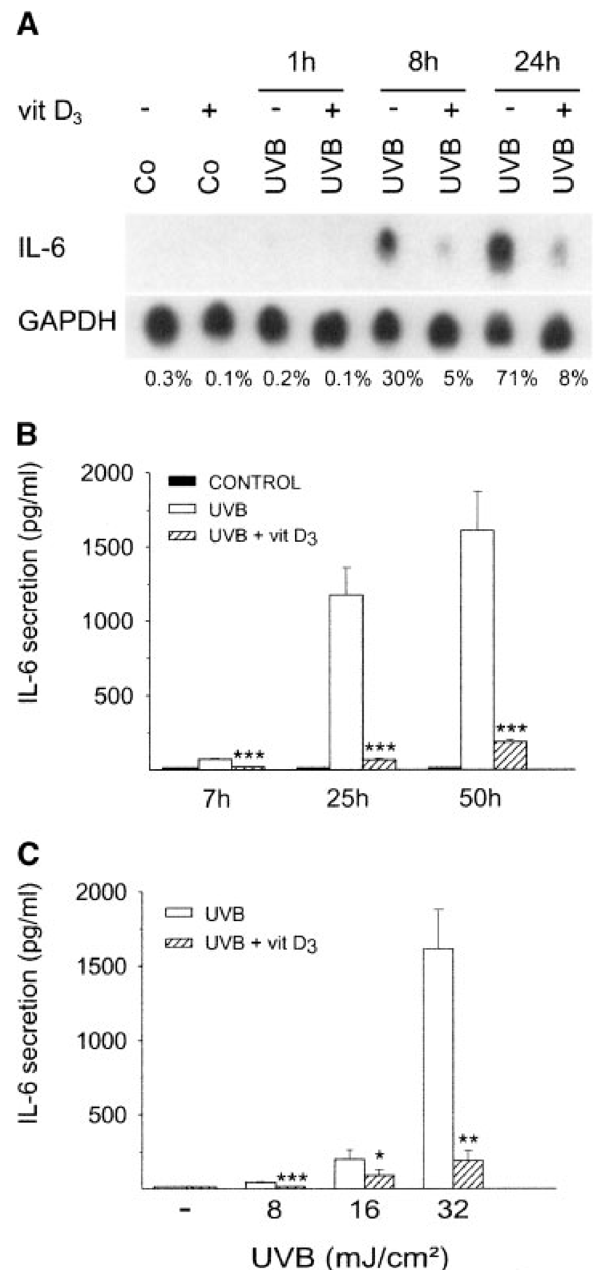

Fig. 6. $1,25(OH)_2D_3$ suppresses UVB-induced IL-6 production in human keratinocytes. **A**: Keratinocytes were pretreated (24 h) with $1,25(OH)_2D_3$ (vit D_3) 1 μM and irradiated with UVB 32 mJ/cm^2. Total RNA (15 μg) was harvested 24 h after irradiation and subjected to Northern blot analysis. The blot was hybridized with probes for IL-6 and GAPDH. Normalized densitometric data are shown. **B**, **C**: Medium was collected from untreated and $1,25(OH)_2D_3$ (vit D_3) 1 μM pretreated (24 h) keratinocytes at various time points after irradiation (32 mJ/cm^2) (B) and 24 h after irradiation with different UVB-doses (C). The concentration of IL-6 was determined by ELISA and results are shown as mean ± SD of triplicate samples. The presented data show one representative of three independently performed experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ when comparing $1,25(OH)_2D_3$ pretreated irradiated cells with untreated irradiated cells.

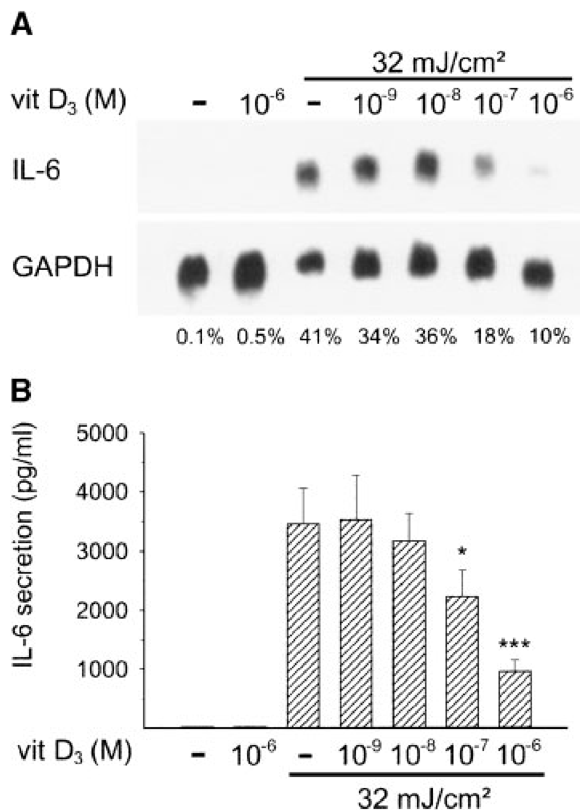

Fig. 7. $1,25(OH)_2D_3$ dose-dependently inhibits UVB-induced IL-6 production. Untreated keratinocytes and keratinocytes pretreated with variable concentrations $1,25(OH)_2D_3$ (vit D_3) were irradiated with UVB 32 mJ/cm^2. **A**: Cells were harvested for RNA 24 h after irradiation and subjected to Northern blot analysis. The blot was hybridized with probes for IL-6 and GAPDH. Normalized densitometric data are shown. **B**: Medium was collected 24 h after irradiation and analyzed for IL-6 secretion by ELISA. Results are shown as mean ± SD of triplicate samples and show one representative of three independently performed experiments. $*P < 0.05$; $***P < 0.001$ when comparing $1,25(OH)_2D_3$ pretreated irradiated cells with untreated irradiated cells.

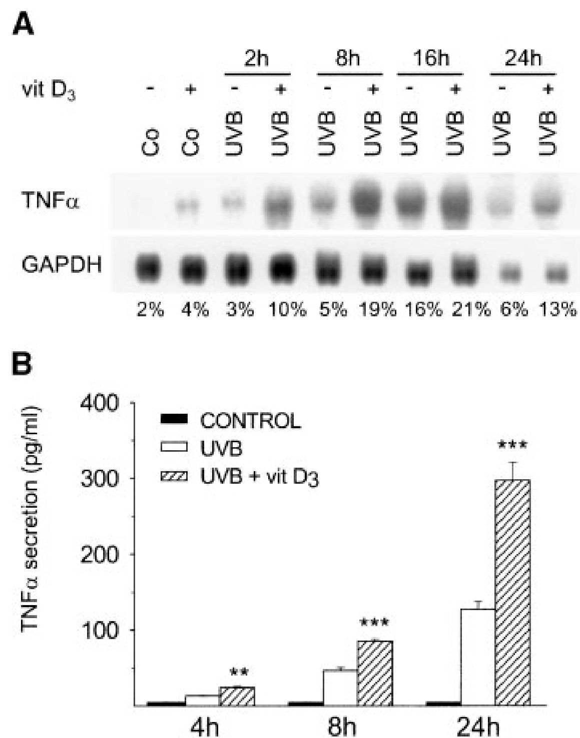

Fig. 8. $1,25(OH)_2D_3$ upregulates basal- and UVB-induced TNFα production. Untreated and $1,25(OH)_2D_3$ (vit D_3) 1 μM pretreated keratinocytes were irradiated with UVB 32 mJ/cm^2. **A**: Cells were harvested at indicated time points after irradiation and subjected to Northern blot analysis. The blot was hybridized with probes for TNFα and GAPDH. Normalized densitometric data are shown. **B**: Medium was collected at different time points after irradiation and analyzed for TNFα secretion by ELISA. Results are shown as mean ± SD of triplicate samples and show one representative of two independently performed experiments. $**P < 0.01$; $***P < 0.001$ when comparing $1,25(OH)_2D_3$ pretreated irradiated cells with untreated irradiated cells.

and protein secretion (Fig. 8B). These results imply that the strong inhibition of UVB-induced IL-6 production by $1,25(OH)_2D_3$ is not secondary to a global suppression of UVB-stimulated cytokine production.

DISCUSSION

In this study we have provided different arguments for the existence of a photoprotective effect of active vitamin D_3 against UVB-mediated events in cultured primary human keratinocytes.

In agreement with previous studies [Hanada et al., 1995; Lee and Youn, 1998], we initially demonstrated that $1,25(OH)_2D_3$ protected keratinocytes against UVB-induced cell death with qualitative (morphological) and quantitative (MTT-assay) data. This protection appeared to be dose-dependent and required pharmacological $1,25(OH)_2D_3$ doses.

We also examined the influence of $1,25(OH)_2D_3$ on apoptosis, the predominant type of cell death seen in keratinocytes upon UVB-irradiation. When cells were pretreated for 8 h with pharmacological concentrations $1,25(OH)_2D_3$, UVB-caused apoptosis was clearly suppressed. Accordingly, mitochondrial cytochrome c release, a hallmark event of UVB-induced apoptosis, was also markedly reduced by $1,25(OH)_2D_3$. In contrast with previous findings [Benassi et al., 1997], $1,25(OH)_2D_3$ itself did not induce apoptosis in keratinocytes, even at doses of 1 μM. The effect of active vitamin D_3 on apoptosis is not unequivocal and depends largely on the cell types and culture conditions examined. Some

reports propose vitamin D as a pro-apoptotic agent [Hsieh and Wu, 1997; Diaz et al., 2000; Park et al., 2000; Narvaez and Welsh, 2001], while others describe a protective role against apoptosis [Wang and Studzinski, 1997; Wang et al., 1999; Manggau et al., 2001; Riachy et al., 2002].

Furthermore, we have shown that $1{,}25(OH)_2D_3$ substantially attenuated UVB-stimulated JNK activation, a major component in the UVB-response, without affecting the activation of p38 MAPK. An inhibitory action by $1{,}25(OH)_2D_3$ has also been observed on JNK activation in response to UVC-irradiation in the GH4 cell line [Caelles et al., 1997] and in response to different stress signals, other than UV-irradiation, in HaCaT cells [Ravid et al., 2002]. In the latter study, $1{,}25(OH)_2D_3$ only affected p38 MAPK activation by certain stress stimuli but not by others.

Finally, we evidenced that $1{,}25(OH)_2D_3$ effectively suppressed UVB-induced IL-6 expression and secretion. This reduction was dose-dependent and, parallel to the anti-apoptotic effect of vitamin D_3, required pharmacological doses.

Although underlying mechanisms of this photoprotective effect are yet to be elucidated, our results evoke a possible connection between the photoprotective and the antiproliferative actions of active vitamin D_3. We clearly demonstrated that $1{,}25(OH)_2D_3$-dependent photoprotection required pharmacological concentrations and an incubation period of several hours. Likewise, the growth inhibitory effects of active vitamin D compounds on keratinocytes in culture are most pronounced with pharmacological doses and take many hours. Treatment of proliferating keratinocytes in culture with high concentrations of $1{,}25(OH)_2D_3$ leads to a marked accumulation of cells in the G_1-phase and a reduction of cells in the S-phase [Bikle and Pillai, 1993; Segaert et al., 1997]. DNA of S-phase cells is possibly more susceptible to UV-induced damage, due to its unfolded and more open structure during DNA-replication [Peterson and Logie, 2000]. Therefore it is tempting to hypothesize that vitamin D_3-arrested keratinocytes in the G_1-phase are less susceptible to UVB-caused DNA-damage and that the photoprotective effect of vitamin D is possibly secondary to its antiproliferative effect.

Since oxidative stress is also involved in the pathogenesis of UV-injury [Kawanishi et al., 2001; Wenk et al., 2001], anti-oxidative properties of $1{,}25(OH)_2D_3$ could also contribute to the observed photoprotective effect. Active forms of vitamin D_3 have been recognized as anti-oxidative agents through their induction of metallothionein, a potent radical-scavenging protein [Karasawa et al., 1987; Hanada et al., 1995]. Accordingly, the prolonged incubation period of $1{,}25(OH)_2D_3$, needed for a significant photoprotective effect, could be explained as the time necessary to accumulate sufficient intracellular metallothionein.

In our experiments we demonstrated a clear anti-apoptotic effect of active vitamin D_3 in UVB-irradiated human keratinocytes. Apoptosis can be considered as a mechanism ensuring the removal of irreversibly damaged and cancer precursor cells [Kulms et al., 1999]. Therefore, suppression of apoptosis by $1{,}25(OH)_2D_3$ may facilitate photocarcinogenesis rather than protect against it. However, if the inhibition of apoptosis results from a reduction of cell damage, vitamin D may be a potent photoprotective agent. Preliminary results demonstrated that $1{,}25(OH)_2D_3$ (0.1 μM) also significantly reduced cisplatin (40 μg/ml) induced apoptosis in primary human keratinocytes (results not shown). Cisplatin is also a well-known DNA-damaging agent, killing cells through formation of covalent, bifunctional DNA adducts [Sherman et al., 1985]. These data, together with the reduction of IL-6, considered as a detector of DNA-damage [Petit-Frère et al., 1998], suggests a possible protective role for vitamin D_3 against DNA-damage.

Protection against sun-induced damage leading to photocarcinogenesis in skin is a highly desirable goal. Primary prevention strategies, such as minimizing UV-exposure and the use of sunscreens, are indispensable. However, due to the realities of sun-exposure and the inadequacies of the primary prevention strategies, development of additional chemopreventive agents is necessary. Retinoic acid is a well-documented photoprotective agent, which is used clinically for prevention against photoaging and photocarcinogenesis [Moon et al., 1997]. There is however a striking analogy between $1{,}25(OH)_2D_3$ and retinoic acid: both are vitamin-derived steroid hormones, which activate a nuclear receptor (retinoic acid receptor and vitamin D receptor) that heterodimerizes with a common retinoid X receptor [Fisher and Voorhees, 1996; Norman, 1998]; they both have potent effects on epidermal proliferation and differentiation

[Bikle and Pillai, 1993; Fisher and Voorhees, 1996] and exhibit a similar therapeutic spectrum in dermatology [Fogh and Kragballe, 1997; Orfanos et al., 1997]. This similarity between active vitamin D compounds and retinoids, together with our presented results, makes $1,25(OH)_2D_3$ an attractive candidate as chemopreventive agent against UV-induced skin cancer.

The photoprotective effect of $1,25(OH)_2D_3$ in keratinocytes in culture could also have important implications for the role of active vitamin D_3 in cutaneous physiology. Epidermal vitamin D is generated in response to UVB-irradiation [Holick, 1994] and is converted to active $1,25(OH)_2D_3$ [Lehmann et al., 2001], which can subsequently influence keratinocytes in an auto- or para-crine way [Bikle and Pillai, 1993]. The cutaneous vitamin D system might therefore act as a natural counterregulatory signal against UVB-induced stress. However, the photoprotective effect of $1,25(OH)_2D_3$ in our cell culture system was only seen with pharmacological doses. Therefore the question remains whether physiological concentrations active vitamin D_3 produced locally in the skin can offer protection against UVB-induced damage. In addition, with the 0.1–1 μM doses required, there is the possibility of loss of receptor specificity and a steroid hormone receptor, other than the vitamin D receptor, might be involved in mediating the photoprotective effect of $1,25(OH)_2D_3$. However, this is can also be said for the well-known antiproliferative effect of $1,25(OH)_2D_3$, requiring the same pharmacological doses [Segaert et al., 1997].

Taken together, our findings certainly create new promising perspectives for the pharmacological use of active vitamin D compounds in the prevention of UVB-induced skin carcinogenesis.

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