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# Role for tumor necrosis factor-α in UVB-induced conversion of 7-dehydrocholesterol to 1α,25-dihydroxyvitamin $D_3$ in cultured keratinocytes☆

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**Abstract**

UVB irradiation of cultured human keratinocytes induces both the conversion of 7-dehydrocholesterol (7-DHC) to calcitriol (1α,25$(OH)_2$ $D_3$) and the release of tumor necrosis factor-α (TNF-α) in these cells. Calcitriol synthesis in human keratinocytes was reduced in the presence if a neutralizing polyclonal antibody directed against human TNF-α. On the other hand, we found a 1.7-fold higher stimulatory effect of UVB on liberation of TNF-α in cultured keratinocytes enriched with 7-DHC compared with irradiated cell cultures in absence of 7-DHC. These observations argue in favor of a synergetic relationship between generation of TNF-α and calcitriol in UVB irradiated keratinocytes. In addition, we found that TNF-α potently increases the conversion rate of Vitamin $D_3$ (cholecalciferol) to calcitriol in this cell system. The UVB-triggered formation of both TNF-α and calcitriol in cultured keratinocytes was wavelength-, time- and dose-dependent. Maximum formation of TNF-α and calcitriol was found at 300 nm and UVB doses of 30 mJ/$cm^2$. The enhancement of both the formation of TNF-α and calcitriol in keratinocytes by UVB may be of relevance for regulation of growth and apoptosis in light-exposed epidermal cells and, in addition, may play a role in the UVB treatment of diseased skin including psoriasis.


*Keywords:* Keratinocyte; Calcitriol; Tumor necrosis factor-α; Ultraviolet radiation

## 1. Introduction

UVB radiation (280–320 nm) induces both the conversion of 7-dehydrocholesterol (7-DHC) via several intermediate products (previtamin $D_3$, Vitamin $D_3$ and 25-hydroxyvitamin $D_3$) to calcitriol (1α,25$(OH)_2D_3$) [1,2] and the release of tumor necrosis factor-α (TNF-α) in human keratinocytes [3]. Calcitriol is a hormonally active metabolite of Vitamin $D_3$ which regulates keratinocyte proliferation and differentiation via Vitamin D receptor (VDR) mediated processes [4–6]. TNF-α is a pleiotropic cytokine [7] that is involved in regulation of cell growth [8], differentiation [8], apoptosis [9,10] as well as in immunological [11] and inflammatory processes [12] in the skin. TNF-α is expressed in both a soluble form and a membrane-associated form [7,13,14]. UVB induces the release of the soluble form of TNF-α in human skin and in cultured keratinocytes [13]. Little is known at present about the membrane-bound form of TNF-α. Biological effects of TNF-α are mediated through either the membrane-bound tumor necrosis factor receptors TNFR 1 (p-55) or TNFR 2 (p-75) [15,16]. Human keratinocytes were shown to lack TNFR 2, indicating that TNF-α responsiveness of these cells critically depend on regulation of TNFR 1 expression [17]. UVB radiation was also found to regulate TNFR 1 expression in keratinocytes by stimulating TNF-α release which then acts in an intracrine, autocrine and paracrine manner [17]. Interestingly, it has been reported that TNF-α stimulates the 1α-hydroxylation of 25-hydroxyvitamin $D_3$ (calcidiol) to calcitriol in proliferating keratinocytes [18] and in human alveolar macrophages [19]. On the other hand, generation of TNF-α is upregulated by calcitriol in human keratinocytes (10). We have recently described an autonomous metabolic Vitamin $D_3$ pathway in human epidermis which results in synthesis of calcitriol independent of hydroxylases located in the liver and the kidney [1,2]. In this study, we investigated (i) the possible influence of TNF-α on the autonomous epidermal Vitamin $D_3$ pathway in cultured keratinocytes; (ii) we monitored the release of TNF-α from keratinocytes during UVB-induced synthesis of calcitriol and (iii) we

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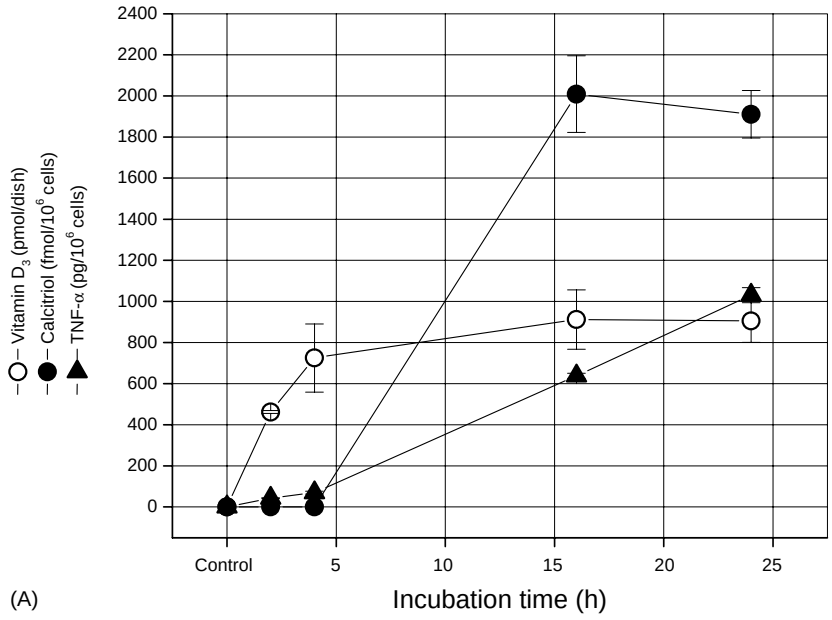
investigated the role of different wavelengths of UVB on these processes.

## 2. Materials and methods

Neonatal human keratinocytes (TEBU, Frankfurt/M, Germany) were cultured in KGM at 95% relative humidity, 5% $CO_2$ ant 37 °C. When cells reached preconfluency, they were incubated in culture medium (KBM) containing 1% (w/v) bovine serum albumin and 7-DHC (25 μM) or Vitamin $D_3$/cholecalciferol (750 nM). In experiments using 7-DHC cells were irradiated with a HI-monochromator/Dermolum at 290, 300, 310 and 320 nm and UV-doses between 7.5 and 45.0 mJ/cm$^2$. Elimination of UVB-induced synthesis of TNF-α was realized by addition of polyclonal anti-human



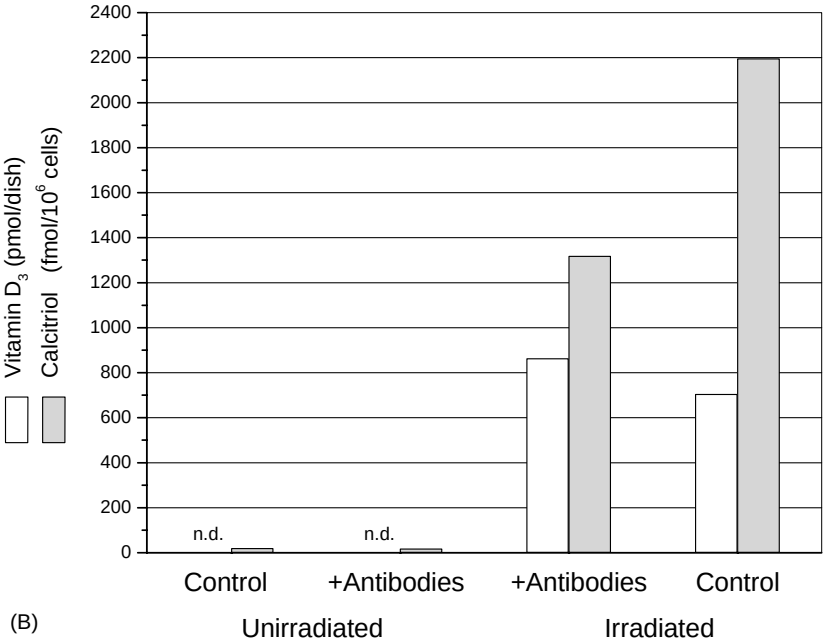




Fig. 1. (A) Time course of the synthesis of Vitamin $D_3$, calcitriol and TNF-α after irradiation at 300 nm and 30 mJ/cm$^2$; culture medium was supplemented with 25 μM 7-DHC. Results are expressed as means ± S.D. ($n = 3$). (B) Reduction of the generation of calcitriol after addition of polyclonal antibodies directed against TNF-α (0.2 μg/ml), concentration of 7-DHC and conditions of irradiation are identical to (A) but followed by 16 h incubation time. Data are depicted as mean of two independent experiments; n.d.: not detectable.

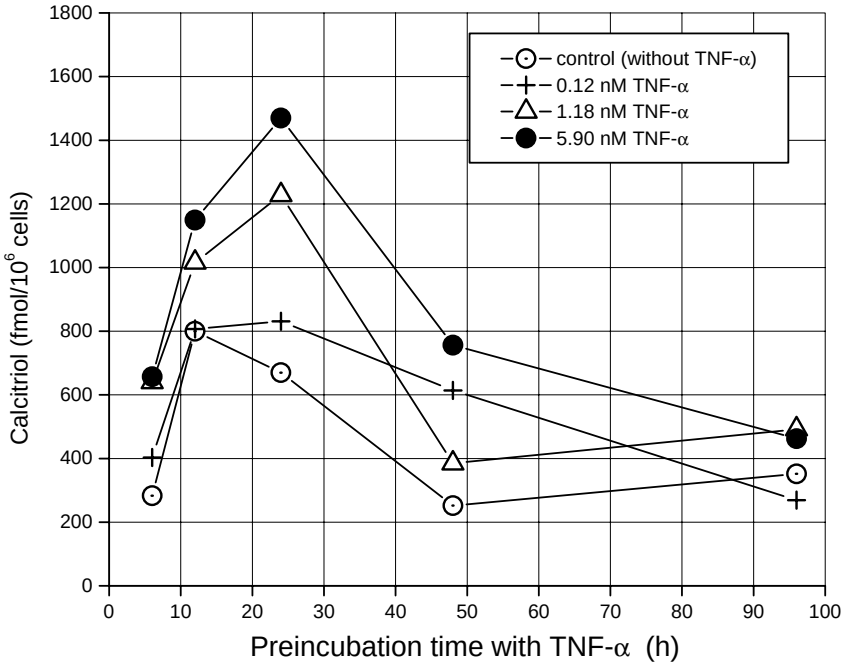



Fig. 2. Influence of different TNF-α concentrations and incubation times on the generation of calcitriol from Vitamin $D_3$ in human keratinocytes. Cells were preincubated in the presence of different concentrations of TNF-α for variable times followed by an incubation time of 5 h with Vitamin $D_3$. Data points are depicted as mean of two independent experiments.

TNF-α antibodies (R&D Systems) to the cell culture (final concentration: 0.2 μg/ml) immediately after irradiation. In experiments using Vitamin $D_3$ cells were preincubated in presence of different concentrations of TNF-α (Boehringer, Mannheim, Germany) for variable times. Thereafter, Vitamin $D_3$ was added to the cell culture as ethanolic solution (final concentration of ethanol: 0.5% (v/v)).

The cell number and the viability was determined by using a CASY 1 cell counter (Schärfe System, Reutlingen, Germany). Viability was always >91%. Cells and supernatant were separately extracted with chloroform:methanol (1:1); chloroform phases were pooled and dried under nitrogen; residues were solubilized in ethanol. Amounts of Vitamin $D_3$ were analyzed by NP-HPLC and UV detection at 265 nm [1,2]. The quantitative determination of calcitriol was carried out by using a radioreceptor assay (Nichols Institute, Bad Nauheim, Germany). The results of calcitriol were converted to fmoles per culture dish and normalized to $10^6$ cells. The amount of TNF-α generated after UVB irradiation was determined by using a current immunoassay (Biosource International, Solingen, Germany) and normalized to $10^6$ cells.

## 3. Results

The de novo synthesis of calcitriol in cultured keratinocytes from 7-DHC following irradiation with monochromatic UVB at 300 nm and 30 mJ/cm$^2$ is shown in Fig. 1A. Maximum levels of Vitamin $D_3$ (912 pmol per dish) and calcitriol (2010 fmol/106 cells) were observed 16 h after irradiation. At this point approximately 640 pg TNF-α/$10^6$ cells were detectable in the culture medium, and the amount further increased up to 24 h. Addition of polyclonal antibodies directed against TNF-α immediately after irradiation at 300 nm with 30 mJ/cm$^2$ reduced the production of calcitriol by about 40% (Fig. 1B). UVB irradiation of cell cultures enriched with 25 μM 7-DHC at 300 nm and 30 mJ/cm$^2$ provoked within 24 h (equivalent to the production of

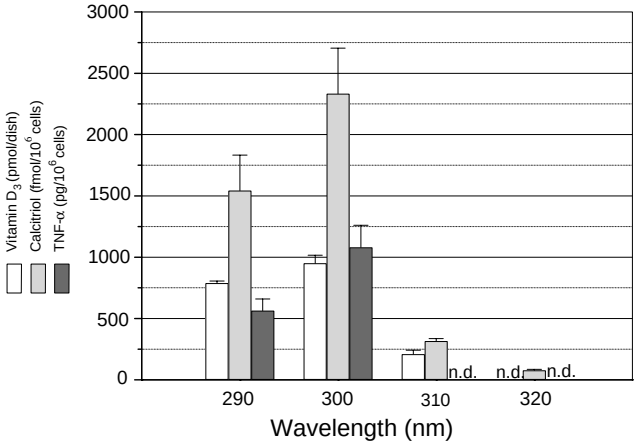



Fig. 3. Formation of Vitamin $D_3$, calcitriol and TNF-α in dependence on the wavelength of UVB; culture medium was supplemented with 25 μM 7-DHC, 24 h incubation time after irradiation at 30 mJ/cm$^2$. Data points are depicted as means ±S.D. ($n = 3$); n.d.: not detectable.

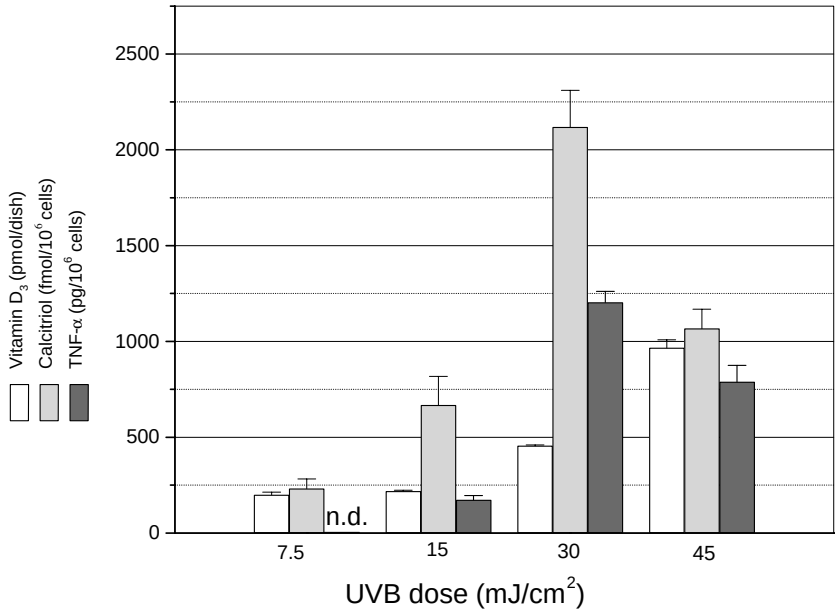



Fig. 4. Formation of Vitamin $D_3$, calcitriol and TNF-α in dependence on the UVB-dose at 25 μM 7-DHC; 24 h incubation time after irradiation at 300 nm. Results are expressed as means ± S.D. ($n = 3$); n.d.: not detectable.

approximately 2000 fmol calcitriol/$10^6$ cells) an about 1.7-fold higher degree of liberation of TNF-α from cells into the supernatant than after irradiation in absence of 7-DHC (1031 ± 36 versus 603 ± 112 pg TNF-α/$10^6$ cells) (time course not shown in detail).

In further experiments we studied the influence of TNF-α on the conversion rate of Vitamin $D_3$ (cholecalciferol) to calcitriol. After 24 h preincubation of cultered keratinocytes with increasing concentrations of TNF-α, Vitamin $D_3$ was added for further 5 h incubation. As shown in Fig. 2 the generation of calcitriol from Vitamin $D_3$ in keratinocytes was dose-dependently upregulated in the presence of TNF-α. Increased concentrations of TNF-α (up to 5.90 nM) augmented the formation of calcitriol after an incubation time of 24 h from 670 fmol/$10^6$ cells (control) to maximum 1470 fmol/$10^6$ cells. Similar concentrations of calcitriol were found after preincubation with 0.12 nM TNF-α and in controls without TNF-α.

In a second series of experiments the influence of the wavelength and dose of UVB on the Vitamin $D_3$ pathway and liberation of TNF-α was studied. Our results indicate that generation of Vitamin $D_3$, calcitriol and TNF-α depends on the UVB wavelength and shows maximum levels of each at 300 nm (Fig. 3). Irradiation at 310 nm resulted in generation of no TNF-α and only small amounts of Vitamin $D_3$ and calcitriol. After irradiation at 320 nm and in unirradiated cultures neither Vitamin $D_3$ nor TNF-α and only traces of calcitriol were detectable. The formation of calcitriol and TNF-α peakes at 300 nm and a UVB dose of 30 mJ/$cm^2$ (Fig. 4). There is a clear decrease in synthesis of calcitriol and TNF-α at 45 mJ/$cm^2$, whereas the production of Vitamin $D_3$ is continued.

## 4. Discussion

Previously, an autonomous Vitamin $D_3$ pathway was demonstrated in human keratinocytes both in vitro [1,2] and in vivo [20] starting with UVB-induced photolysis of 7-DHC to previtamin $D_3$ and isomerization to Vitamin $D_3$, followed by two subsequent enzymatic hydroxylation steps (25- and 1α-hydroxylation) which finally result in the production of 1α,25$(OH)_2D_3$. This study shows that UVB-induced synthesis of calcitriol at 300 nm (dose: 30 mJ/$cm^2$) in cultured keratinocytes is paralleled by increased generation of TNF-α in the same cells. Both the synthesis of calcitriol and release of TNF-α are time-dependent processes. Polyclonal antibodies directed against TNF-α clearly reduce the synthesis of calcitriol indicating that UVB-induced production of calcitriol and TNF-α are connected. The UVB increased synthesis of TNF-α in cultured keratinocytes enriched with 7-DHC to a higher degree (1.7-fold) than in cell cultures in absence of 7-DHC suggesting a mutual interaction of UVB-induced formation of TNF-α and calcitriol. This hypothesis is supported by Geilen et al. [10] who found that calcitriol stimulates mRNA and protein expression of TNF-α in the human keratinocyte line HaCaT.

In further experiments we could demonstrate that in cultured keratinocytes TNF-α clearly enhances the conversion rate of Vitamin $D_3$ to calcitriol. Maximum generation of calcitriol was observed when keratinocytes were preincubated for about 22 h with TNF-α (1.18 or 5.9 nM) before Vitamin $D_3$ was added for a second incubation period of 5 h. Metabolic conversion of Vitamin $D_3$ to calcitriol in cultured keratinocytes has already been described in previous reports [21–23]. It is not clear at present whether 25- or

1α-hydroxylation or both metabolic steps are upregulated by TNF-α. Furthermore, the molecular mechanism(s) of UVB regulation are unknown yet. Our findings are extending the observations by Bikle et al. [18] that generation of $1\alpha,25(OH)_2D_3$ from $25OHD_3$ in preconfluent keratinocytes is stimulated by TNF-α.

Interestingly, we found that in addition to Vitamin $D_3$ and calcitriol, TNF-α also displayed a wavelength-dependent production with a common maximum at 300 nm. The synthesis of Vitamin $D_3$ and calcitriol as well as the release of TNF-α are UVB dose-dependent processes. Decreased amounts of calcitriol and TNF-α after irradiation at high UVB doses (>30 $mJ/cm^2$) can be explained by photolytic inactivation of the hydroxylases required for synthesis of calcitriol and, possibly, a higher portion of membrane-bound TNF-α at expense of the concentration of the soluble form of TNF-α [14]. Furthermore, it cannot be ruled out that UVB radiation dose-dependently affects the TNF-α synthesis at the level of transcription or regulates mRNA stability and translational efficiency. In contrast, the formation of Vitamin $D_3$ is a non-enzymatic photochemical reaction which only depends on the availability of 7-DHC, the wavelength and intensity of UVB irradiation.

In conclusion, these results indicate that UVB-induced generation of calcitriol and of TNF-α in keratinocytes may be synergetic processes in which TNF-α acts as a positive regulator in synthesis of calcitriol and vice versa. The stimulating effect of UVB on both the formation of TNF-α and calcitriol in keratinocytes may be of importance for the growth, differentiation and immunmodulatory processes in light-exposed epidermal cells and in diseased skin including psoriasis.

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