Arch Dermatol Res (2003) 295 : 71–79
DOI 10.1007/s00403-003-0403-x

ORIGINAL PAPER

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Premature keratinocyte death and expression of marker proteins of apoptosis in human skin after UVB exposure

Received: 22 November 2002 / Revised: 12 February 2003 / Accepted: 9 April 2003 / Published online: 17 May 2003

Abstract Epidermal keratinocytes undergo a process of terminal differentiation or cornification that in many aspects resembles apoptosis. It is characterized by the elimination of cell nuclei within the granular layer, whereas the cytoplasm is transformed into horn cells. Premature death of keratinocytes can be induced by extrinsic factors such as UV irradiation. We investigated the time-dependent expression of apoptotic marker proteins in the skin of one healthy human volunteer after irradiation with a fourfold minimal erythema dose (MED) of UVB. The data were supplemented by including healthy skin areas of biopsies from patients UVB-irradiated for therapeutic reasons. Punch biopsies were analysed by in situ end-labelling (ISEL) for DNA strand breaks and by immunohistochemistry for expression of *p53*, *bcl-2*, active caspase-3 and its proform, and deoxyribonuclease I (DNase I). Keratinocytes with pyknotic nuclei were first detected 6 h after UVB exposure, and apoptotic keratinocytes (sunburn cells) 12 h after exposure. These aggregated to sunburn bodies after 24 h. In control skin, nuclei with DNA strand breaks were only occasionally detected in the granular layer but 6 h after UVB irradiation in the spinous layer. After 12 h, many sunburn cells were ISEL-positive and positively stained for active caspase-3, P53, and DNase I. Morphometric evaluation of the immunohistochemical data demonstrated that maximal upregulation of P53, DNase I and activation of caspase-3 occurred 12 h after irradiation and in advance of the peak of apoptotic cell death reached after 24 h as verified by ISEL. In contrast, strong Bcl-2 immunostaining appeared restricted to presumed melanocytes and basal cells but was not increased after UVB irradiation.

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Keywords Apoptosis · UVB irradiation · P53 · Caspase-3 · Endonuclease

Introduction

In the epidermis, keratinocytes are constantly generated from replicating stem cells located in the basal and/or parabasal layer [1]. During their migration to the epithelial surface, keratinocytes undergo terminal differentiation and finally cornification that in some aspects resembles apoptosis [2]. It is characterized by the elimination of the cell nuclei within the granular layer, whereas the cytoplasm is transformed into dead horn cells after a phase of extensive crosslinking of its constituents into an elastic but mechanically resistant envelope catalysed by a number of sequentially activated transglutaminases [2]. The constant transformation of keratinocytes into horn cells and their shedding at the epithelial surface are necessary for tissue homeostasis (reviewed in references 1, 3 and 4). This differentiation process is morphologically characterized first by cellular enlargement followed by shrinkage of the cytoplasm and nucleus within the upper part of the spinous layer. Their chromatin is finally degraded and the remaining nuclear structure dissolved within the stratum granulosum [4]. These changes have been termed anoikosis and may result from the activity of enzymes being also part of the apoptotic pathway. It has been suggested that apoptotic endonucleases and the epidermis-specific caspase-14 are upregulated and/or activated during this process [5].

Premature death of keratinocytes can be induced by extrinsic factors such as UV irradiation [6]. The ultraviolet spectrum of sunlight comprises wavelengths from 200 to 400 nm and due to its natural and unavoidable presence is an important source of skin damage and cancer [7, 8]. The UV spectrum is divided into three sections with different biological effects. The shortest wavelengths ranging from 200 to 280 nm (UVC) have the highest potential for biological effects, but are mainly absorbed by the ozone layer of the atmosphere. Of more relevance are UVB (280–320 nm) and UVA (320–400 nm) that are not

completely absorbed by the atmosphere and have biological effects. UVA penetrates the epidermis and is absorbed by the dermal and subcutaneous layers and is believed to be responsible for skin ageing and cancer [9, 10]. UVB radiation is mostly absorbed by the epidermal layers and is thought to damage the DNA of keratinocytes leading to the activation of the tumour suppressor gene product P53 [11] (for review see references 12 and 13). P53 is known to sense DNA damage caused by irradiation and to subsequently halt the cell cycle in the G_1 phase, providing time for DNA repair. If the damage to the genome is too serious and repair likely to fail, P53 acts as an inducer of apoptosis. Furthermore, it has been shown that UV irradiation of cultured cells directly activates CD95 independently of its ligand CD95L, thereby inducing the signal cascade leading to the apoptotic demise of the affected cells [14, 15, 16]. Alternatively, ionizing radiation could also affect cellular membranes leading to activation of sphingomyelinase producing ceramide that is known to signal the initiation of programmed cell death [17]. Thus there is general agreement that after UVB irradiation keratinocytes prematurely enter the classical pathway of apoptotic death forming so-called sunburn cells [13, 18, 19, 20].

The initiation of apoptosis is furthermore dependent on proteins belonging to the Bcl-2 family. Pro- and antiapoptotic members of this family can be distinguished: Bcl-2 and Bcl-X_L act as inhibiting factors, whereas Bax, Bak and Bcl-X_S have an apoptosis-promoting effect. The ratio of pro- and antiapoptotic factors will finally decide the cell's destiny [21, 22]. If the decision for cell death has been taken, special cysteinyl aspartate proteases, the caspases, are activated from their precursors, the procaspases. In humans, there are 11 different caspases, some of which function as signalling caspases – for example caspase-9 – and others as executing caspases. The most important executioner is caspase-3 which hydrolyses proteins essential for cell survival (for review see references 23 and 24). It has, however, been reported that during normal keratinocyte differentiation caspase-3 is not activated in mouse and human skin [5].

During apoptosis, the activity of caspases is supplemented by endonucleases. The Ca^{2+}/Mg^{2+}-dependent endonuclease(s) catalyse chromatin degradation first into large fragments and finally at internucleosomal sites [25, 26]. In most instances, the apoptotic endonucleases are generated by the dying cell itself (cell-autonomously). However, there are also indications that chromatin degradation of dying cells may be catalysed or at least be completed by DNases of phagocytotic cells [27]. A number of candidate apoptotic endonucleases have been identified such as caspase-activated DNase (CAD) initially isolated from lymphatic cells; however, its expression in humans appears to be restricted to a few tissues [28, 29]. Therefore, different endonucleases might catalyse apoptotic chromatin degradation in other tissues. Deoxyribonuclease I (DNase I) has been proposed as another candidate enzyme [26, 30], but studies in DNase I-deficient mice recently generated in our laboratory have indicated that DNase I is more likely involved in extracellular chromatin clearance [31]. DNase I-like enzymes exhibiting about 60% sequence identity with DNase I have been shown to possess almost identical enzymatic properties to DNase I [32, 33, 34]. These enzymes have been demonstrated to be intracellularly expressed, to possess a nucleus localization signal and to be involved in cell-autonomous apoptotic DNA degradation [34].

In the study reported here we analysed the effect of a single dose of UVB radiation on human skin and its time-dependence in the induction of premature death of keratinocytes and the concomitant expression of a number of proteins related to the induction and execution of apoptosis such as P53, pro- and active caspase-3, Bcl-2, and DNase I or DNase I-like enzymes. We present data collected from a single volunteer that allowed a direct comparison of the analysed parameters. These data were supplemented and confirmed by single time-point analyses of healthy skin areas from archival biopsies taken from dermatological patients UVB-irradiated for therapeutic reasons.

Materials and methods

UVB irradiation of human skin

Determination of the minimal erythema dose (MED) was performed on a healthy male volunteer (aged 41 years), whose skin type was classified to type III according to the Fitzpatrick classification [35]. Informed consent was given by the volunteer. The UVB MED was determined on the right inner forearm using a Multitester SBB LT 400 (Saalmann, Herford, Germany). This UV source is fitted with a metal halide lamp that emits wavelengths in the UVB range from 285 to 320 nm with peaks at 300 and 313 nm (UVB intensity 6 mW/cm^2). The UVB doses for MED determination ranged from 0.05 to 0.09 J/cm^2 (incremental dose 0.01 J/cm^2). After 24 h the MED (0.07 J/cm^2) was read and five adjacent sites (diameter 1 cm^2) on the inner side of the left forearm were irradiated with UVB at fourfold MED (0.28 J/cm^2). Punch biopsies measuring 4 mm in diameter were taken before and 3, 6, 12, 24 and 36 h after UVB irradiation. A further biopsy was taken at 48 h, but only a small amount of material became available for immunostaining. A second healthy male volunteer (aged 27 years) was irradiated identically, but only the biopsy 36 h after irradiation was taken. The skin samples were fixed with 4% paraformaldehyde for 24 h and paraffin-embedded by standard procedures. Finally, sections of 4 µm thickness were spread on aminosilan-coated slides. In addition, healthy areas of archival material from patients UVB-irradiated for therapeutic reasons and biopsied for diagnostic reasons were included.

Histological procedures

For the identification of the epidermal morphology and the detection of sunburn cells, sections were stained with haematoxylin-eosin (HE).

Immunohistochemical procedures were used for the detection of P53, procaspase-3 (CPP 32), active caspase-3, Bcl-2, and DNase I using specific antibodies. A polyclonal antibody against DNase I was raised in rabbits as described previously [36]. This antibody detects DNase I and/or DNase I-like endonucleases as shown by immunoblots of kidney homogenates of wild-type and DNase I-deficient mice (not shown). P53 was identified using a commercial monoclonal mouse antibody (DAKO, Hamburg, Germany; code no. M 7001) recognizing an epitope on the N-terminus of the hu-

man P53 protein. Procaspase-3 was detected using rabbit anti-CPP32 antibody (DAKO, code no. A3537). Active caspase-3 was detected with affinity-purified rabbit IgG (R&D Systems, Wiesbaden, Germany; catalogue no. AF835) which binds to the p17 subunit of active human caspase-3. For immunostaining of Bcl-2 a monoclonal antibody obtained from DAKO (code no. 0887) was used.

The sections were deparaffinized, rehydrated and, after washing in phosphate-buffered saline (PBS), blocked with 10% goat serum in PBS. After incubation with the primary antibodies for at least 12 h at about 4°C, antibodies not bound were removed by washing the slides five times with PBS. Subsequently the sections were incubated with a secondary antibody specific for the primary antibody (rabbit anti-mouse immunoglobulins for the anti-P53 primary antibody, goat anti-rabbit antibodies for all the other primary antibodies). All secondary antibodies were alkaline phosphatase conjugated, so that New Fuchsin chromogen (DAKO, code no. K596) was used resulting in an insoluble fuchsia-coloured reaction product. Counterstaining was achieved with fast green, which stains the cytoplasm.

Fragmented DNA was labelled at the 3′-OH ends by in situ end-labelling (ISEL) on sections of paraffin-embedded tissues using fluorescein isothiocyanate (FITC)-dUTP (FITC-dUTP) and terminal deoxynucleotidyl transferase (tdT) [4, 36, 37, 38]. The sections were deparaffinized and rinsed in PBS. After incubation in 2× SSC (0.3 *M* NaCl, 30 m*M* trisodium citrate, pH 7.0) at 70°C and washing in PBS, the slides were treated with 0.5% pepsin in 0.01 *M* HCl for 20 min followed by subsequent washing in PBS. The 3′-OH ends were labelled by covering the sections with the reaction mixture consisting of 4 nmol FITC-dUTP (Amersham, Freiburg, Germany), 25 U tdT (Amersham, product no. E2230Z) and 200 µl tdT buffer (30 m*M* Tris-HCl, 140 m*M* sodium cacodylate, 1 mM $CoCl_2 \cdot 6H_2O$, pH adjusted to 7.3). The best results were achieved by incubating the sections overnight in a humidified chamber at 37°C. The transferase reaction was stopped with 2× SSC at room temperature and the slides were rinsed in water, counterstained with Hoechst 33342 (0.5 µg/ml PBS) and examined with a Zeiss Axiophot (Zeiss, Göttingen, Germany) light microscope equipped with epifluorescence optics to visualize ISEL-positive cells. Photographs were taken either on Kodak Select 400 film or by means of an AxioCam HRc digital camera (Zeiss, Göttingen, Germany).

For quantitative analysis of the results, ten pictures in series were taken for each immunohistochemical preparation. The epidermal area was measured and both total and specifically stained cells were counted. The data obtained were finally related to a standard epidermal area of 10,000 µm² or the percentile increase in positively stained cells was calculated by setting the number of positive control cells to one.

Results

Expression of apoptosis-related marker proteins before UVB radiation

We analysed sections of paraffin-embedded skin biopsies of the volunteer before UVB irradiation using all histochemical and immunohistological procedures employed (Fig. 1). HE staining demonstrated normal epidermal morphology (Fig. 1A). ISEL revealed only a few positive cells within the stratum granulosum (Fig. 1B). These cells were located directly underneath the stratum corneum, at the location of the physiologically occurring elimination of the cell nuclei during the transformation process into horn cells (anoikosis). All other epidermal layers were devoid of ISEL-positive keratinocytes.

Subsequently we tested by immunohistochemistry the expression of marker proteins of apoptosis. We hardly detected any cells stained with the anti-P53 antibody. There were only a few cells in the middle and upper stratum spinosum, close under the stratum granulosum, showing weak expression of *p53* (Fig. 1C). In contrast, a much higher expression of procaspase-3 was observed (Fig. 1D). From the basal layer up to the granular layer, the staining intensity gradually increased. The cells staining positively for procaspase-3 seemed to have accumulated the inactive protease in their cytoplasm. The highest expression was found in the upper stratum granulosum, where a continuous red staining indicated that all cells of this layer highly expressed procaspase-3 (Fig. 1D). In contrast, cells positively stained for active caspase-3 were very rarely observed and were located within the spinous layer (Fig. 1E). Immunohistochemistry revealed expression of DNase I or DNase I-like enzymes within the suprabasal keratinocytes that continuously increased to the granular layer where it reached a maximum (Fig. 1F). DNase immunoreactivity was occasionally still observed in horny cells of the stratum corneum.

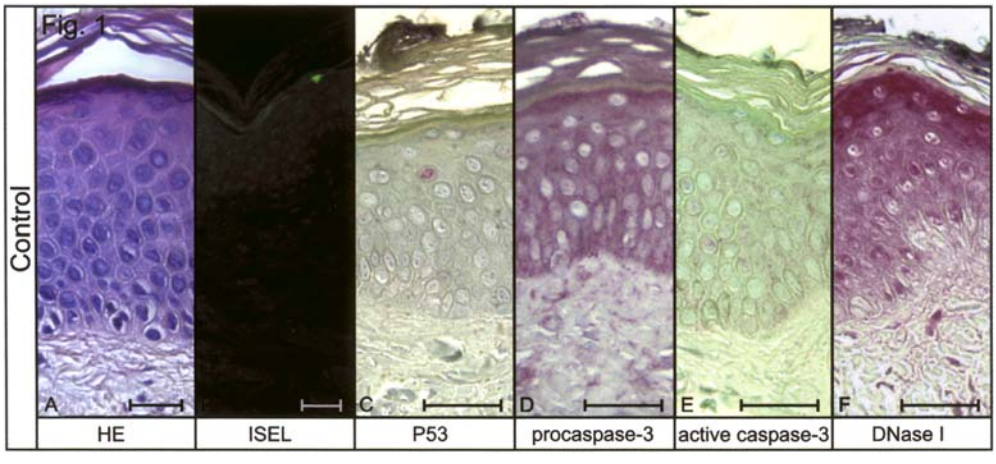

Fig. 1A–F Immunohistochemical analysis of control skin, i.e. before irradiation (**A** HE stain, **B** ISEL, **C** anti-P53, **D** anti-procaspase-3 , **E** anti-active caspase-3, **F** anti-DNase I) (*bars* **A** 15 µm, **B–F** 20 µm)

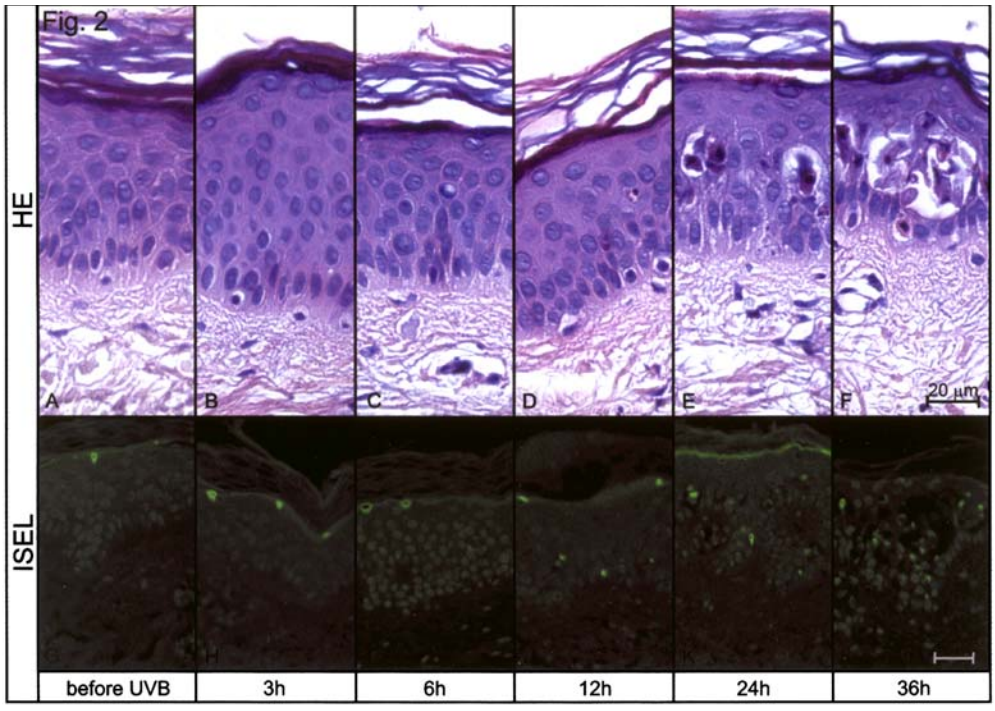

Fig. 2A–L Time series of UVB-irradiated skin. Sections were stained with HE (**A–F**) or ISEL (**G–L**) before (**A**, **G**), and 3 h (**B**, **H**), 6 h (**C**, **I**), 12 h (**D**, **J**), 24 h (**E**, **K**) and 36 h (**F**, **L**) after UVB irradiation (*bar* in **F** 20 µm, for **A–L**)

Increase in keratinocyte cell elimination after UVB irradiation

Punch biopsies of about 4 mm in diameter were taken 3, 6, 12, 24, and 36 h after the a single dose of UVB radiation, fixed in 4% paraformaldehyde and paraffin-embedded (see Materials and methods). Due to the small size of the biopsies, only a limited number of sections were available. HE staining revealed an increase in the number of cells with condensed nuclei surrounded by a bright halo. These cells were suspected to be undergoing apoptosis (Fig. 2A–F). After 24 h, sunburn bodies comprising a number of cells within a cavity became visible located within the lower spinous layer, but appeared to have moved towards a more superficial position after 36 h (Fig. 2E, F). Staining with the nuclear dye Hoechst 33342 revealed an increasing number of cells with condensed nuclei within the spinous layer and after 24 h within sunburn bodies. However, about half of the cells within sunburn bodies lacked Hoechst 33342-stainable nuclei (data not shown).

After irradiation, ISEL-positive cells were detected in the granular layer as in control skin, but from 3 h onwards also at various heights in the spinous layer (Fig. 2G–L). Their number increased dramatically after 12 h (Fig. 2J) reaching a maximum at 24 h and remained at a high level at 36 h (Fig. 2K, L). Cells of sunburn bodies were frequently ISEL-positive, although a number of cells within sunburn bodies were not stained, possibly due to the absence of chromatin as revealed by Hoechst 33342 staining (Fig. 2K, L).

Changes in the expression of apoptosis-related marker proteins after UVB irradiation

Since it has been proposed that keratinocytes damaged by UVB irradiation undergo apoptosis, we analysed by im-

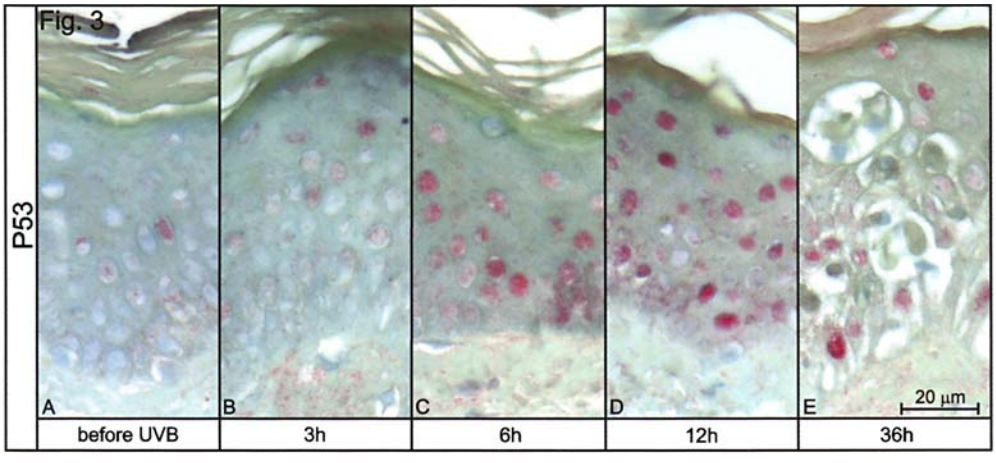

Fig. 3A–E Staining of sections from UVB-irradiated skin with monoclonal anti-P53. Sections of biopsies obtained before (**A**, area different from Fig. 1C), and 3 h (**B**), 6 h (**C**), 12 h (**D**) and 36 h (**E**) after UVB irradiation (*bar* 20 µm)

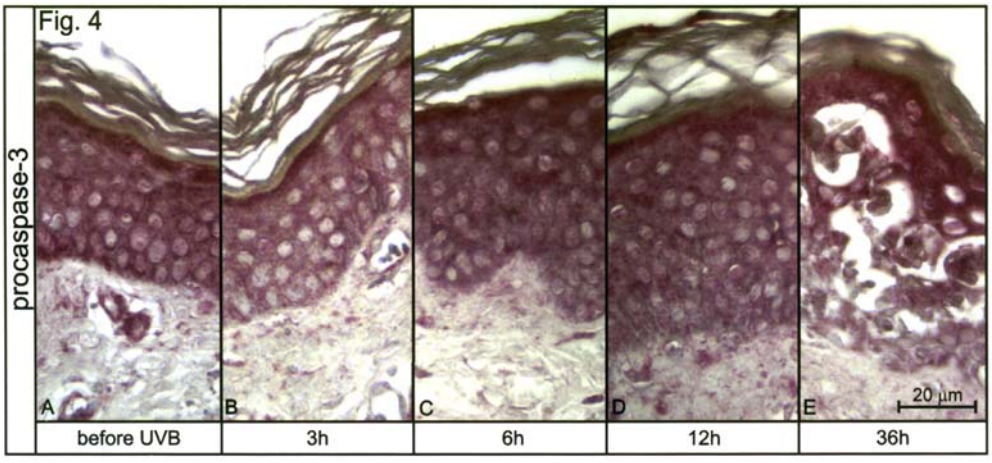

Fig. 4A–E Staining of sections from UVB-irradiated skin with antibody against procaspase-3. Sections of biopsies obtained before (**A**, area different from Fig. 1), and 3 h (**B**), 6 h (**C**), 12 h (**D**) and 36 h (**E**) after UVB irradiation (*bar* 20 µm)

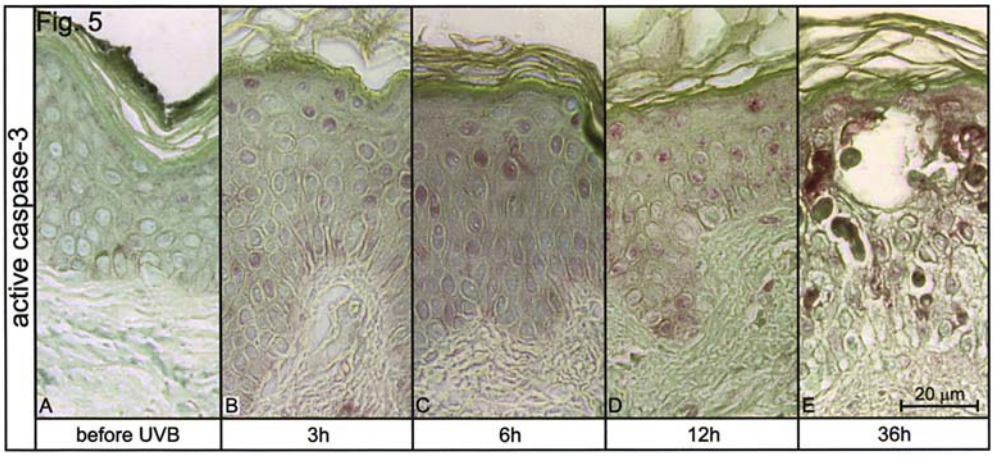

Fig. 5A–E Staining of sections from UVB-irradiated skin with antibody against active caspase-3. Sections of biopsies obtained before (**A**, area different from Fig. 1), and 3 h (**B**), 6 h (**C**), 12 h (**D**) and 36 h (**E**) after UVB irradiation (*bar* 20 µm)

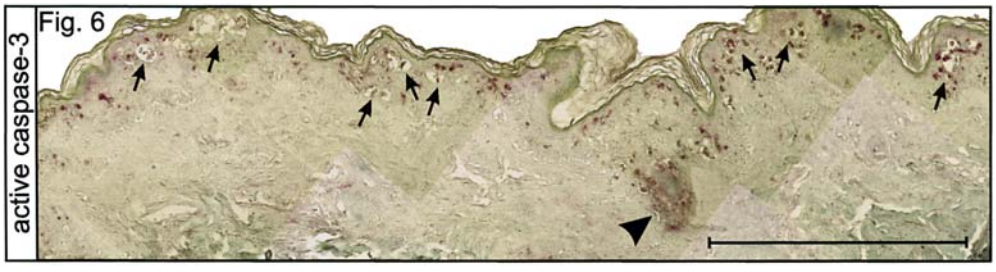

Fig. 6 Staining of UVB-irradiated skin after 36 h with anti-active caspase-3. Composite overview to demonstrate the distribution of active caspase-3-positive cells within the epidermis (*arrows* sunburn bodies containing positively stained sunburn cells, *arrowhead* upper part of a hair canal; *bar* 300 µm)

munohistochemical procedures the expression of apoptosis-related proteins.

P53 expression started to increase 3 h after irradiation (Fig. 3). At this time-point, a higher number of cells faintly positive for P53 extended from the suprabasal layer to the stratum granulosum. At more superficial locations, these cells became more strongly stained by anti-P53 (Fig. 3C). Thereafter P53 was found to continuously increase. After 6 h, cells strongly P53-positive were spread through all epidermal layers. The maximal expression of P53 was reached at 12 h after UVB exposure, and in most cases the P53 immunoreactivity appeared to be concentrated within the keratinocyte nuclei (Fig. 3D). In contrast, epidermal sections at 36 h showed a decrease in P53 (Fig. 3E). Cells in the neighbourhood of sunburn bodies were strongly stained, whereas the sunburn cells themselves appeared to be P53-negative or only faintly stained.

As demonstrated in control skin, immunoreactivity for the inactive procaspase-3 was present in the cytoplasm of keratinocytes of all layers (Fig. 4A) and at all time-points after irradiation (Fig. 4B–E). After 6 and 12 h its expression appeared to slightly increase, but to decrease at 24 h (not shown) and 36 h (Fig. 4E) with formation of a staining gradient towards superficial layers. Most cells within sunburn bodies were strongly stained, although a few also appeared CCP32-negative (Fig. 4E).

A completely different behaviour was observed for active caspase-3 (Fig. 5). Generally, control epidermis was not stained by anti-active caspase-3. Positively stained keratinocytes were rarely detected within the spinous layer

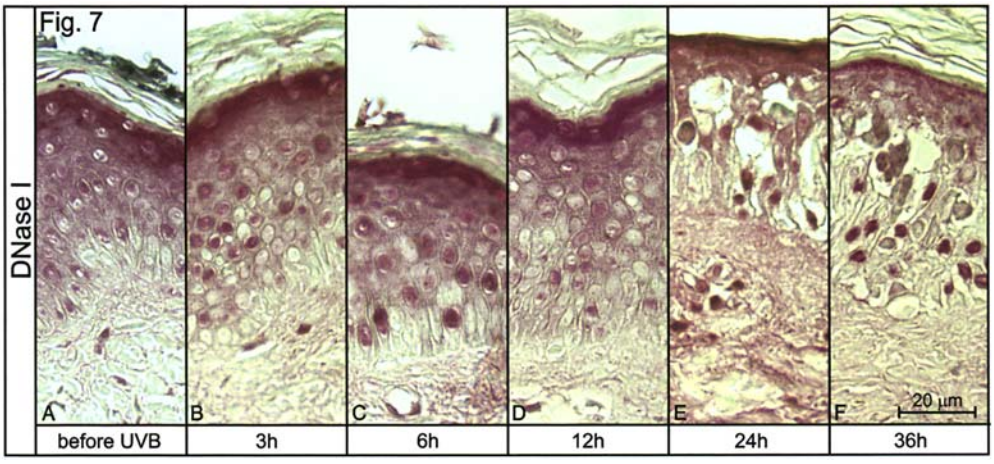

Fig. 7A–F Staining of sections from UVB-irradiated skin with antibody against DNase I. Sections of biopsies obtained before (**A**, area different from Fig. 1), and 3 h (**B**), 6 h (**C**), 12 h (**D**) and 36 h (**E**) after UVB irradiation (*bar* 20 µm)

of the epidermis (Fig. 5A), which might have been due to sun exposure before the UVB irradiation. Indeed, when we analysed healthy areas of non-irradiated skin collected from a number of patients for diagnostic reasons, we did not detect positive staining for active caspase-3 (not shown). The number of keratinocytes positive for active caspase-3 increased with time after irradiation reaching a maximum after 12 h (Fig. 5B–D). Thereafter, it slightly declined, but remained at a high level (Fig. 5E). Active caspase-3-positive cells were predominantly located within the spinous layer which comprised many sunburn bodies and also cells within the epidermal invagination forming the upper part of the hair canal as shown after 36 h in a composite overview (Fig. 6).

In control skin, DNase I immunoreactivity was detected in keratinocytes of all layers but was highest in the granular layer (Fig. 7A). Its distribution was mainly cytoplasmic, but also nuclear in some keratinocytes in the spinous layer and many cells of the granular layer in agreement with previous observations [4]. Shortly after irradiation, the number of keratinocytes with strongly DNase-positive nuclei increased within the spinous layer (Fig. 7B, C) peaking around 6 to 12 h (Fig. 7C, D). After 24 h and 36 h, the number of positive cells clearly decreased (see also Fig. 8). Many of the keratinocytes with strongly stained nuclei showed an apoptotic morphology with condensed chromatin as verified by nuclear staining using Hoechst 33342 (not shown), and in particular sunburn cells (Fig. 7E, F).

The data were quantitatively evaluated by a morphometric analysis. Figure 8 shows the percentage of positively stained cells per unit area. The results clearly demonstrate a rapid increase in P53 expression after UVB irradiation peaking at 12 h. A similar behaviour was obtained for DNase expression and caspase-3 activation. Due to lack of material, it was not possible to perform anti-P53 and anti-active caspase-3 staining for the 24 h time-point. However, in comparison to the anti-DNase staining, a decrease after 12 h appeared plausible. In agreement with previous reports, we observed the maximal number of ISEL-positive cells after 24 h, suggesting that the expression and activation of apoptosis executioner proteins occurs in advance of nuclear degradation [37, 38]. These data were supplemented by staining the biopsy of the second volunteer

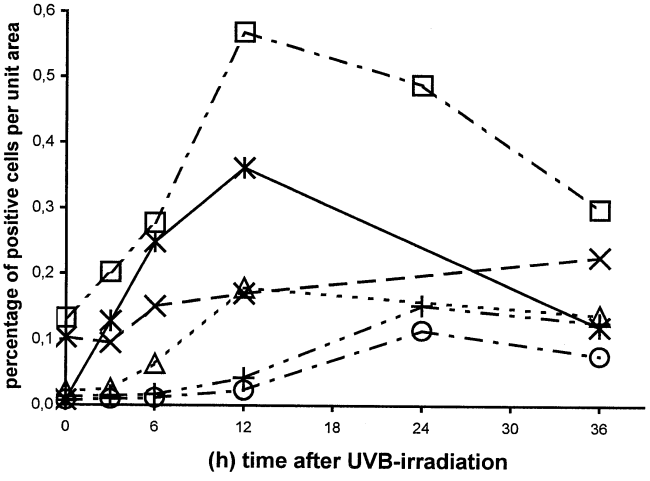

Fig. 8 Morphometric analysis of staining data. Change in the percentage of positively stained in relation to the total cell number in unit area (see Materials and methods) (✻ P53, × procaspase-3, △ activated caspase-3, □ DNase, + condensed nuclei as verified by Hoechst 33342 staining, ○ ISEL-positive nuclei)

taken before and 36 h after irradiation. The data obtained employing all the antibodies used in this study (not shown) fully corroborated the results presented.

In addition, we stained healthy areas of archival skin biopsies of patients before (n=6) and after UVB irradiation (n=4; including time-points at 6, 12 and 24 h) with the antibodies against Bcl-2 (Fig. 9), P53, active caspase-3 and DNase I (Fig. 10). Similar to the results obtained from the first volunteer (not shown), immunostaining with anti-Bcl-2 of skin biopsies of patients before and at different time-points after UVB irradiation indicated the presence of a few strongly stained Bcl-2-positive cells attached to or within the basal layer as shown previously [39, 40, 41] and a faint staining of cells within upper layers (Fig. 9). Their number and location did not change up to 24 h after UVB irradiation. Sunburn bodies detected after 24 h were Bcl-2-negative (Fig. 9D).

The immunohistochemical data from these biopsies before irradiation for the marker proteins of apoptosis were identical to the results obtained from the first volun-

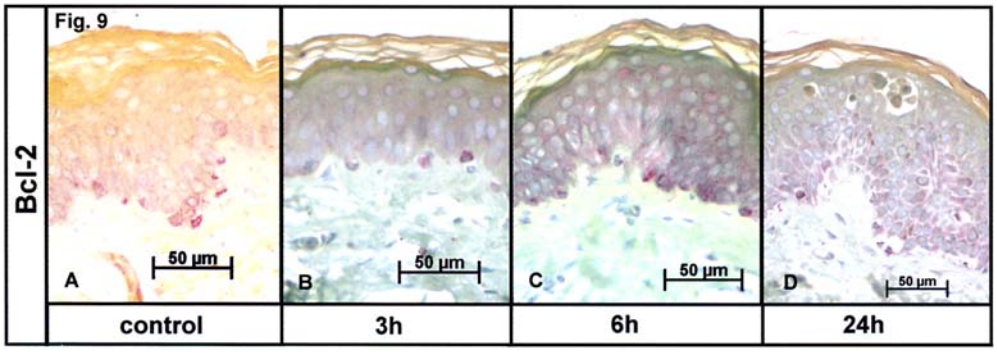

Fig. 9A–D Sections of UVB-irradiated skin stained with anti-Bcl-2. Sections of biopsies taken before (**A**), and 3 h (B), 6 h (C) and 24 h (**D**) after UVB irradiation and stained with anti-Bcl-2 using secondary antibody tagged to alkaline phosphatase (**B–D** counterstained with fast green)

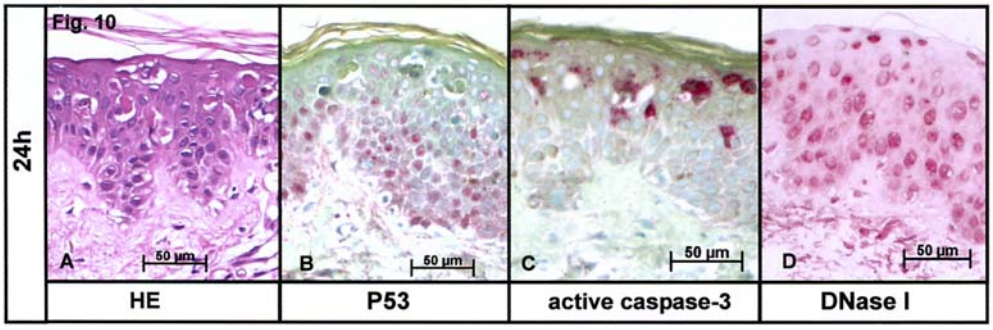

Fig. 10A–D Sections of skin 24 h after UVB irradiation. **A** Healthy skin (HE stained). **B–D** Immunostaining with anti-P53 (**B**), anti-active caspase-3 (**C**), and anti-DNase I (**D**)

teer. Although after irradiation fewer time-points were available, we observed increasing nuclear staining for P53 in basal and suprabasal keratinocytes (as shown for 24 h in Fig. 10B) and the appearance of sunburn bodies and keratinocytes strongly stained for active caspase-3 containing condensed chromatin (Fig. 10C). Similarly, we observed an increased DNase reactivity and nuclear staining 24 h after UVB irradiation (Fig. 10D). Thus these results completely corroborated the data obtained from the first volunteer.

Discussion

Here we present a histochemical study on the effects of a single UVB dose at fourfold MED on a number of apoptotic marker proteins within human skin. The data presented were collected from a single human volunteer, but they were completely corroborated by histochemistry of healthy areas of archival material from patients treated with UVB irradiation. We mainly present the data collected from the volunteer, since they give a clear coherent synopsis of a large number of apoptosis indicators allowing a direct comparison of the time-dependent changes in these parameters. Our study included for the first time a histochemical analysis of DNase I and active caspase-3 in human skin confirming that caspase-3 is not activated during normal keratinocyte differentiation [5] but after UVB irradiation. Our data are in agreement with the findings of a number of previously reported studies showing P53 upregulation and TUNEL staining of human skin after UVB irradiation [18, 19, 42, 43] and with the findings of a study (published while this manuscript was in preparation) using human skin organ cultures [43].

In agreement with these previous reports, we demonstrated that UVB irradiation led to increased keratinocyte death at various locations within the epidermis as visualized by ISEL of chromatin breaks. However, the reliability of this procedure for identifying solely apoptotic cells has repeatedly been challenged, because phagocytosed or necrotic cells can also be stained by this technique (own unpublished data; [44]). To reliably demonstrate keratinocyte apoptosis, we used anti-P53 and an antibody specific for active caspase-3. The positive staining of a rapidly increasing number of keratinocytes with anti-P53 illustrates that UVB irradiation elicited an immediate upregulation of this nuclear transcription factor. UV irradiation is also known to directly damage DNA. Indeed, UVB radiation induces the formation of pyrimidine and/or thymine dimers [43] that are, however, subsequently rapidly excised and repaired. It therefore appears unlikely that the increase in ISEL-positive cells observed after 24 h resulted from strand breaks directly induced by the UVB irradiation itself. Indeed, the delay between *p53* expression and the appearance of ISEL-positive cells (see Fig. 8) suggests that P53 induced the apoptosis executing machinery, in particular caspase-3 and an endonuclease.

The immunohistochemical appearance of active caspase-3 followed an identical time-course to that of P53. Whereas procaspase-3 was found evenly distributed within all epidermal layers in control skin, active caspase-3 was only rarely detected in control skin, but clearly increased after UVB treatment and was concentrated in cells presumably initiating or undergoing apoptosis. After 36 h, active caspase-3 was in many instances present in cells of apoptotic morphology and in sunburn bodies strongly suggesting that these represent cells undergoing apoptosis. Recent findings indicate that caspase-14, but not cas-

pase-3, is activated during normal keratinocyte differentiation, although procaspase-3 is expressed [5]. Our results confirm these findings but clearly demonstrate that active caspase-3 is only activated in keratinocytes after UVB exposure, suggesting that procaspase-3 might be expressed solely for use in "emergency" situations. Thus caspase-3 activation appears to be restricted to keratinocytes undergoing damage induced apoptosis. It would be interesting to analyse the expression of caspase-14 after UVB irradiation in future experiments. The occasional appearance of active caspase-3-positive cells in control skin was attributed to unintentional sun exposure of the volunteer before UVB treatment.

In contrast, after irradiation we did not observe an alteration in the extent of expression of Bcl-2 within the epidermis of the first volunteer (not shown) nor of archival material (Fig. 9). Our data demonstrated high expression of Bcl-2 in a few cells scattered along the basal epidermal layer. Their distribution pattern suggested that these cells were melanocytes that are known to highly express Bcl-2, whereas keratinocytic cells in culture have recently been shown to be almost devoid of proteins of the Bcl-2 family [45].

As for procaspase-3, we detected in keratinocytes increasing DNase I immunoreactivity towards the granular layer of normal skin as described previously [4]. However, since then a number of DNase I-like enzymes have been discovered which exhibit about 60% sequence identity to the classical DNase I. DNase I-like endonucleases have been identified in humans (DNAS1L3 or nhDNase) and rats (DNase γ or DNaseY). DNase γ has been suggested to participate in DNA cleavage during apoptosis of thymocytes [32, 34]. Classical DNase I is an extracellular endonuclease, whereas the DNase I-like enzymes are intracellularly localized and therefore appear better suited for cell-autonomous DNA degradation. Unfortunately, there are presently no specific antibodies available which discriminate between DNase I itself and other members of the DNase I family.

We therefore have to assume that our antibody against DNase I cross-reacted with DNase I-like endonucleases as was indeed demonstrated by immunoblots of kidney homogenates of wild-type and DNase I-deficient mice (not shown). Furthermore, when employing the anti-DNase I antibody on sections of skin of these mice, we observed a similar staining gradient in the epidermis of control and DNase I-deficient mice (data not shown). It is therefore probable that members of the DNase I family participated in the chromatin degradation after UVB irradiation, since DNase I immunoreactivity was clearly concentrated in cell nuclei of a number of keratinocytes shortly after UVB exposure and of apoptotic sunburn bodies. We cannot, however, exclude the possibility that CAD also participated in chromatin degradation. CAD is the main apoptotic endonuclease in lymphatic cells [28]. In humans, however, CAD expression is confined to a few tissues and is reduced or lacking in tumour cell lines derived from epithelia [29]. Alternatively, extracellular DNase I could have penetrated the keratinocytes through the plasma membrane damaged either directly by UVB irradiation or due to subsequent secondary necrosis. Although sunburn cells expressed apoptotic marker proteins, they did not exhibit nuclear fragmentation typical of apoptosis and furthermore remained within the epidermis for prolonged periods (48 h in our experiments) without being phagocytosed by neighbouring cells or invading macrophages.

In summary, our results demonstrate the specific upregulation of a number of apoptosis-related marker proteins after UVB irradiation and suggest that premature keratinocyte elimination occurs via apoptosis. Morphometry demonstrated that their increased expression or activation occurs in advance of actual cell elimination. During the observation period of up to 48 h sunburn cells were not eliminated by phagocytosis. Our principal data were derived from a single volunteer, but were corroborated by staining of archival biopsy material. We therefore believe that this analysis is of general interest, because it presents a comparison of a large number of apoptotic markers in relation to apoptotic events including active caspase-3. Data acquisition would certainly be easier using skin equivalents or organ cultures due to the availability of larger amounts of material, but subtle alterations or lesions may remain unnoticed. For example, in skin organ culture the aggregation of sunburn cells to bodies is not seen [43]. Therefore, in vivo studies similar to our investigation will still remain indispensable.

Acknowledgements We gratefully acknowledge the financial support of the DFG (Deutsche Forschungsgemeinschaft, Bonn, Germany) and of the Medical Faculty of the Ruhr-University Bochum via a grant from FoRUM. This work is part of the doctoral thesis of P. Maß.

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