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Alterations in cell death and cell cycle progression in the UV-irradiated epidermis of *bcl-2*-deficient mice

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Abstract

The effect of *bcl-2* gene ablation on epidermal cell death induced by UV-B irradiation was investigated in mice. Exposure of depilated back skin of *bcl-2*$^{-/-}$ mice to 0.5 J/cm^2 UV-B caused a prolonged increase in the number of epidermal cells showing nuclear DNA fragmentation compared to wild-type littermates. Consistently, skin explants from *bcl-2*-deficient mice exhibited a higher number of sunburn cells per cm epidermis (16.6$\pm$2.1 *vs* 7.0$\pm$1.5) following exposure to 0.1 J/cm^2 UV-B *in vitro*. Furthermore, UV irradiation failed to increase pre-melanosomes in skin explants from mutant animals, and primary menalocyte cultures derived from *bcl-2* null mutants were highly susceptible to UV-induced cell death compared to cultures from wild-type littermates. An accelerated reappearance of proliferating cells, showing nuclear immunoreactivity for Ki-67 and c-Fos, was observed in the UV-irradiated epidermis of *bcl-2*-deficient mice. Taken together, these findings suggest that effects of UV radiation on epidermal cell death and cell cycle progression are influenced by survival-promoting Bcl-2.

Keywords: ultraviolet radiation; *bcl-2* gene targeting; DNA damage; apoptosis; proliferation; melanocytes; keratinocytes

Abbreviations: TUNEL, terminal deoxynucleotidyltransferase and biotin-16-dUTP nick end-labeling; UV, ultraviolet

Introduction

Ultraviolet (UV) radiation in the UV-B or UV-A wavelength range (290–320 nm or 320–400 nm) is the major source of cellular damage in the mammalian epidermis.[1] In epidermal cells UV-B radiation is primarily absorbed by DNA leading to the formation of cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidone photoproducts.[2–4] Exposure to UV light induces formation of apoptotic keratinocytes ('sunburn cells'), which have been defined by morphologic criteria and *in situ* end-labeling of fragmented DNA.[5,6] Furthermore, chromatin margination, nuclear fragmentation and oligonucleosomal DNA cleavage have been detected in keratinocytes and melanocytes after UV irradiation *in vitro* indicating that apoptotic cell death is triggered by genotoxic stress.[7,8] The function of apoptosis in the UV-exposed skin may be the deletion of aberrant cells that have acquired harmful mutations.

The *bcl-2* proto-oncogene product inhibits apoptosis induced by various stimuli in many different cell types.[9] In the mammalian epidermis distinct Bcl-2 immunoreactivity has been detected in basal keratinocytes and melanocytes, and deregulated expression of Bcl-2 has been linked to malignant transformation.[10–12] Following cutaneous UV irradiation Bcl-2 expression in keratinocytes is down-regulated, whereas overexpression of Bcl-2 in transfected HaCaT keratinocytes confers resistance to UV-B-induced apoptosis.[7,13] Targeted expression of human *bcl-2* in the epidermis of transgenic mice prevents apoptotic death of keratinocytes after UV-B exposure strongly suggesting a functional role for Bcl-2 in epidermal cell death after genotoxic stress.[14] Additionally, three lines of *bcl-2*-deficient mice have been reported which develop similar phenotypic abnormalities after birth, including hair hypo-pigmentation, suggesting that *bcl-2* may also be essential for melanocyte function or maintenance under physiological conditions.[15–17] In the present study, we investigated the effects of *bcl-2* gene-ablation in mice on UV-induced epidermal cell death *in vivo* and *in vitro*.

Results

Gene ablation of *bcl-2* increases the rate of epidermal cell death following UV irradiation

Hair pigmentation of *bcl-2*$^{-/-}$ mice was initially indistinguishable from wild-type littermates. At 5–6 weeks postnatally the coats of *bcl-2*-deficient mice turned gray as has been described previously.[15–17] Immunostaining of skin sections with a monoclonal antibody that recognizes a membrane antigen of pre-melanosomes (NKI/beteb)[12] revealed no significant differences in the number of NKI/beteb-positive melanocytes between mutant and wild-type mice at 4 weeks of age (data not shown).

No spontaneous cell death was detected by the TUNEL technique in the interfollicular epidermis of both *bcl-2*$^{-/-}$ and wild-type littermates (Figure 1a,b). To investigate the role of Bcl-2 in genotoxic stress, depilated back skin of 4-week-old mice was exposed to 0.5 J/cm^2 of UV-B radiation. Six hours after exposure the number of TUNEL-positive cells per mm epidermis significantly increased to 53$\pm$8 in *bcl-2*-deficient mice and to 45$\pm$7 in wild-types (Figure 1b,e). At 24 h the number of epidermal cells showing DNA fragmentation remained elevated in mutant mice (57$\pm$5),

whereas the epidermis of wild-type mice was almost completely cleared of TUNEL-positive cells (Figure 1c,f). To exclude an influence of systemic abnormalities (e.g. lymphoid hypoplasia, renal malfunction), which develop postnatally in *bcl-2*$^{-/-}$ mice,[15–17] similar experiments were performed using skin explants. The rate of spontaneous epidermal cell death as assessed by hematoxylin-eosin staining tended to increase in skin organ cultures from mutant mice compared to wild-type animals, which however, did not reach significance. More importantly, 24 h after transient UV-B irradiation (0.1 J/cm^2) in culture the number of epidermal sunburn cells, which are characterized by shrunken size, eosinophilic cytoplasm and a pyknotic nucleus, was significantly higher ($P<0.01$, Mann-Whitney *U*-test) in *ex vivo* explants from null mutant mice (Figure 2).

Immunostaining of skin sections revealed a twofold increase in the number of NKI/beteb-positive, dendritic cells in UV-irradiated explants from *bcl-2*$^{+/+}$ mice at 24 h post-irradiation, whereas the number of NKI/beteb-immunoreactive, epidermal melanocytes did not change in explants from null mutant animals. Since UV exposure causes both an increase in the number of melanosomes and a proliferation of precursor melanocytes[18,19] our findings in skin explants could be attributed to either impaired melanogenesis or enhanced radiation sensitivity in melanocytes lacking Bcl-2. Therefore, primary melanocyte cultures were prepared from back skin of 4-week-old animals. No conspicuous differences in cellular morphology or cell number were observed between cultures from mutant and wild-type animals (Figure 3a,b). However, a significant difference in viability became apparent in melanocytes derived from *bcl-2*$^{-/-}$ mice compared to cultures from wild-type littermates following *in vitro* exposure to UV-B (Figure 3c,d). Twelve hours after irradiation 67$\pm$11% of the *bcl-2*-deficient cells showed condensed or fragmented nuclei (Figure 4b,d), whereas only 12$\pm$5% of wild-type cells exhibited chromatin condensation (Figure 4a,c)

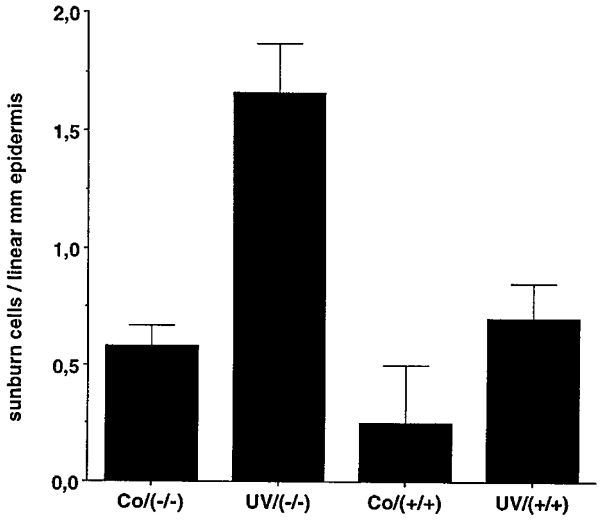

Figure 2 Number of apoptotic keratinocytes ('sunburn cells') in skin explants from *bcl-2*$^{-/-}$ mice and *bcl-2*$^{+/+}$ littermates (mean$\pm$S.D., *n*=5 per group). *Ex vivo* skin organ cultures were either sham-treated (Co, Control) or UV-exposed (UV). Twenty-four hours later tissue sections were stained by hematoxylin-eosin

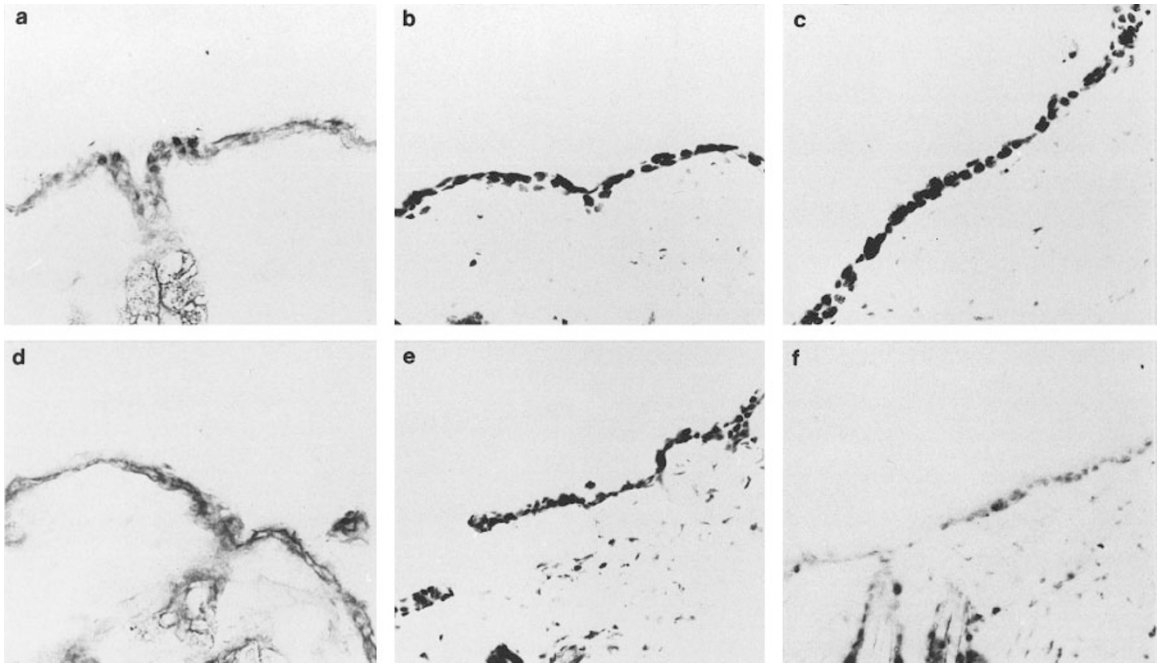

Figure 1 End-labeling of nuclear DNA fragments in sections from back skin of *bcl-2*$^{-/-}$ mice (**a,b,c**) and wild-type littermates, respectively (**d,e,f**). Mice were either sham-treated (**a,d**) or exposed to 0.5 J/cm^2 UV-B radiation and killed after 6 h (**b,e**) or 24 h (**c,f**). Persistent cell death is detectable in the UV-irradiated epidermis of $-/-$ mice

indicating that Bcl-2 deficiency renders melanocytes more vulnerable to UV-B exposure.

Bcl-2 modulates cell cycle progression after UV exposure

To investigate the influence of Bcl-2 on epidermal cell proliferation, back skin sections from 4-week-old mice were stained with an antibody against the proliferation-associated antigen Ki-67. In the basal cell layer of the epidermis numerous keratinocytes exhibited nuclear staining for Ki-67 (Figure 5a,b), and cell counts revealed similar numbers of Ki-67-positive epidermal cells in null mutant and wild-type mice (Figure 6). Six hours after high-dose UV exposure interfollicular, epidermal Ki-67 labeling slightly declined both in *bcl-2*-deficient mice and in wild-types (data not shown). Epidermal cells in wild-type animals showed prolonged cell cycle arrest as indicated by the significant decrease in Ki-67-positive epidermal cells at 24 h after irradiation (Figures 5d and 6). Interestingly, the number of Ki-67-labeled nuclei increased by twofold in the UV-exposed epidermis of *bcl-2*$^{-/-}$ mice already at 24 h suggesting an accelerated cell cycle re-entry compared to wild-types. Consistent with this notion, co-induction of transcription factor c-Fos was observed exclusively in epidermal cells of null mutant mice at 24 h post-irradiation (data not shown), which has been shown to be necessary for cell cycle progression of skin cells after UV exposure.[20–22] Double staining of skin sections for Ki-67 and

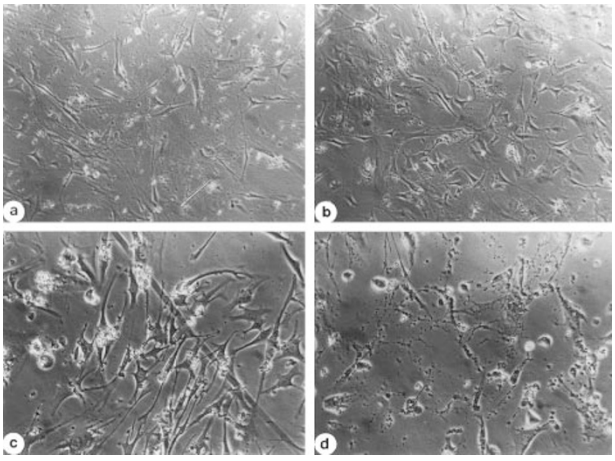

Figure 3 Morphologic appearance of primary melanocyte cultures derived from wild-type (**a,c**) or *bcl-2*-deficient mice (**b,d**) 12 h after treatment. Cell cultures were either sham-treated (**a,b**) or exposed to 0.1 J/cm^2 UV-B (**c,d**). Note extensive cell loss in UV-irradiated cultures from mutant mice

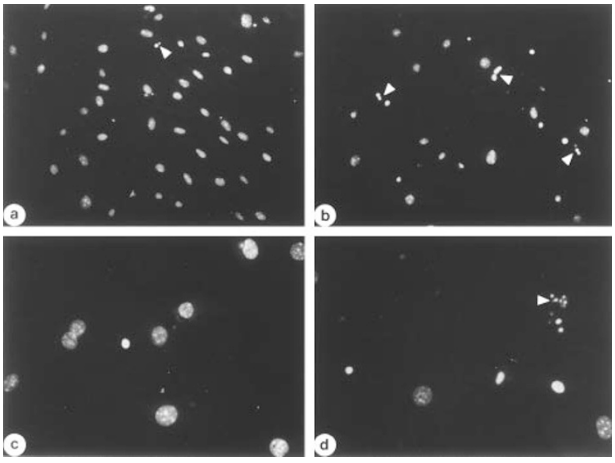

Figure 4 Nuclear morphology of melanocytes derived from wild-type (**a,c**) or *bcl-2*$^{-/-}$ mice (**b,d**) 12 h after UV-B irradiation. Numerous cells showing chromatin condensation and nuclear fragmentation are visible in cultures from null mutant animals (arrowheads)

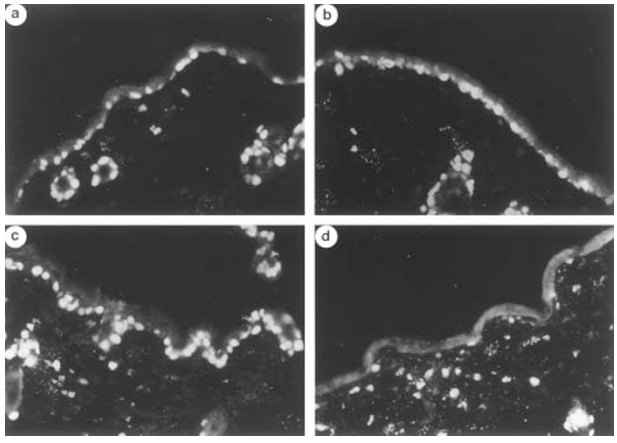

Figure 5 Immunolocalization of the proliferation-associated antigen Ki-67 in dorsal skin from *bcl-2*-deficient mice (**a,c**) and wild-type littermates (**b,d**). Animals were killed 24 h after sham-treatment (**a,b**) or UV-B irradiation (**c,d**). Increased proliferation of basal keratinocytes can be observed in the UV-exposed epidermis of mutant mice, whereas UV-induced cell cycle arrest is obvious in wild-types

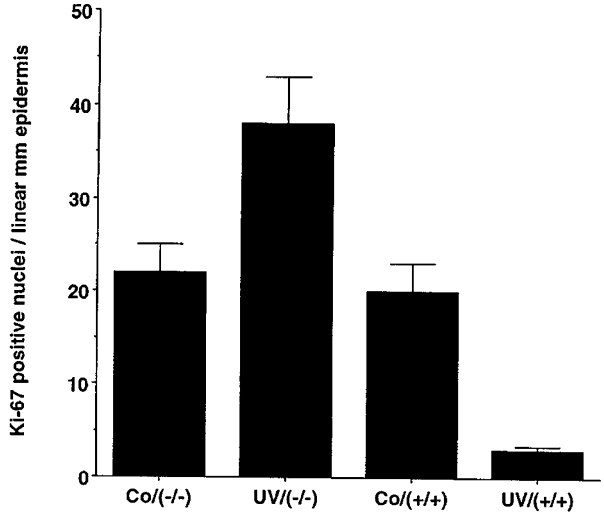

Figure 6 Number of epidermal cells showing nuclear immunoreactivity for the proliferation-associated antigen Ki-67 in skin sections from *bcl-2*$^{-/-}$ mice and from *bcl-2*$^{+/+}$ littermates (mean$\pm$S.D., *n*=5 animals per group). Animals were killed 24 h after sham-exposure (Co, Control) or UV-B irradiation (UV)

DNA fragmentation (TUNEL) revealed lack of co-localization in epidermal cells at 24 h after UV-B irradiation (Figure 7).

Discussion

At 4 weeks of age, the rate of spontaneous cell death and histologic appearance was unchanged in the epidermis of *bcl-2*-deficient mice indicating that normal cell turnover and differentiation in keratinocytes proceeds via a Bcl-2 independent mechanism. Furthermore, the number of NKI/betab-immunoreactive, epidermal melanocytes was similar between wild-type animals and *bcl-2*$^{-/-}$ mice suggesting that development and melanogenesis of epidermal melanocytes is not affected by *bcl-2* gene ablation. In *bcl-2* transgenic mice epidermal cell death under physiological conditions is unaltered as well.[14] On the other hand, the response of epidermal cells to genotoxic stress is influenced by the level of >*bcl-2* expression. Following UV-B irradiation expression of *bcl-2* in epidermal keratinocytes and melanocytes is rapidly down-regulated,[7,13,23] which according to current concepts lowers the threshold for apoptosis.[9,24] Consistently, a decrease in the number of apoptotic keratinocytes ('sunburn cells') by approximately 50% has been reported after UV-B exposure of *bcl-2* transgenic mice or *bcl-2* overexpressing keratinocyte cultures.[7,14] In our *bcl-2*$^{-/-}$ mice the number of epidermal cells showing DNA fragmentation did not differ from wild-type littermates at 6 h after UV-B, whereas at 24 h the number of TUNEL-positive keratinocytes was much higher in *bcl-2*-deficient animals. The similar rate of early UV-induced cell death in wild-types and null mutants may be attributed to the higher dose of UV radiation applied *in vivo*. The second, delayed phase of epidermal cell death however, seems to be sensitive to *bcl-2* gene ablation and may represent damaged cells which die by apoptotic cell death. Consistently, the number of keratinocytes showing apoptotic morphology ('sunburn cells') was significantly higher in skin explants from *bcl-2*-deficient mice after low dose UV-B irradiation *in vitro*. Similarly, high dose UV-B exposure of human cell lines leads to rapid cell death by necrosis, whereas moderate doses evoke apoptotic cell suicide.[25] However, neither TUNEL-technique nor hematoxylin-eosin staining clearly discriminates between an apoptotic and a necrotic type of cell death. Additionally, UV-B-induced pyrimidine dimers and (6–4) photoproducts are converted into transient single-strand breaks during nucleotide excision repair,[26] which may become labeled by TUNEL-technique. Finally, we cannot formally exclude that *bcl-2* gene ablation affects removal of epidermal sunburn cells which are shed by desquamation or phagocytosed by neighbouring keratinocytes.[5,6] Time course for the appearance of TUNEL-positive cells and their frequency in our study differs from the findings by,[27] who showed fewer TUNEL-labeled cells in the UV-irradiated murine epidermis peaking at 24 h. In the latter study, mice were exposed to 0.1 J/cm^2 of UV-B at an irradiance of 11 J/m^{2}xs and DNA fragmentation was detected by direct incorporation of labeled nucleotides, whereas in our study animals were exposed to 0.5 J/cm^2 at 140 J/m^{2}xs and DNA breaks were detected using biotinylated nucleotides and avidin-biotin amplification. Thus, a higher UV dose and/or dose rate may lead to a more rapid apoptotic response and TUNEL with avidin-biotin-based detection may result in a higher sensitivity of detection.

UV exposure of skin organ cultures derived from wild-type mice lead to an increase in the number of NKI/betab-immunoreactive, epidermal melanocytes which was absent in cultures from mutant mice. These findings could be attributed to either premature cell death or impaired melanin synthesis of melanocytes similar to the postnatal hair hypopigmentation in *bcl-2*$^{-/-}$ mice.[15,16,28] Primary melanocyte cultures derived from *bcl-2* null mutants were highly susceptible to UV-induced cell death compared to cultures from wild-type littermates strongly suggesting that Bcl-2 promotes melanocyte survival under stressful conditions. Noteworthy, administration of nerve growth factor has been shown to rescue cultured melanocytes from UV-induced apoptosis, and this rescuing effect was found to be mediated by up-regulation of Bcl-2 levels.[8]

Mammalian cells respond to DNA damage induced by UV radiation with transient arrest in G_1 and G_2 to allow for DNA repair before entering the phases of DNA replication and mitosis, respectively. Recovery of DNA synthesis and cell cycle progression appears after a delay of several hours depending on the dose of UV.[2–4,29,30] Expression of the proliferation-associated nuclear protein Ki-67 is reduced during cell cycle arrest *in vitro* and is induced in the human epidermis 48 h after UV-B exposure.[30,31] In the present study, we found that keratinocytes in the UV-exposed epidermis of *bcl-2*$^{-/-}$ mice stopped proliferation with similar kinetics compared to wild-type animals as indicated by the decrease in immunolabeling for proliferation-associated Ki-67 after 6 h. Twenty four hours after UV-B irradiation there was a greater percentage of Ki-67-positive cells in the epidermis of null mutants compared to pre-irradiation levels which may be due to a synchronization of cell cycling or an increased rate of proliferation. More importantly, Ki-67-immunoreactive epidermal cells were not detected in wild-type animals at 24 h post-irradiation indicating an accelerated cell cycle re-entry of *bcl-2*-

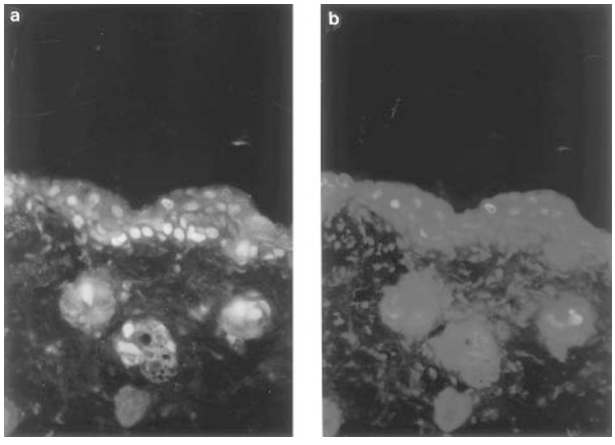

Figure 7 Double staining of skin sections from *bcl-2*-deficient mice for (**a**) proliferation-associated antigen Ki-67 (green-yellow) and (**b**) nuclear DNA fragments (TUNEL, red-yellow). Strongly Ki-67 immunopositive cells are concentrated in the basal epidermis, whereas TUNEL-positive cells are detectable in the upper layers. Co-localization is visible in scattered cells only

deficient keratinocytes. That UV-irradiated keratinocytes in *bcl-2*$^{-/-}$ mice start cell cycle progression earlier is further indicated by the massive induction of transcription factor c-Fos at 24 h which appears to be necessary for cell cycle re-entry of growth-arrested skin cells after UV.[20–22]

There is increasing evidence for an influence of Bcl-2 on cell cycling. T cells from *bcl-2*$^{-/-}$ mice enter S-phase more rapidly after mitogen stimulation.[32] Conversely, expression of a *bcl-2* transgene in lymphocytes and fibroblasts delays cell cycle re-entry after serum stimulation. It has also been shown that proliferation rate under optimal culture conditions and kinetics of withdrawal from the cell cycle following growth factor deprivation are not affected by overexpression of Bcl-2[33–36] in agreement with our *in vivo* findings. Moreover, our study for the first time provides evidence that Bcl-2 influences cell cycle progression also after UV-induced genotoxic stress. It has been hypothesized that Bcl-2 mediated retardation of G_0/G_1 to S-phase progression may provide additional time for repair of DNA damage or may inhibit aberrant cell cycle progression, thus contributing to the cell death-preventing activity of Bcl-2.[33,35] In *bcl-2* null mutant mice, Ki-67-immunoreactive epidermal cells reappeared at 24 h after UV-B exposure, when numerous TUNEL-positive cells were present. It may be speculated that in the absence of Bcl-2 keratinocytes re-enter the cell cycle before UV-induced DNA lesions are completely repaired, which then activates apoptotic cell death. However, we could not detect significant co-localization of nuclear Ki-67-immunoreactivity and DNA fragments at 24 h post-irradiation in *bcl-2*$^{-/-}$ keratinocytes speaking against this hypothesis. Unfortunately, cell cycle kinetics have not been investigated in *bcl-2* overexpressing keratinocytes following UV exposure,[7,14] however, accelerated cell cycle re-entry of *bcl-2*-deficient keratinocytes occurs too rapid to be attributed solely to indirect homeostatic feedback mechanisms that compensate for increased UV-induced cell loss.[29] This may be deduced from recent findings in UV-irradiated *bcl-x$_s$* transgenic mice, where increased epidermal cell death leads to a dramatic reduction of keratinocytes at 24 h.[37]

Materials and Methods

Animals

The targeted disruption of the bcl-2 gene and the generation of the knockout mice has been described previously.[17] Litters produced by matings between heterozygous mice were genotyped by Southern blot analysis of genomic DNA extracted from pieces of tail using standard molecular biology techniques.

Tissue and cell culture

About 4 mm^2 explants of depilated mouse back skin were placed on sterile gauze and immersed in RPMI 1640 medium (Gibco). Primary melanocyte cultures were prepared from epidermal sheets of back skin as described.[38] Melanocytes were cultured on glass coverslips in 24-well plates using melanocyte growth medium supplemented with 0.4% bovine pituitary extract, 1 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone and 10 ng/ml phorbol myristate acetate.

Ultraviolet irradiation

Tissue and cell cultures (n=6 each) were irradiated through the petri dish plastic cover at 0.1 J/cm^2 UV-B which is sufficient for induction of apoptotic cell death.[7,8,14] During exposure culture medium was replaced with phosphate-buffered saline (PBS) to avoid formation of cytotoxic photoproducts. Sham-irradiated cultures were processed in parallel but covered with aluminium foil. Back skin of mice (n=5 per group, 4 weeks of age) was depilated using depilatory cream and received a single exposure of 0.5 J/cm^2 UV-B. This dose has been shown to induce inflammation and cell death in the murine epidermis.[13]

Immunocytochemistry

After fixation in 4% paraformaldehyde in PBS tissue sections (15 μm) were incubated overnight at 4°C in one of the following antisera: (i) a monoclonal mouse anti-melanoma-associated antigen antiserum (clone NKI/beteb, 1 : 50, Monosan), (ii) a polyclonal rabbit anti-Ki-67 antiserum (1 : 50, Dianova), or (iii) a polyclonal rabbit anti-c-Fos antiserum (1 : 1000, generously donated by R. Bravo). Sections were subsequently incubated in biotinylated secondary antibody and antibody binding was localized either by avidin-biotin-peroxidase technique (Vector) or by streptavidin-cychrome-3 (Sigma). For quantitative analysis, positive cells in the interfollicular epidermis were counted in five different microscopic fields per section with the investigator being unaware of treatment and the mean counts per linear millimeter of epidermis were calculated.

Cell death assay

Degenerating cells were identified in tissue sections by *in situ* end-labeling of nuclear DNA fragments using terminal deoxynucleotidyl-transferase and biotin16-dUTP (TUNEL) or by standard histological detection of sunburn cells after staining with hematoxylin and eosin. For co-localization analysis, sections were stained by the TUNEL technique followed by immunolabeling for Ki-67 as described above. Incorporated biotin-dUTP was detected by streptavidin-cychrome-3 and antibody binding was localized by FITC-conjugated secondary antibody. In cell culture, nuclear morphology was analyzed after staining with the chromatin-dye HOECHST 33342 (5 μg/ml). The number of homogenously stained nuclei *versus* condensed/fragmented nuclei was determined in five representative microscopical fields per well.

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