Molecular events associated with apoptosis and proliferation induced by ultraviolet-B radiation in the skin of hairless mice

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Summary Background: It is recognized that UV radiation produced apoptotic cells (sun burn cells) in the epidermis of mice. However, the relationship between apoptosis and cell proliferation after UV exposure in the skin of hairless mice are still unclear. Objective: To investigate the effects of ultraviolet (UV) radiation on molecular events associated with apoptosis and proliferation in SKH1-hr mouse skin. Methods: Mice were irradiated with daily UVB exposure of 0.1 or 0.25 J/cm² for 14 days. The skin tissues were analyzed at 2 and 24 h after the end irradiation for the presence of apoptotic cells and Bromodeoxyuridine (BrdU)-positive cells. We measured the expression of p53, p21, bcl-2, bax and E2F-1. Results: The results indicated that UVB irradiation caused to increase apoptotic cells in the epidermis of mice. The expression of p53 and p21 was increased at 2 and 24 h after irradiation compared with the control. UV radiation induced high levels of bax at 2 and 24 h after irradiation with a concomitant decrease in bcl-2 expression. The expression of E2F-1 in the skin was also increased at 2 and 24 h after irradiation. Coinciding with these changes, BrdU positive cells increased at 2 and 24 h after UVB exposure at the epidermis of hairless mice, which observed the apoptotic expression. Conclusion: These results suggest that UVB irradiation of mouse skin induces apoptosis and is mediated by the p53/p21/E2F-1/bax pathway and that the dead cells are replaced by hyperproliferative cells, leading to epidermal hyperplasia.

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1. Introduction

The majority of human skin cancers are associated with exposure to ultraviolet (UV) radiation in sunlight. The incidence of skin cancer is increasing, and additional increases are predicted because of an increase in recreational exposure to sunlight and also because of the depletion of the stratospheric ozone layer [1]. The UV spectrum can be divided into three parts by wavelength: UVA (320–400 nm), UVB (280–320 nm) and UVC (200–280 nm). UVB light and to a much lesser extent UVA light are responsible for these cancers [2]. Wave-
lengths less than 280 nm (UVC) do not occur at the earth’s surface, since they are absorbed, predominantly by ozone, in the stratosphere. Although UVB wavelengths represent only approximately 1.5% of the solar energy received at the earth’s surface, they can produce erythema, burns and eventually skin cancers. Studies using laboratory animals have shown that UVB radiation is very efficient in inducing skin cancer [3,4]. UVB radiation exerts its carcinogenic and cytotoxic actions primarily through the direct formation of cyclobutane pyrimidine dimers (thymidine dimers) and pyrimidine (6-4) pyrimidone photodimers (6-4 photoproducts) in DNA which then leads to a cascade of events including cell cycle arrest, DNA repair, mutation, and transformation [5].

The p53 suppressor gene is considered the guardian of the genome and is one of the most frequently mutated genes in UV-induced human and mouse skin cancers. UV radiation could induce high levels of p53 which in turn activates the transcription of downstream genes responsible for cell cycle arrest at the G1-S transition. The G1-S arrest results partly from p53 transactivation of p21, which binds to and inactivates the cyclin-dependent kinases required for cell cycle progression. This growth arrest allows the cells to repair the DNA damage [6–12]. Members of the E2F family of transcription factors are thought to regulate cell cycle progression by activating the transcription of a set of genes necessary for the induction of S-phase. It is suggested that deregulated E2F-1 activity gives rise to proliferative and apoptotic signals [13–16].

It is recognized that UV radiation produced apoptotic cells (sun burn cells) in the epidermis of mice [17]. However, there are not many studies for the effect of UV on changes in expression of apoptosis-regulatory molecules repeated exposure of UVB in hairless mice. Moreover, the relationship between apoptosis and cell proliferation after UV exposure in the skin of hairless mice has not been reported.

The present study was undertaken in order to investigate changes in expression of apoptosis regulatory molecules such as p53, p21, bax, bcl-2, E2F-1 and cell proliferation in the skin of SKH1-hr hairless mice treated with 2 week repeated exposure of UVB.

2. Materials and methods

2.1. Experimental animal and UV radiation

Specific pathogen-free hairless male mice of SKH1-hr (6-weeks-old) were obtained from National Institute of Toxicological Research of Korea Food and Drug Administration (Seoul, Korea). Animals used were cared for in accordance with the Accreditation of Laboratory Animal Care (AAALAC). These animals were housed in polycarbonate cage in a room with controlled temperature and humidity, and given food and water ad libitum. A total of 24 mice were divided into three groups of eight animals. One group of eight mice was not exposed to UV and was used as a control. The other 16 mice were irradiated for 14 days at a dose of 0.1 or 0.25 J/cm² UVB, respectively. UV irradiation was carried out using UV lighter (RMX 3 W lighter, Dong Sung Lab. Tech., Seoul, Korea) equipped with a F40M UVB lamp emitting maximally at 312 nm (Vilbert-Lourmat, Merne-La-Vallee Cedex 2, France). The radiation intensity was monitored by a VLX-3W radiometer (Vilbert-Lourmat) equipped with VLX-312 (UVB) sensors. The UVB detector was placed in a cage with a wire top under the lights, and the UVB was measured.

2.2. Preparation of skin samples

Groups of four mice were killed at 2 and 24 h after UVB radiation. The dorsal skin (ca. 2 × 4 cm) was excised from each mouse and cut into two pieces. One piece was immediately fixed in 4% neutral buffered formaldehyde for paraffin embedding. The other piece was placed on ice and immediately frozen at −80 °C for Western blotting. The paraffin-embedded skin was cut into 5-µm sections, deparaffinized, hydrated, dehydrated, and stained with hematoxylin and eosin.

2.3. Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay

UV-irradiated mouse skin was examined for the presence of apoptotic cells by the TUNEL assay. This assay provides a relatively reliable measure of apoptosis and readily identifies fragmented DNA. The TUNEL assay was performed using a commercial kit according to the manufacturer’s protocol (Boehringer Mannheim, Germany). Briefly, the 5-µm sections were deparaffinized and hydrated with deionised water. Then, they were treated with 20 mg/ml proteinase K for 30 min and permeabilized by incubation with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. After being rinsed twice with PBS for 5 min, the slides were incubated with reaction buffer containing terminal deoxynucleotidyl transferase and anti-fluorescein Ab conjugated with POD in a humid atmosphere at 37 °C for 1 h. After a wash in
PBS, they were reacted with diaminobenzidine as the chromogen. Counterstaining was performed with Hematoxylin. The TUNEL positive cells in the epidermis were calculated from the number of stained TUNEL-positive cells per 100 epidermal cells counted from entire 20-mm length of epidermis for each section.

2.4. DNA isolation and electrophoresis

The epidermis was incubated with lysis buffer [30 mM Tris, pH 8.0, 800 mM guanidine HCl, 5% Tween 20, 0.5% Triton X-100, 20 mM EDTA, 1 mg/ml proteinase, 200 μg/ml RNase] at 40 °C for 4 h. At the end of incubation, the mixture treated with an equal volume of phenol–chlorform–isoamyl alcohol and water phase containing DNA were precipitated with 0.3 M sodium acetate and 2.5 volumes of ethanol at −70 °C and rinsed once with 70% ethanol. The precipitate was dried under vacuum and resuspended in TE buffer supplemented with RNAse A and incubated at 37 °C for 1 h. Extracted DNA was separated on a 1.6% agarose gel and stained with ethidium bromide.

2.5. Western blotting

The epidermis of mice was homogenized with lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% sodium dodecyl sulfate (SDS), 100 μg/ml phenylmethylsulfonyl fluoride (PMFS), 1 μl/ml aprotinin, 1% gelatin 630 (Sigma Chem. Co., St. Louis, MO), 0.5% deoxychoate], and centrifuged at 23 000 × g for 1 h. Equal amount of proteins was separated on a SDS-12%-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were then blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% tween-20. The membrane was then incubated for 3 h at room temperature with specific antibodies. The antibodies used were mouse anti-p53 monoclonal antibody Pab240, mouse anti-E2F-1 monoclonal antibody, rabbit polyclonal anti-p21 antibody, rabbit polyclonal anti-bax antibody, and rabbit polyclonal anti-bcl-2 antibody. All antibodies using western blotting were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Detection of immunoreactive proteins was performed with the ECL Western blotting detection system. The relative density of the protein bands was quantified by densitometry using Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Com., Rochester, NY).

2.6. BrdU Incorporation into DNA and labeling index

All animals received injections of BrdU (100 mg/kg body weight) i.p. and were killed 2 h later. Immunohistochemical assays performed according to previous report. After deparaffinization, 5 μm sections were treated with proteinase K and denatured with 2 N HCl. Endogenous peroxidase was blocked by incubating the tissue sections in 3% hydrogen peroxide in methanol for 30 min at room temperature. The sections were incubated with blocking serum for 20 min at room temperature and covered with biotinylated mouse monoclonal anti-BrdU antibody (Beckton–Dickson, USA) at room temperature at 60 min. Then sections were incubated with biotinylated horse anti-mouse serum and incubated with streptavidin-peroxidase for 30 min. Color development was achieved by incubation with a DAB substrate kit (Sigma, USA) containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide. The slides were weakly counterstained in Mayer’s hematoxylin for 2 min, cleared with xylene, mounted with a coverslip, and scored under a light microscope. The labeling index (LI) of BrdU-labeled cell in the epidermis was calculated from the number of stained BrdU-positive cells per 100 epidermal cells counted from entire 20-mm length of epidermis for each section.

2.7. Statistics

The data was expressed as the mean ± standard derivation. The data was analyzed with a one way analysis of the variance followed by Fisher LSD’s method as a post hoc test [18]. Statistical analysis was performed using SIGMA STAT VER 2.0.

3. Results

3.1. Effect of UVB on the skin of hairless mice

UVB-irradiated mouse skin was revealed the presence of sunburn cells that exhibited the classic feature of apoptosis, pyknotic nuclei (Fig. 1). In the unirradiated mouse skin, a few cells undergoing normal cell death or differentiation were randomly distributed across the epidermis. However, in UV-irradiated skin, there were many sunburn cells, and the number increased at 24 h after the termination of irradiation. Some of the sunburn cells at 2 h after the end of irradiation had contracted nuclei.
and clear cytoplasm, whereas the sunburn cells at 24 h had contracted nuclei and pink cytoplasm, suggesting that they were more likely related directly to UV-induced damage. UV-irradiated skin at 2 and 24 h after UVB exposure was also revealed an increase in epidermal hyperplasia in UV-irra-

Fig. 1 The morphological analysis of skin in SKH1-hr mice treated with UVB radiation of 0, 0.1 and 0.25 J/cm² for 14 days. H&E stained sections reveal sunburn cell and epidermal hyperplasia of skin treated with 0.25 J/cm² radiation of UVB at 2 h after the end of irradiation (b), but not in control skin (a) (original magnification × 400). The TUNEL stained sections show apoptotic cells in the skin treated with 0.25 J/cm² radiation of UVB at 2 h after the end of irradiation(d), but not in control skin (c) (original magnification × 400). BrdU stained section reveal proliferation of keratinocytes in skin treated with treated with 0.1 J/cm² radiation of UVB at 2 h after the end of irradiation (f), but a few positive cell observed in control skin (e). (original magnification × 400).
diated skin. Focal hyperplasia of the skin occurred at 24 h when the epidermis was four to six cells thick (data not shown). In addition to formation of sunburn cells, inflammatory cells were also observed in UV-irradiated mouse skin. These cells were mainly focal in the dermis, around blood vessels.

3.2. TUNEL assay and DNA fragmentation for apoptosis in UVB-irradiated mouse skin

The UV-irradiated mouse skin was examined for the presence of TUNEL-positive cells. Less than 2% of unirradiated control mice were TUNEL-positive. UV radiation induced increases in the number of epidermal TUNEL-positive cells by about 45% after the termination of irradiation. There were more increases at 24 than 2 h after irradiation in both doses of UVB 0.1 and 0.25 J/cm² (Fig. 1). To determine whether TUNEL positive cells were actually undergoing apoptosis, total cellular DNA was extracted from epidermis at 2 h after UV irradiation. The extracted DNA was separated by electrophoresis on an agarose gel to detect the internucleosomal DNA ladders that are a hallmark of apoptosis. Control irradiated contained high-molecular weight DNA. In contrast, UVB irradiation produced internucleosomal DNA ladders (Fig. 2).

3.3. Expression of p53, p21, bax, bcl-2, and E2F-1 in UVB-irradiated mouse skin

Western blot data of representative UV-irradiated and unirradiated mouse skin at 2 and 24 h after the termination of irradiation are shown in Fig. 3. The level of p53 increased at 2 and 24 h in the dose of UVB 0.1 J after irradiation. And the level of UVB 0.25 J slightly more increased than 0.1 J of UVB. Similarly, p21 expression closely followed p53 expression. Expression of bax was increased at 24 h after UVB irradiation, whereas the level of bcl-2 expression had substantially decreased at 24 h after irradiation (Fig. 4).

The expression of E2F-1 at 2 and 24 h was elevated in UV-treated skin compared with that in the untreated control skin (Fig. 5).

3.4. Expression of BrdU positive cell in the skin of mice irradiated with UVB

The UV-irradiated mouse skin was examined for the presence of BrdU-positive cells. Less than 5% of unirradiated control mice were BrdU-positive. UV radiation induced increases in the number of epidermal BrdU-positive cells by about 40% after the termination of irradiation. There were more increases at 24 than 2 h after irradiation in both doses of UVB 0.1 and 0.25 J/cm² (Fig. 6).

4. Discussion

In this study, we observed that exposure of hairless mouse skin to a repeated dose of UVB caused the formation of sunburn (apoptotic) cells at 2 and 24 h after the termination of irradiation. One of the most characteristic histologic change of acutely UVB-damaged skin is the appearance of sunburn cells. The mechanisms involved in the generation of sunburn cells remain not well defined. It has been suggested DNA repair, and reactive oxygen intermediates may be important [3,4]. In the present study, we observed the characteristic ladder pattern of fragmented DNA in the skin of mice irradiated UVB (Fig. 2).

Several studies have shown that p53 is involved in UV-induced apoptosis [5–7]. The protein product of the p53 gene responds to cellular stresses such as DNA damage and hypoxia and plays important roles in regulating cell cycle progression, genomic stability, and apoptosis [19]. Mutations in the p53 gene have been detected in about 50% of all human cancers, demonstrating the universality of this tumor suppressor. DNA damage can be caused by a number of genotoxic agents, and UVB radiation is one of the most biologically relevant inducers of DNA damage. UVB radiation in sunlight is the carcinogen responsible for most human skin cancers. The importance of UV radiation in the development of cutaneous squamous cell carci-
noma and the frequency of p53 mutations in this form of cancer, implicates p53 in control of the keratinocyte protective response after UV damage [20–23]. However, it was reported that UVB-induced keratinocyte apoptosis was not affected by overexpressed mutant p53 protein using a murine transgenic model in which mice carry extra copies of a mutant p53 gene [24]. In this study, UVB radiation caused increase in the level of p53 in the skin of mice. Our data could support that in response to the DNA damage induced by UVB, the p53 protein is stabilized and translocated the nucleus, where it triggers an arrest of the cell cycle or induces apoptosis.

The p21 protein, one of key cell cycle regulators, subsequently binds and inhibits cyclin-dependent kinase substrates and blocking cell cycle progression [11]. Gene targeting strategies have recently established p21 as a critical establishment of p53-mediated G1 arrest response [25]. Liu and Pelling [21] demonstrated that UV induction of p21 in mouse keratinocytes is mediated by p53 because UV irradiation does not induce p21 in p53-deficient cells. However, it is reported that a recent study has shown that UV can induce p53-independent p21 protein expression in mouse keratinocytes in vivo and in vitro [26]. Our data could support that UV induction of p53 preceded induction of p21 protein suggest that UV induces p53, which, in turn, transactivates p21 and causes cell cycle arrest to permit the repair of UV-induced DNA damage.

E2F, a family of heterodimeric transcription factors composed of E2F-like and DP-like subunits, is crucial for transcriptional activation of genes that regulate S phase entry and genes that function to engage DNA synthesis [27]. E2F-1, the first cloned member of this family, plays a key role in the regulation of cell proliferation and apoptosis. Qin et al. [13] reported that deregulated E2F-1 activity gives rise to proliferative and apoptotic

![Western blot analysis of p53 and p21](image1)

**Fig. 3** Western blot analysis of p53 and p21 of the skin in SKH1-hr hairless mice. Animals were treated with UVB radiation of 0, 0.1 and 0.25 J/cm² for 14 days. Values represent the mean ± S.E.M. (n = 4).

![Western blot analysis of bax and bcl-2](image2)

**Fig. 4** Western blot analysis of bax and bcl-2 of the skin in SKH1-hr hairless mice. Animals were treated with UVB radiation of 0, 0.1 and 0.25 J/cm² for 14 days. Values represent the mean ± S.E.M. (n = 4).
signals. Degregori et al. [16] reported that E2F-1 overexpression induces cells to undergo apoptosis despite the fact that at least two other E2F family members, E2F2 and E2F3, are equally capable of inducing S phase. In this study, we observed that level of E2F-1 increased in the epidermis of mice treated UVB. Our data could support that the increase of E2F-1 could be relevant to UV-induced apoptosis and E2F-1 may function as a specific signal for the initiation of an apoptosis pathway that must normally be blocked for a productive proliferation event.

Bax, a bcl-2 family member, functions as a death agonist within a common apoptotic pathway. Bax forms homodimers and also heterodimerizes with death antagonists, bcl-2 and bcl-xL. The bcl-2 gene promotes cell survival by blocking apoptosis. The high levels of bcl-2 promote cancer by inhibiting apoptosis, thereby prolonging cell survival [28]. Bcl-2 protects against diverse cytotoxic insults-for example, γ- and UV-irradiation, dexamethasone, and cytotoxic drugs [29]. Bax is induced in some cells as part of the p53-mediated damage response [29,30]. In this study, we observed the induction of apoptosis by UV closely followed the time course induction by p53 and p21. In addition, UV-induced bax expression was inversely correlated with bcl-2 expression (Fig. 4). These results suggest that the activation of apoptosis by UV irradiation is most likely mediated by the p53 pathway, which involves up-regulation of bax and down-regulation of bcl-2.

UVB irradiation of mouse skin causes a number of cellular and pathological changes, including DNA damage, cell-cycle arrest, sunburn, apoptosis and hyperplasia [5,31–33]. In normal human and mouse epidermis, cells are constantly turning over; stem cells divide and generate into keratinocytes that differentiate and desquamate on the surface of the skin. Thus, differentiated cells are constantly replaced by proliferating cells from the basal layer. BrdU, an analog of thymidine, is incorporated into DNA during the S-phase of the cell cycle, and is a useful alternative to labeling proliferating cells...
with tritiated thymidine [34–36]. In this study, we observed the hyperplasia of epidermis in the hairless mice irradiated with UVB. The level of BrdU expression in epidermis dramatically increased compared with un-irradiated control and slightly increased at 24 h after the end of irradiation than at 2 h. Similar to the pattern of BrdU staining, the pattern of TUNEL positive cell was dramatically increased compared with un-irradiated control and increased at 24 h after the termination of UVB irradiation than at 2 h. This result is consistent with the results of Ouhtit et al.’s study [12] and may support that UVB causes severe hyperplasia in the skin to replace the dead cells, suggesting that the processes of apoptosis and proliferation are closely linked and tightly regulated and that chronic UV irradiation may uncouple and dysregulate these two events, leading to the development of cancer.

In summary, our data give support to increasing evidence that exposure of mouse skin to repeated dose of UVB results in induction of apoptosis with the coordinated induction of p53, p21, bax and EZF-1 as well as the proliferation of keratinocyte in epidermis of the mice.

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