Temporal Events in Skin Injury and the Early Adaptive Responses in Ultraviolet-Irradiated Mouse Skin

Allal Ouhtit,* H. Konrad Muller,[‡] Darren W. Davis,[†]
Stephen E. Ullrich,* David McConkey,[†] and
Honnava N. Ananthaswamy*

From the Departments of Immunology* and Cancer Biology,[†]
University of Texas M. D. Anderson Cancer Center, Houston,
Texas; and the Department of Pathology,[‡] University of
Tasmania, Hobart, Tasmania, Australia

We examined the effects of ultraviolet (UV) radiation on the time course for induction of sunburn (apoptotic) cells and expression of proteins known to be associated with growth arrest and apoptosis in SKH-hr1 mouse skin. Mice were irradiated with a single dose (2.5 kJ/m²) of UV from Kodacel-filtered (290–400 nm) FS40 sunlamps and the skin tissues were analyzed at various times after irradiation for the presence of apoptotic cells and expression of p53, p21^{Waf-1/Cip1}, bcl-2, bax, and proliferating cell nuclear antigen. The results indicated that p53 expression was induced early in the epidermis, reaching maximum levels 12 hours after irradiation, and p21^{Waf-1/Cip1} expression in the epidermis peaked at 24 hours after irradiation. In contrast, UV radiation induced high levels of bax at 24 to 72 hours after irradiation with a concomitant decrease in bcl-2 expression. Coinciding with these changes, apoptotic cells began to appear 6 hours after irradiation and reached a maximum at 24 hours after irradiation. Interestingly, proliferating cell nuclear antigen expression, which was initially confined to the basal layer, became dispersed throughout the basal and suprabasal layers of the skin at 48 hours and paralleled marked hyperplasia. These results suggest that UV irradiation of mouse skin induces apoptosis mediated by the p53/p21/bax/bcl-2 pathway and that the dead cells are replaced by hyperproliferative cells, leading to epidermal hyperplasia. This implies that UV-induced apoptosis and hyperplasia are closely linked and tightly regulated and that dysregulation of these two events may lead to skin cancer development. (*Am J Pathol* 2000, 156:201–207)

Nonmelanoma skin cancers are the most frequent type of human cancer.^{1–2} Epidemiological, clinical, and biologi-

cal studies have indicated that solar ultraviolet (UV) radiation is the major etiological agent in skin cancer development.^{1–4} Wavelengths in the UVB (280–320 nm) region of the solar spectrum are absorbed by the skin, producing erythema, burns, and eventually skin cancer.^{5–8} Studies using laboratory animals have shown that UVB radiation is very efficient in inducing skin cancer.^{9–11} The first step in skin carcinogenesis by UV radiation involves the induction of DNA damage, mainly formation of cyclobutane pyrimidine dimers¹² and pyrimidine (6–4) pyrimidone photoproducts,¹³ which then leads to a cascade of events including cell cycle arrest, DNA repair, mutation, and transformation.

The p53 tumor suppressor gene is considered the guardian of the genome¹⁴ and is one of the most frequently mutated genes in UV-induced human and mouse skin cancers.^{15–17} DNA-damaging agents such as UV and ionizing radiation induce high levels of p53,^{18–27} which in turn activates the transcription of downstream genes responsible for cell cycle arrest at the G₁-S transition.²⁸ The G₁-S arrest results, at least in part, from p53 transactivation of p21^{Waf-1/Cip1}, which binds to and inactivates the cyclin-dependent kinases required for cell cycle progression.^{29–31} This growth arrest allows the cells to repair the DNA damage.^{32–34} However, p53 can also cause apoptosis of cells with excessive unrepaired DNA damage^{35–36} by activation of bax and/or down-regulation of bcl-2 expression.^{37–39}

In vitro and *in vivo* studies from different laboratories have shown that UV induces the expression of p53^{18–27,40} and p21^{Waf-1/Cip1}^{22,25,29–31} and the formation of sunburn cells.³⁶ However, the effects of UV radiation on the time course for induction of p53 and its downstream effectors, formation of sunburn (apoptotic) cells, changes in expression of apoptosis-regulatory molecules, and hyperplasia have not been investigated in the same system. This is quite important to understanding the temporal events, at the cellular and molecular level, in skin injury

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Address reprint requests to Dr. Honnava N. Ananthaswamy, Department of Immunology, Box 178, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030. E-mail: hanantha@notes.mdacc.tmc.edu.

(sunburn) caused by exposure to UV radiation and the adaptive responses that the skin employs to cope with this injury. In the study described here, we examined the time course for induction of sunburn cells and epidermal hyperplasia in UV-irradiated mouse skin. In addition, we investigated the temporal changes in expression, not only of proteins known to be associated with growth arrest such as p53 and p21^{Waf-1/Cip1}, but also those associated with apoptosis, bax and bcl-2, and proliferation, proliferating cell nuclear antigen (PCNA).

Materials and Methods

Mice and UV Irradiation

Female SKH-hr1 mice 7 to 8 weeks old were obtained from Charles River (Wilmington, MA) and housed in cages in a room with controlled temperature and humidity and alternating 12-hour light and dark cycles. The room was lit with yellow fluorescent lamps (Mazda S.10) in ceiling fixtures with plastic diffusers to eliminate all ambient UV radiation. The mice were fed with a commercial diet and had water *ad libitum*.

Twenty-one mice were used in this experiment. One group of 3 mice was not exposed to UV and was used as a control. The other 18 mice were placed into standard cages (≤ 5 mice/cage) separated into individual compartments with Plexiglas dividers. The animal cages were placed on a shelf 20 cm below the light source, and mice were irradiated with a single dose of 2.5 kJ/m² UV from a bank of six Kodacel-filtered (290–400 nm) FS40 sunlamps, as described previously.⁴¹ The irradiance of the sunlamps was measured with an IL-700 radiometer with an SEE 240 UVB detector equipped with an A127 quartz diffuser (International Light, Newburyport, MA). The UVB detector was placed in a cage with a wire top under the lights, and the UVB was measured. Under this protocol the mice were exposed to 2.5 W/m² UVB radiation.

Isolation of Skin Samples

Groups of 3 mice were killed at 3, 6, 12, 24, 48, and 72 hours after UV irradiation. The dorsal skin (approximately 2 × 4 cm) was excised from each mouse and cut into 2 pieces. One piece was immediately fixed in 4% buffered formaldehyde for paraffin embedding. The other piece was floated dermis side down on 0.5 mol/L buffered EDTA solution, pH 7.4, for 1 hour at 37°C, and the epidermis was separated from the dermis. Cell lysates were prepared from the epidermis in the cold (on ice) and immediately frozen at –80°C. The paraffin-embedded skin was cut into 5- μ m sections, deparaffinized, hydrated, dehydrated, and stained with hematoxylin and eosin.

Terminal Deoxynucleotidyl Transferase Nick End Labeling (TUNEL) Assay

In addition to defining the sunburn cells histologically, UV-irradiated mouse skin was examined for the presence

of apoptotic cells by the TUNEL⁴² 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 (Promega Corp., Madison, WI). Briefly, the 5- μ m sections were deparaffinized and fixed in 4% paraformaldehyde at room temperature for 5 minutes. Then, they were treated with 20 μ g/ml proteinase K for 10 minutes and permeabilized by incubation with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes at room temperature. After being rinsed twice with PBS for 5 minutes, the slides were incubated with reaction buffer containing terminal deoxynucleotidyl transferase and fluorescein-12-dUTP in a humid atmosphere at 37°C for 1 hour. EDTA was added to the slides for 5 minutes to stop the reaction, and the slides were washed three times with PBS for 5 minutes and stained with 10 μ g/ml propidium iodide for 10 minutes. Finally, after 3 washes with PBS for 5 minutes, coverslips were mounted with Prolong reagent (Molecular Probes, Eugene, OR) to prevent fluorescence bleaching during analysis, and fragmented DNA was identified by measurement of incorporated fluorescein-12-dUTP. The slides were examined with an Olympus Inverted System Microscope IX70 (Olympus, Melville, NY), and pictures were taken with a Nikon 35-mm camera. The number of TUNEL-positive cells per 100 cells in a field were counted under a microscope. Four such fields were counted for each of the three mice used for each time point. The mean values and standard deviations were calculated using StatView 4.0.

Western Blot Analysis

The epidermis of each mouse was homogenized, and proteins were extracted with a lysis buffer (120 mmol/L NaCl, 25 mmol/L Tris, pH 7.5, and 1% Triton X-100) containing protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) for 1 hour on ice. Equal amounts of protein extract (100 μ g) from each lysate were electrophoresed on 12% polyacrylamide-sodium dodecyl sulfate gels, transferred onto a nitrocellulose membrane, and incubated with specific antibodies. The antibodies used were mouse anti-p53 monoclonal antibody PAb240 (cat. no. NCL-p53-240, NovoCastra, Newcastle, UK), monoclonal anti-p21^{Waf1/Cip1} antibody Ab-4 (cat. no. OP76, Oncogene Science, Inc., Uniondale, NY), monoclonal anti-bcl-2 antibody (cat. no. 15616E, Pharmingen, San Diego, CA), rabbit polyclonal anti-mouse bax antibody (cat. no. sc-493, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-human PCNA antibody clone PC10 (cat. no. M0879, DAKO, Carpinteria, CA) diluted 1:100 in PBS. Only p53 proteins were initially immunoprecipitated with anti-p53 monoclonal antibody Ab-3 (cat. no. OP29, Oncogene Science) before the electrophoresis. After incubation with the appropriate secondary antibody, signals were detected using immunochemiluminiscent reagents (Amersham Life Science, Poole, UK) and autoradiography. Equal protein loading in each lane was confirmed by hybridization with a 1:2000 dilution of

α -actin antibody (Sigma Chemical Co., St. Louis, MO). The band intensities were quantified by densitometric scanning of autoradiographs using Image Quant (Molecular Dynamics, Sunnyvale, CA). Three optical density values, corresponding to the three mice used per each time point, were normalized for α -actin and then averaged. The resulting mean values were compared with the control (unirradiated) skin to determine the increase or decrease of protein expression level. Statistical analysis was performed using StatView 4.0.

Immunohistochemical Analysis

Immunohistochemical assays were performed according to Berg et al,⁴³ with slight modifications. After deparaffinization, 5- μ m sections were treated with target retrieval solution (DAKO), washed 3 times with PBS and incubated in H₂O₂/methanol/PBS solution (1:50:50) for 15 minutes to block endogenous peroxidase activity. After 3 washes in PBS with 0.5% Tween, the sections were preincubated for 10 minutes in 10% normal goat serum in PBS and then incubated with the first antibody overnight at 4°C. The antibodies used were: (i) rabbit polyclonal anti-mouse p53 antibody (cat. no. NCLp53-CM5, Novocastra) diluted 1:1000 in PBS, (ii) rabbit polyclonal anti-mouse p21 antibody (cat. no. sc-471, Santa Cruz Biotechnology) diluted 1:500 in PBS, (iii) rabbit polyclonal anti-mouse bcl-2 antibody (cat. no. 15021A, Pharmingen) diluted 1:500 in PBS, (iv) rabbit polyclonal anti-mouse bax antibody (cat. no. sc-493, Santa Cruz Biotechnology,) diluted 1:500 in PBS, and (v) mouse anti-human PCNA antibody clone PC10 (cat. no. M0879, DAKO) diluted 1:50 in PBS. After 3 washes in PBS plus 0.5% Tween, the sections were incubated for 1 hour at room temperature with the following secondary antibodies: (i) biotin-conjugated goat anti-rabbit antibody solution for p53 (Vector Laboratories, Burlingame, CA), (ii) donkey anti-rabbit horseradish peroxidase-linked F(ab)₂ fragment (cat. no. NA9340, Amersham Life Science) diluted 1:100 in PBS for p21, bcl-2, and bax, and (iii) anti-mouse horseradish peroxidase-linked antibody (cat. no. NA931, Amersham Life Science) diluted 1:100 in PBS for PCNA. After a wash in PBS, p53 staining was performed with the Vectastain Elite ABC kit with diaminobenzidine as the chromagen, as recommended by the manufacturer (Vector Laboratories) or by direct diaminobenzidine staining for p21, bcl-2, bax, and PCNA. Counterstaining was performed with hematoxylin. As a negative control, tissue sections were stained only with the secondary antibody.

Results

Induction of Sunburn Cells in UV-Irradiated Mouse Skin

UV-irradiated mouse skin was examined for the presence of sunburn cells at various times after irradiation. Hematoxylin-and-eosin-stained photomicrographs of representative unirradiated mouse skin and UV-irradiated skin 24

to 72 hours after exposure, shown in Figure 1, revealed the presence of sunburn cells that exhibited the classic feature of apoptosis, pyknotic nuclei. 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, the number (59 ± 5 per 100 cells) peaking at 24 hours after irradiation. Some of the sunburn cells at early time points after irradiation had contracted nuclei and clear cytoplasm, whereas the sunburn cells during the peak of this response (24 hours) had contracted nuclei and pink cytoplasm, suggesting that they were more likely related directly to UV-induced damage. In one case, at 48 hours after UV, the entire epidermal outer layer showed marked parakeratosis and many apoptotic nuclei (data not shown). In addition to formation of sunburn cells, inflammatory cells were also observed in UV-irradiated mouse skin. These ranged from a few dermal mononuclear cells in the control skin to many mononuclear cells and neutrophil polymorphs in the UV-irradiated skin. These cells were mainly focal in the dermis, around blood vessels, and their numbers seemed to peak at 48 hours after irradiation (data not shown).

TUNEL Assay for Programmed Cell Death in UV-Irradiated Mouse Skin

In addition to defining the sunburn cells histologically, the UV-irradiated mouse skin was examined for the presence of TUNEL-positive cells. Immunofluorescence pictures of representative unirradiated mouse skin and UV-irradiated skin 24 to 72 hours after irradiation, shown in Figure 2, indicated a gradual increase in apoptotic cells with increasing time after UV irradiation. Although a few TUNEL-positive cells were observed in unirradiated skin, the number of apoptotic cells in the epidermis dramatically increased and peaked at 24 hours (68 ± 4 per 100 cells), then significantly decreased at 72 hours (4 ± 0.08 per 100 cells) after UV exposure. Interestingly, apoptotic cells were also present in the dermis of UV-irradiated skin, suggesting that UV radiation had penetrated and damaged the cells in the dermis.

Kinetics of p53, p21^{Waf1/Cip1}, bax, and bcl-2 Expression in UV-Irradiated Mouse Skin

The time course for induction of p53 and p21^{Waf1/Cip1} in UV-irradiated mouse epidermis was first investigated by Western blot analysis with specific antibodies. Western blot data of representative unirradiated mouse skin and UV-irradiated skin 3 to 72 hours after irradiation is shown in Figure 3. Maximum induction of p53 occurred 12 hours after UV (20.4 ± 2.3 -fold) but declined to basal levels at 72 hours (0.005 ± 0.003 -fold). Similarly, p21^{Waf1/Cip1} expression closely followed p53 expression, reaching maximal levels 24 hours (5.6 ± 0.9 -fold) after UV irradiation.

The time course for induction of a proapoptotic protein (bax) and the down-regulation of an anti-apoptotic protein (bcl-2) was also examined in UV-irradiated mouse

Figure 1. Induction of sunburn cells and epidermal hyperplasia in unirradiated and UV-irradiated mouse skin at various times after UV irradiation. **Arrows** indicate representative sunburn cells. Original magnification, $\times 400$ for unirradiated and 24 hours post-UV and $\times 200$ for 48 and 72 hours post-UV.

skin. Expression of bax was maximal at 24 hours (4.0 ± 0.3 -fold) after irradiation, whereas the level of bcl-2 expression had substantially decreased between 3 and 24 hours (0.17 ± 0.009 -fold) after irradiation (Figure 3).

Localization of p53, p21^{Waf1/Cip1}, bax, and bcl-2 Expression in UV-Irradiated Mouse Skin

Immunohistochemical analysis was then used to determine whether UV irradiation had any effect on the cellular distribution of p53 and p21^{Waf1/Cip1}, bax, and bcl-2 expression at different time points after UV irradiation. Although no positive signal for p53 was detected in unirradiated skin (Figure 4), intense p53 nuclear immunostaining was observed along the basal layer of the epidermis 12 hours after UV exposure

Figure 2. TUNEL assay for detection of apoptotic cells in unirradiated and UV-irradiated mouse skin at various times after UV irradiation. Original magnification, $\times 200$.

Figure 3. Western blot analysis of p53, p21^{Waf1/Cip1}, bax, bcl-2, PCNA, and α -actin protein expression in unirradiated (N) and UV-irradiated mouse skin at various time points.

(Figure 4) and also appeared to accumulate in the hair follicles. However, at 24 hours after irradiation, p53 was expressed in both the basal and the suprabasal layers. In addition, analogous to the presence of apoptotic cells in the dermis (Figure 2), cells in the dermis also expressed high levels of nuclear p53 immunostaining 12 to 24 hours after UV irradiation (Figure 4), suggesting further that UV radiation had indeed penetrated and damaged the cells in the dermis. Although the p21^{Waf1/Cip1} expression was very low in the epidermis of unirradiated skin, it was expressed at high levels, peaking at 24 hours, in and around the nuclei in the upper layers of the epidermis (mature and differentiated cells) in irradiated skin (Figure 5). On the other hand, bax immunoreactivity was both perinuclear and cytoplasmic and was high in the upper layers of the epidermis 24 hours after UV (Figure 5). In contrast to bax immunoreactivity, bcl-2 immunoreactivity was high in unirradiated skin but decreased gradually over time after UV exposure (Figure 5).

Induction of Hyperplasia and PCNA Expression in UV-Irradiated Mouse Skin

Histological examination of UV-irradiated skin 3 to 72 hours after exposure revealed a gradual increase in epidermal hyperplasia in UV-irradiated skin. Focal hyperplasia of the skin occurred as early as 6 hours after irradiation when the epidermis was 4 to 6 cells thick (data not shown); the hyperplasia became more uniform and was maximal (about 14 to 18 cells thick) 48 to 72 hours after irradiation (Figure 1). In addition, epidermal hyperplasia was accompanied by increased keratin production at 24 hours and hyperkeratosis at 48 and 72 hours after irradiation. At 24 hours, cells in focal areas of the epidermis were quite swollen and had fine granular cytoplasm.

To determine whether the cells in hyperplastic epidermis were proliferating, the skin of UV-irradiated mice was analyzed for PCNA expression, a marker of proliferation, by Western blot and immunohistochemical analyses. The

Figure 4. Immunohistochemical analysis for p53 and PCNA proteins in unirradiated and UV-irradiated mouse skin. Original magnification, $\times 200$.

Western blot data shown in Figure 3 revealed that PCNA expression increased gradually, reaching maximal levels at 48 hours (50.3 ± 5.0 -fold) after irradiation and then decreased at 72 hours after irradiation. At the cellular level, although PCNA immunoreactivity was limited to the basal layer in unirradiated skin, a strong nuclear immunostaining was detected throughout the epidermis 48 hours after irradiation (Figure 4). However, the number of PCNA-positive cells in the epidermis as well as epidermal hyperplasia decreased to normal levels about 2 weeks after UV irradiation (data not shown).

Discussion

Acute UV irradiation of human and mouse skin causes a number of cellular and pathological changes, including DNA damage, cell-cycle arrest, sunburn, apoptosis, and hyperplasia. In this study we investigated the time course for induction of some these events and the pathways or molecules that regulate them. Our results demonstrated that exposure of hairless mouse skin to a single dose (2.5 kJ/m^2) of UV caused the maximum formation of sunburn (apoptotic) cells at 24 hours after irradiation (Figures 1 and 2), suggesting that the UV-induced DNA damage was excessive and was not efficiently repaired, leading to activation of the apoptotic pathway, which eliminates severely damaged cells. Several studies have shown that p53 is involved in UV-induced apoptosis.^{15,36,44} Interestingly, wild-type p53 can simultaneously induce the genetic programs of both G₁ growth arrest and apoptosis in

Figure 5. Immunohistochemical analysis for p21^{Waf1/Cip1}, bcl-2, and bax proteins in unirradiated and UV-irradiated mouse skin. Original magnification, $\times 200$.

the same cell type.⁴⁵ Recently, Cotton and Spandau showed that high doses of UV radiation induce human keratinocytes to undergo apoptosis, whereas low doses do not but appear instead to activate the repair of UV-induced DNA damage.²⁶

The time course for induction of apoptosis by UV closely followed the time course for induction of p53 and p21^{Waf1/Cip1}. In addition, UV-induced bax expression was inversely correlated with bcl-2 expression (Figure 3). These results suggest that 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. Our results on induction of p53 expression and its nuclear accumulation in response to UV irradiation are consistent with previous reports both *in vitro*^{20,22,23,26,27} and *in vivo*.^{18,19,25} Interestingly, immunohistochemical examination of p53 expression in human skin exposed to equally erythemogenic doses of UVC (200–280 nm), UVB, and UVA (320–400 nm) showed that UVA-induced p53 expression is confined to the innermost basal layer of the epidermis and UVC-induced p53 expression to the granular and stratum spinosum layers, whereas UVB-induced p53 expression, which is probably the most relevant to human skin cancer, occurs uniformly through all layers of the epidermis.¹⁸ Because the Kodacel-filtered FS40 sunlamps we used emitted 53% UVB and 47% UVA and nuclear expression of p53 was found in both the basal and suprabasal layers of the epidermis and in the dermis, we can conclude that both UVA and UVB were responsible for induction of p53 in hairless mouse skin. In addition, the presence of apoptotic cells in the dermis (Figure 2) further suggests that both UVB and UVA play a role in induction of p53 and apoptosis.

As mentioned before, p53 also plays a key role in the regulation of cell cycle events.⁴⁶ Indeed, when p53 protein expression is elevated, it turns on the transcription of one of its important downstream genes, p21^{Waf1/Cip1}.^{22,29-31} The p21^{Waf1/Cip1} protein subsequently binds and inhibits cyclin-dependent kinases, preventing phosphorylation of critical cyclin-dependent kinase substrates and blocking cell cycle progression, presumably to provide extra time for the cell to repair DNA damage. Our findings that UV induction of p53 preceded induction of p21^{Waf1/Cip1} protein (Figure 3) suggest that UV induces p53, which, in turn, transactivates p21^{Waf1/Cip1} and causes cell cycle arrest to permit the repair of UV-induced DNA damage. This conclusion is supported by studies of Liu and Pelling,⁴⁷ who demonstrated that UV induction of p21^{Waf1/Cip1} in mouse keratinocytes is mediated by p53 because UV irradiation does not induce p21^{Waf1/Cip1} in p53-deficient cells. Contrarily, a recent study has shown that UV can induce p53-independent p21^{Waf1/Cip1} protein expression in mouse keratinocytes *in vivo* and *in vitro*.⁴⁸ In addition, UV-induced p21^{Waf1/Cip1} expression was confined mainly to the upper layer of the epidermis, further supporting a role for p21^{Waf1/Cip1} protein as a marker of terminally differentiated keratinocytes.⁴⁹

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. PCNA, a subunit of DNA polymerase γ , is known to play a role in DNA replication and repair and serves as a biomarker of proliferation. It is interesting to note that DNA-damaging agents induce both p53 and PCNA expression.^{19,21,28} More importantly, the wild-type p53 protein is known to transcriptionally activate the PCNA promoter,⁵⁰ providing a mechanism whereby p53 induces PCNA expression as a cellular response to DNA damage. On the other hand, p21^{Waf1/Cip1} can inhibit cell cycle progression by forming a quaternary complex with cyclin, cyclin-dependent kinase, and PCNA.⁴⁵ In our study, the level of PCNA expression increased dramatically and was maximal at 48 hours after irradiation (Figure 3), and the PCNA-positive cells were seen throughout the basal and suprabasal layers of the skin (Figure 4) and coincided with the increase in hyperplasia (Figure 1). This observation is consistent with the results of a previous study⁴³ and suggests that UV also activates a proliferative pathway, probably mediated by secretion of growth factors in the skin, to replace apoptotic cells.

In summary, our results demonstrate that exposure of mouse skin to a single dose of UV results in the coordinated induction of p53, p21^{Waf1/Cip1}, and bax as well as the formation of sunburn (apoptotic) cells. In addition, UV 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 skin cancer.

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