Attenuation of UVB-Induced Sunburn Reaction and Oxidative DNA Damage with no Alterations in UVB-Induced Skin Carcinogenesis in *Nrf2* Gene-Deficient Mice

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UV radiation is an important environmental factor in the pathogenesis of skin aging and cancer. Many harmful effects of UV radiation are associated with generation of reactive oxygen species. Cellular antioxidants prevent the occurrence and reduce the severity of UV-induced photoaging and diseases of the skin. The transcription factor Nrf2 (NF-E2-related factor 2) and its negative regulator protein, Keap1 (Kelch-like-ECH-associated protein 1), are central regulators of cellular antioxidant responses. We used *nrf2*-null mice to investigate the roles of the Nrf2–Keap1 system in protection of skin from harmful effects of UVB irradiation. A single irradiation with UVB induced stronger and longer lasting sunburn reaction in *nrf2*-null mice. Histological changes, including epidermal necrosis, dermal edema, inflammatory cell infiltration, sunburn cell formation, TUNEL-positive apoptotic cell formation, and accumulation of oxidative DNA products such as 8-hydroxy-2′-deoxyguanosine after UVB irradiation, were more prominent in *nrf2*-null mice. These findings indicate that the Nrf2–Keap1 pathway plays an important role in protection of the skin against acute UVB reactions, including cutaneous cell apoptosis and oxidative damage. However, there were no significant differences in skin carcinogenesis between *nrf2*-null and wild-type mice exposed to chronic UVB irradiation, suggesting that there is a complex and subtle balance between factors promoting and preventing photocarcinogenesis.

Journal of Investigative Dermatology (2008) **128,** 1773–1779; doi:10.1038/sj.jid.5701245; published online 17 January 2008

INTRODUCTION

As the skin acts as a physiological barrier protecting the organism against pathogens and chemical or physical damage, it is the organ directly exposed to the hazardous effects of UV radiation. UV irradiation of the skin leads to acute inflammatory reactions such as erythema, sunburn (Ley, 1985), and chronic reactions, including premature skin aging (Fisher *et al.*, 1996) and skin tumors (Ananthaswamy and Pierceall, 1990). UV radiation, particularly UVB, has strong cytotoxic and mutagenic effects (Ichihashi *et al.*, 2003), as the bases in DNA directly absorb incident photons. This absorption can result in DNA damage, particularly the formation of cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts ((6–4) PDs), which induce DNA mutation in skin cells, leading to the development of skin tumors. UV radiation is also a potent generator of oxidative stress in the skin. Exposure of mammalian skin to UV increases the cellular levels of reactive oxygen species (ROS), which damages lipids, proteins, and nucleic acids in both epidermal and dermal cells and probably contribute to the sunburn reaction as well as photocarcinogenesis and photo-aging (Scharffetter-Kochanek *et al.*, 1997). Photoexcited sensitizers are likely to generate ROS, including superoxide and singlet oxygen. In addition, photo-oxidation reactions of DNA, including the formation of 8-oxo-7, 8-dihydroguanine (8-oxo-dG) and 8-hydroxy-2′-deoxyguanosine (8-OH-dG), occur on UV irradiation of cellular DNA (Wamer and Wei, 1997).

Cells can be protected against the adverse effects of UV irradiation by a number of enzymatic and nonenzymatic antioxidants. Antioxidants, such as polyphenols (Vayalil *et al.*,

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Abbreviations: CPD, cyclobutane pyrimidine dimer; Keap1, Kelch-like-ECH-associated protein 1; Nrf2, NF-E2-related factor 2; ROS, reactive oxygen species; SBC, sunburn cell; 8-OhdG, 8-hydroxy-2′-deoxyguanosine

Received 3 August 2007; revised 25 October 2007; accepted 19 November 2007; published online 17 January 2008

2003) and vitamin E (Packer and Valacchi, 2002), applied topically or in the diet, show protective effects against photooxidative damage of the skin. The endogenous antioxidant capacity of the skin is a major determinant in its response to oxidative stress-mediated damage. Low intracellular levels of glutathione result in elevated sensitivity to UV irradiation (Tyrrell and Pidoux, 1988). Thus, antioxidants constitute an important group of pharmacological agents capable of preventing the occurrence and reducing the severity of UV irradiation-induced skin diseases and skin aging. As it is the outermost organ exposed directly to the pro-oxidative environment, including solar UV radiation, the skin is equipped with an elaborate system of antioxidant substances and enzymes, including a network of redox-active antioxidants.

Nrf2 (NF-E2-related factor 2) and Keap1 (Kelch-like-ECH-associated protein 1) are key proteins in the coordinated transcriptional induction of various antioxidant-metabolizing enzymes. Nrf2 is a member of the NF-E2 family of nuclear basic leucine zipper transcriptional activators, and Keap1 is a cytoplasmic protein homologous to the *Drosophila* actin-binding protein Kelch (Chui *et al.*, 1995; Itoh *et al.*, 1999b). Under normal physiological conditions, Nrf2 is largely bound to Keap1 and degraded rapidly in the cytoplasm (McMahon *et al.*, 2003). On disruption of the Nrf2–Keap1 complex by inducers, Nrf2 undergoes rapid translocation to the nucleus, where it activates its target genes in heterodimeric combinations with other transcription factors, such as small maf proteins (Itoh *et al.*, 1999a). Keap1 negatively regulates Nrf2 by both enhancing its rate of proteasomal degradation and altering its subcellular distribution (Itoh *et al.*, 2003; McMahon *et al.*, 2003).

After translocation into the nucleus, Nrf2 recognizes and binds to antioxidant response elements in the promoter regions of its target genes and induces phase II detoxification enzymes and antioxidant proteins, such as glutamate cysteine ligase, previously referred to as γ-glutamylcysteine synthetase (Wild *et al.*, 1999), cystine/glutamate exchange transporter (Sasaki *et al.*, 2002), glutathione *S*-transferase (Hayes *et al.*, 2000), nicotinamide adenine dinucleotide phosphate quinone oxidoreductase-1 (Venugopal and Jaiswal, 1996), heme oxygenase-1 (Alam *et al.*, 1999), and thioredoxin (Ishii *et al.*, 1999). Furthermore, Nrf2 was recently shown to confer protection against apoptosis induced by Fas signaling (Morito *et al.*, 2003) and mitochondrial toxins (Lee *et al.*, 2003b). We have demonstrated that Nrf2 is activated by UVA irradiation and plays a pivotal role in protection of cells from UVA-induced apoptosis (Hirota *et al.*, 2005). Therefore, we focused on the Nrf2–Keap1 pathway as a putative major component of the protective machinery involved in protection of the skin against UV-induced oxidative damage.

In this study, we used *nrf2*$^{-/-}$ mice to examine the protective effects of Nrf2 against UV-induced acute sunburn reaction, epidermal cell apoptosis, and DNA mutations. Furthermore, we compared the skin carcinogenesis induced by chronic UVB exposure between *nrf2*$^{+/+}$ and *nrf2*$^{-/-}$ mice.

RESULTS

nrf2$^{-/-}$ mice developed stronger and longer lasting sunburn reaction after UVB irradiation

A single exposure to UVB irradiation at a dose of 200 mJ cm^{-2} resulted in more obvious erythema reactions in the *nrf2*$^{-/-}$ mice than in wild-type controls at 24 hours (Figure 1a, day 1) or 48 hours (Figure 1a, day 2) after irradiation. Dermatoscopy of the lesions showed congestion and teleangiectasis of the ear skin, both of which were more prominent in the *nrf2*$^{-/-}$ mice than in the controls (Figure 1a, day 1). Yellowish crust formation was seen on the ear skin of the *nrf2*$^{-/-}$ mice by day 4 after irradiation, whereas the wild-type controls recovered almost normal appearance of the ears 4 days after irradiation with UVB (Figure 1a, day 4). The ears of *nrf2*$^{-/-}$ mice became deformed and necrotic crust formation was observed on dermatoscopy (Figure 1a, day 7). To quantify the severity of the inflammatory reaction in ear sunburn, the ear swelling responses in wild-type and *nrf2*$^{-/-}$ mice after irradiation with UVB at a dose of 200 mJ cm^{-2} were measured. As shown in Figure 1b, *nrf2*$^{-/-}$ mice developed significant ear swelling, which was still increasing on day 5, whereas wild-type mice showed only slight ear swelling at the same UVB dose. The ear swelling in *nrf2*$^{-/-}$ mice was significantly greater than that in wild-type controls throughout the study. Histologically, the ear skin samples from *nrf2*$^{-/-}$ mice showed significant dermal edema and necrosis in the epidermis 4 days after irradiation with UVB at a dose of 200 mJ cm^{-2}, whereas the skin samples from wild-type controls showed little change in the epidermis (Figure 1c). Moreover, *nrf2*$^{-/-}$ mice showed more inflammatory infiltrates of lymphoid cells and vasodilatation in the dermis (Figure 1c).

UVB irradiation induced more prominent sunburn cell and TUNEL-positive epidermal cell formation in *nrf2*$^{-/-}$ mice

Biopsy specimens were taken from the ears of *nrf2*$^{-/-}$ and wild-type mice at 36 hours after irradiation with UVB at a dose of 100 mJ cm^{-2}. The number of sunburn cells (SBCs) recognized within three independent visual fields with the same magnification (× 400) was counted in sections stained with hematoxylin and eosin. UVB irradiation significantly enhanced SBC formation in the epidermis of *nrf2*$^{-/-}$ mice (17.0 ± 3.9 per field) as compared with wild-type controls (5.0 ± 1.7 per field) (Figure 2a and b). Similarly, the number of TUNEL-positive cells in *nrf2*$^{-/-}$ mice was almost fivefold greater than that in wild-type controls (Figure 3a and b).

UVB irradiation increased 8-OHdG-positive epidermal cell formation in *nrf2*$^{-/-}$ mice

8-Hydroxy-2′-deoxyguanosine (8-OHdG), a sensitive marker of oxidative DNA damage (Yarborough *et al.*, 1996), can be generated via a variety of agents such as chemicals, X-irradiation, and exposure to UV and visible light in the presence of a photosensitizer. UVB exposure has been reported to increase 8-OHdG formation in epidermal cells (Hattori *et al.*, 1996). We examined 8-OHdG formation in epidermal cells of *nrf2*$^{-/-}$ and wild-type mice 4 hours after irradiation with UVB at a dose of 100 mJ cm^{-2}. The *nrf2*$^{-/-}$

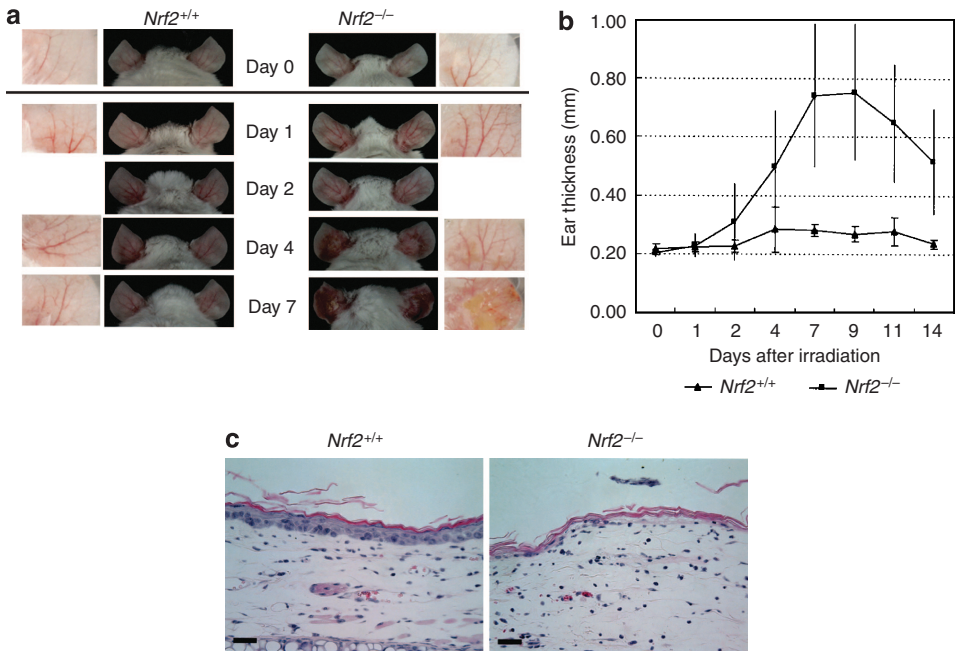

Figure 1. Enhanced photosensitivity in *nrf2*-deficient mice. (**a**) The macroscopic appearance of the ears in sham-irradiated wild-type and $nrf2^{-/-}$ mice is shown as control (day 0). The macroscopic and dermatoscopic appearance of the ears at 1 (day 1), 2 (day 2), 4 (day 4), or 7 days (day 7) after UVB irradiation at a dose of 200 mJ cm^{-2} were compared between wild-type (left panels) and $nrf2^{-/-}$ mice (right panels). (**b**) $nrf2^{-/-}$ mice developed stronger and longer lasting edema after UVB irradiation. The ears of the mice were exposed to UVB and ear thickness was measured immediately before and 1, 2, 4, 7, 9, 11, and 14 days after irradiation. Data are expressed as mean intensity (mm) of ear thickness (± SD) in eight mice per group. (**c**) UVB irradiation induced more prominent histological changes in $nrf2^{-/-}$ mice than in wild-type controls. Skin samples were taken 4 days after UVB irradiation. The $nrf2^{-/-}$ mice showed epidermal necrosis, dermal edema, and inflammatory changes, whereas wild-type controls showed few changes (hematoxylin and eosin staining, × 400). Bar = 50 μm.

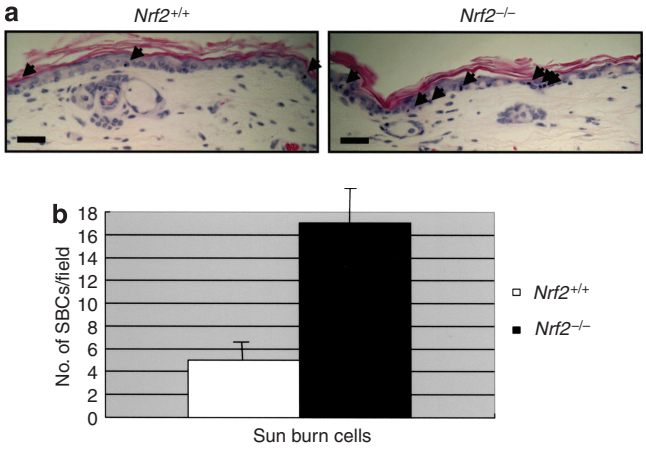

Figure 2. Enhanced SBC formation after UVB irradiation in *nrf2*-deficient mice. (**a**) Biopsy specimens were taken from the ears of $nrf2^{-/-}$ and wild-type mice 36 hours after UVB irradiation at a dose of 100 mJ cm^{-2}, and stained with hematoxylin and eosin. The SBCs in the epidermis are indicated by arrows. Bar = 50 μm. (**b**) The numbers of SBCs recognized within three independent visual fields with the same magnification (× 400) were counted. Data are expressed as mean number of SBCs ± SD per field.

mice showed a significant increase in formation of 8-OHdG-positive cells in the epidermis (Figures 4a and b), whereas there were no apparent differences in CPD or (6–4) PD formation between wild-type and $nrf2^{-/-}$ mice (Figure 4c). These results indicate that the Nrf2–Keap1 pathway suppresses oxidative DNA damage on UVB irradiation and has no effect on the formation of CPD or (6–4) PD, which are produced directly by UVB irradiation.

Skin carcinogenesis did not differ significantly between chronic UVB-irradiated wild-type and $nrf2^{-/-}$ mice

To evaluate the effects of Nrf2 on UVB-induced skin carcinogenesis, the shaved backs of wild-type and $nrf2^{-/-}$ mice were irradiated with UVB at a dose of 300 mJ cm^{-2} three times a week for 36 weeks, and the number of tumors developing each week was counted. There were no significant differences between wild-type and $nrf2^{-/-}$ mice in either the mean number of tumors per animal (Figure 5b) or in the onset of tumors (Figure 5c). Histological examination with hematoxylin and eosin staining indicated that all malignant tumors that developed in these mice were spindle-shaped tumors (Figure 5a, middle panels), and immunohistochemical staining showed that the spindle cells were positive for cytokeratin (Figure 5a, lower panels) but negative for vimentin (data not shown). Thus, we diagnosed these tumors as spindle-cell carcinomas.

DISCUSSION

UV radiation is the major environmental cause of skin damage. UVB radiation is the main cause of sunburn and is probably the most carcinogenic component of sunlight (de Gruijl *et al.*, 1993). As exposure of skin to UVB results in an

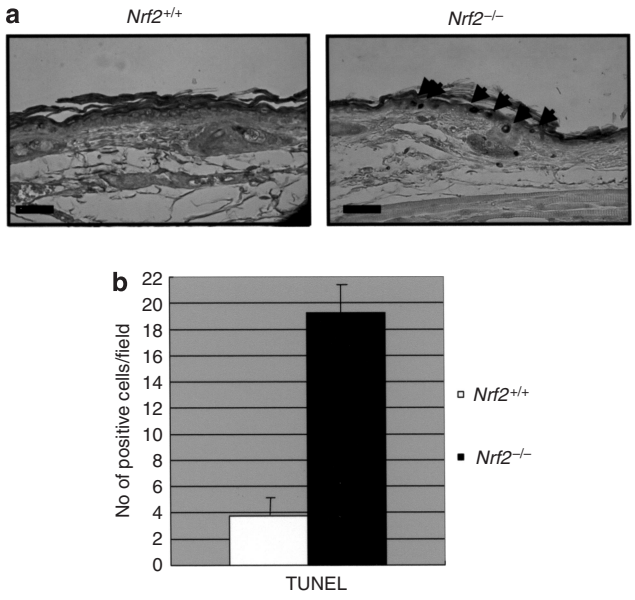

Figure 3. More prominent apoptotic epidermal cell formation in *nrf2*$^{-/-}$ mice as compared with wild-type controls. (**a**) Biopsy specimens were taken from the ears of *nrf2*$^{-/-}$ and wild-type mice at 36 hours after UVB irradiation at a dose of 100 mJ cm^{-2}, and examined for apoptotic nuclei by TUNEL analysis. The TUNEL-positive apoptotic cells in the epidermis are indicated by arrows. Bar = 100 μm. (**b**) *nrf2*$^{-/-}$ mice showed significant increase in the number of apoptotic cells as compared with wild-type controls.

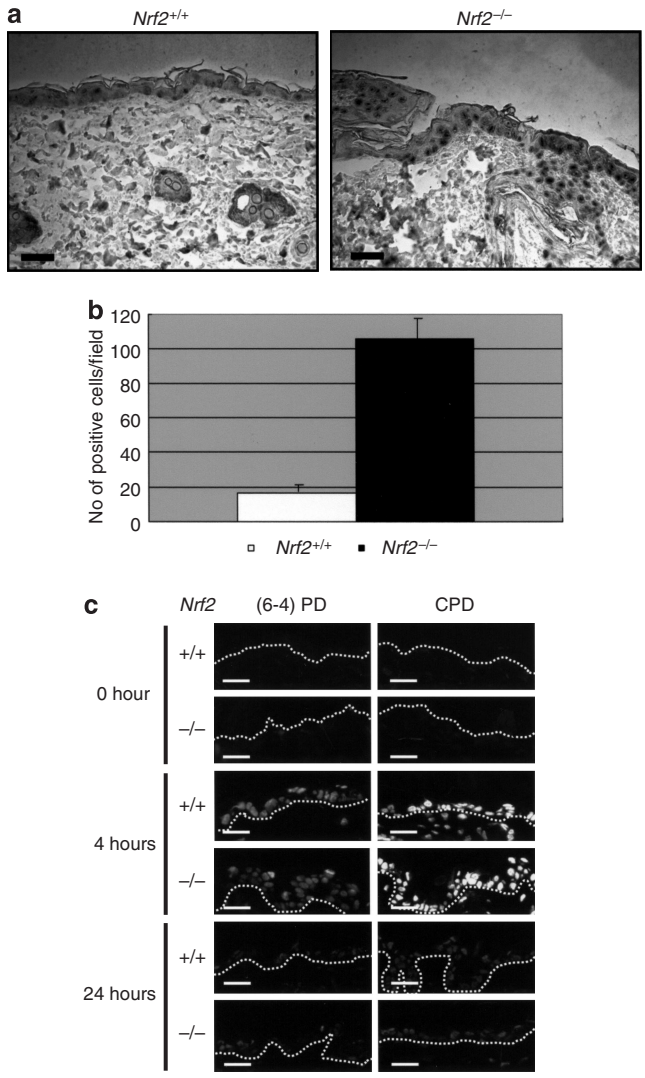

Figure 4. Enhanced 8-OHdG-positive epidermal cell formation in *nrf2*$^{-/-}$ mice, whereas no apparent differences in CPD or (6–4) PD formation between wild-type and nrf2-deficient mice. (**a**) 8-OHdG immunostaining of biopsy specimens from the ears of *nrf2*$^{-/-}$ and wild-type mice at 4 hours after UVB irradiation at a dose of 100 mJ cm^{-2}. The nuclei of 8-OHdG-positive cells in the epidermis were stained violet. (**b**) *nrf2*$^{-/-}$ mice showed a statistically significant increase in the number of 8-OHdG-positive cells as compared with wild-type controls. Bar = 50 μm. (**c**) CPD (upper panels) and (6–4) PD (lower panels) immunofluorescence staining of biopsy specimen from the ears of *nrf2*$^{-/-}$ and wild-type mice at 0 hour (control), 4, and 24 hours after UVB irradiation at a dose of 100 mJ cm^{-2}. The dotted line represents the basement membrane zone. Bar = 50 μm.

increase in ROS generation, which contributes to several pathological conditions including acute sunburn reaction and chronic photocarcinogenesis, scavenging of ROS generated by exposure to UVB may protect the skin from these acute and chronic adverse effects. The Nrf2–Keap1 pathway is a system that plays a key role in the coordinated transcriptional induction of various antioxidant-metabolizing enzymes, which neutralize the ROS. Therefore, we postulated that Nrf2 would have attenuating effects on acute and chronic cutaneous reactions to UV irradiation, and we reported recently that the Nrf2–Keap1 pathway protected dermal fibroblasts from UVA irradiation-induced apoptosis *In Vitro* (Hirota *et al.*, 2005). In this study, we investigated the role of the Nrf2–Keap1 pathway in the acute and chronic cutaneous responses to UVB *In Vivo* using *nrf2*$^{-/-}$ mice.

First, we found that a single dose of UVB irradiation induced stronger and longer lasting sunburn reaction in *nrf2*$^{-/-}$ mice than in wild-type controls. This result indicated that Nrf2 is involved in attenuation of acute inflammatory reactions in the skin. Although it is still unclear how oxidative stress provoked by UV irradiation exacerbates inflammatory reactions, antioxidants have been shown to suppress UV-induced activation of mitogen-activated protein kinase, leading to AP-1 and NF-κB activation (Ichihashi *et al.*, 2003; Li and Nel, 2006). The Nrf2–Keap1 pathway may contribute to attenuation of acute inflammatory reactions by increasing cellular antioxidant levels and resulting in inhibition of AP-1 and NF-κB pathways, transcription factors involved in the activation of inflammatory processes. Histological changes, including epidermal SBC formation and TUNEL-positive apoptotic cell formation after UVB radiation, were more prominent in *nrf2*$^{-/-}$ mice than in wild-type controls. Recent studies indicated that *nrf2*-null mutant cells are prone to apoptosis induced by H_2O_2 stimulation (Lee *et al.*, 2003a). Similarly, neurons of *nrf2*$^{-/-}$ mice are sensitive to mitochondrial toxin-induced apoptosis (Calkins *et al.*, 2005). Overexpression of Nrf2 was shown to protect the cells from Fas-induced apoptosis (Kotlo *et al.*,

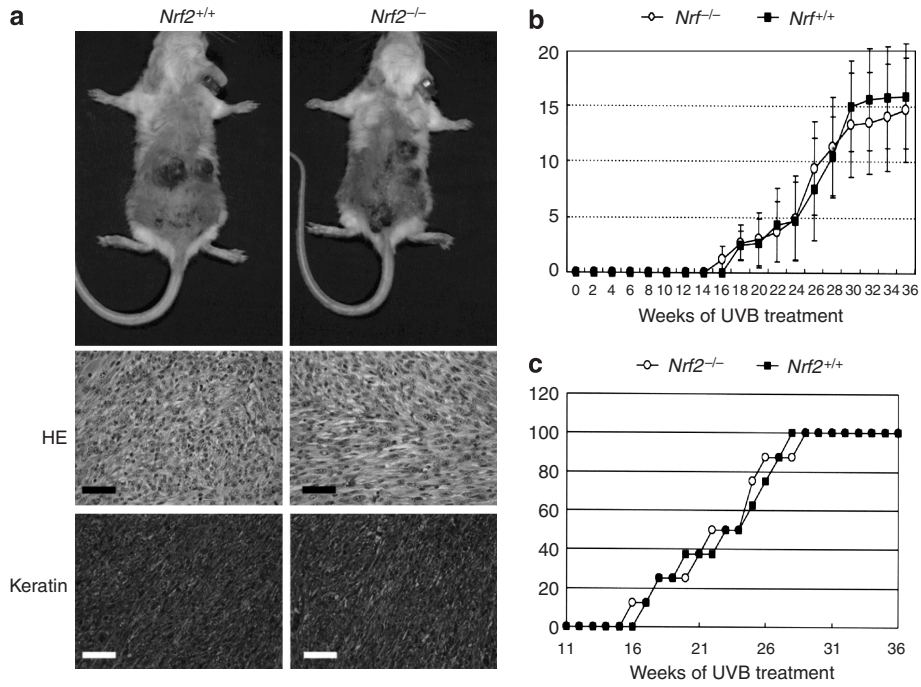

Figure 5. No significant difference in chronic UVB-induced skin carcinogenesis between wild-type and *nrf2*-deficient mice. (**a**) After 36 weeks of UVB irradiation (300 mJ cm^{-2} × three times per week), wild-type (upper left) and *nrf2*$^{-/-}$ (upper right) mice had multiple skin tumors in which histologically spindle-shaped cell proliferation was seen with hematoxylin and eosin staining (wild-type: middle left; *nrf2*$^{-/-}$: middle right). The spindle-shaped cells showed positive staining for pancytokeratin (wild-type: lower left; *nrf2*$^{-/-}$: lower right), but were negative for vimentin (data not shown). Bar = 100 μm. (**b**) Time courses of tumor induction per mouse after UVB irradiation. There were no significant differences in the mean number of tumors per animal between wild-type and *nrf2*$^{-/-}$ mice. (**c**) Time course of changes in the percentage of tumor-bearing mice in each genotype. There were no significant differences in the onset of tumors between wild-type and *nrf2*$^{-/-}$ mice.

2003). It has been reported that Fas-induced apoptosis of Jurkat T cells is accompanied by rapid and specific export of glutathione from apoptotic cells (van den Dobbelsteen *et al.*, 1996), and conversely Nrf2 regulates the sensitivity of cells to Fas-inducible apoptosis by affecting intracellular glutathione levels (Morito *et al.*, 2003). The results of oligonucleotide microarray analyses have shown that *nrf2*$^{-/-}$ mice have lower basal levels of expression of Nrf2 target genes than wild-type controls (Thimmulappa *et al.*, 2002). Catalase, superoxide dismutase, and glutathione peroxidase, all of which eliminate ROS, are also dependent on Nrf2 (Lee *et al.*, 2003b). Decreases in the levels of expression of antioxidant enzymes and proteins may result in the increased susceptibility to UVB irradiation seen in *nrf2*$^{-/-}$ mice.

Our immunohistochemical analysis revealed a significant increase in the formation of 8-OHdG-positive cells in the epidermis of *nrf2*$^{-/-}$ mice as compared with wild-type controls at 4 hours after irradiation with UVB. As 8-OHdG is widely recognized as a useful marker for estimation of DNA damage produced by ROS (Nakae *et al.*, 1995), and 8-OHdG has been reported to play an important role in UV-related skin carcinogenesis (Nishigori *et al.*, 2004; Kunisada *et al.*, 2005), we anticipated that *nrf2*$^{-/-}$ mice would show increased susceptibility to UV-induced skin carcinogenesis. However, there were no significant differences in skin carcinogenesis between wild-type and *nrf2*$^{-/-}$ mice associated with chronic UVB irradiation of the skin. Activation of the Nrf2–Keap1 pathway has been shown to protect cells from apoptosis, and Nrf2 deficiency increases the susceptibility of cells to apoptosis caused by ROS (Leung *et al.*, 2003; Hirota *et al.*, 2005). Furthermore, recent studies demonstrated that elevated Nrf2 activity may also help cancer cells survive ROS-induced apoptosis and result in the evolution of cancer (Hayes and McMahon, 2006; Padmanabhan *et al.*, 2006). That is, more prominent accumulation of 8-OHdG in the DNA of *nrf2*$^{-/-}$ mice accelerates carcinogenesis, whereas the increased susceptibility to apoptosis may prevent carcinogenesis. Thus, the lack of differences in UV-induced skin carcinogenesis between wild-type and *nrf2*$^{-/-}$ mice may be due to the above two antagonistic effects. A recent study indicated that p53, which is a key molecule in UV-induced apoptosis, suppresses the Nrf2-dependent transcription of antioxidant response genes (Faraonio *et al.*, 2006). Nrf2 has also been reported to be an important protective factor for chemical carcinogenesis, but our results indicated no alteration of UVB-induced skin carcinogenesis in *nrf2* gene deficiency. This discrepancy may have been due to differences in the carcinogens, that is, UV and chemical carcinogens. UV seems to induce apoptosis of exposed cells more strongly than chemical carcinogens. As Nrf2 deficiency has been shown to accelerate apoptosis of cells, the cancer-promoting

effect of Nrf2 deficiency may be antagonized by its apoptosis-promoting effect much more effectively in photocarcinogenesis than in chemocarcinogenesis. The findings of this study indicate that there is a complicated and subtle balance between factors that promote and prevent photocarcinogenesis.

MATERIALS AND METHODS

Mice

Wild-type BALB/c mice were purchased from Charles River Breeding Laboratories Japan (Yokohama, Japan). *Nrf2*-null mutant mice in the BALB/c background were established by specific deletion of the *nrf2* gene segment (Itoh *et al.*, 1997). All mice used in this study were maintained in our animal facilities under specific pathogen-free conditions. All experiments were approved by the Institutional Review Board and performed in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tsukuba, Japan.

UVB irradiation regime

Ten-week-old female wild-type or *nrf2*-null mutant mice were exposed to a single dose of 100 or 200 mJ cm^{-2} of UVB irradiation using FL20SE lamps (Toshiba, Tokyo, Japan), with emission at 280–320 nm and a peak at 312.5 nm. The dose of UVB irradiation was measured by a radiometer (UVR-305/365; Toshiba). In UV carcinogenesis experiments, the shaved backs of wild-type and *nrf2*-null mutant mice were irradiated with UVB at a dose of 300 mJ cm^{-2} three times a week. Each group consisted of eight mice.

Dermatoscopy of the sunburn lesions

Dermatoscopic images from each lesion were obtained using a lens (Dermatoscope Delta 20; Heine Optotechnik, Herrsching, Germany) mounted on a digital camera (Nikon, Tokyo, Japan).

Ear swelling response to UVB irradiation

The dorsal side of the ear of wild-type or *nrf2*-null mutant mice was irradiated with UVB at a dose of 150 mJ cm^{-2}. Ear thickness was measured immediately before and 1, 2, 4, 7, 9, 11, and 14 days after treatment using a dial gauge (Shinwa Rules, Tokyo, Japan). Data are expressed as the mean thickness ± SD of 10 mice.

Histological examination and SBC counting

The dorsal sides of the ears or the shaved back of mice were exposed to 100 or 200 mJ cm^{-2} of UVB. Biopsy specimens were taken 36 hours after irradiation and stained with hematoxylin and eosin. The number of SBCs in the interfollicular epidermis was counted in three different specimens, and the average number of SBCs per ×200 microscopic field was analyzed statistically.

TUNEL assay

The dorsal sides of the ears or shaved back area of mice were exposed to UVB at a dose of 100 mJ cm^{-2}, and biopsy specimens were taken 36 hours after irradiation. TUNEL assays were performed with a commercial kit in accordance with the manufacturer's protocol (Promega, Madison, WI).

Immunohistochemistry

For detection of 8-OHdG in mouse skin, specimens were collected 4 hours after UVB irradiation at a dose of 100 mJ cm^{-2}, fixed in 10% neutralized formalin, and embedded in paraffin. Sections were cut, deparaffinized, dehydrated, and washed in phosphate-buffered saline. Sections were microwaved in 10 mmol l^{-1} citrate buffer (pH 6) for 5 minutes. After blocking endogenous peroxidase activity, nonspecific binding sites were blocked by incubation of the sections with protein blocking serum (Dako, Kyoto, Japan). Sections were incubated for 1 hour at 40 °C with a primary mouse monoclonal antibody against 8-OHdG, N45.1 (Hattori *et al.*, 1996). After washing with phosphate-buffered saline, phosphate-buffered saline, with biotin-conjugated anti-mouse IgG (Dako) at room temperature, followed by incubation for 15 minutes with streptavidin-conjugated alkaline phosphatase (Vector Laboratories, Burlingame, CA), and finally mounted in mounting medium (Dako). Substrate for alkaline phosphatase was obtained from Vector Laboratories.

Immunofluorescence staining for CPD and 6–4 PD

For detection of CPD and (6–4) PD in mouse skin, skin specimens were collected 24 hours after UVB irradiation at a dose of 100 mJ cm^{-2}, embedded in Tissue-Tek II OCT compound (Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen. Sections 5-μm thick were cut on a cryostat and placed on uncoated slides. These sections were air-dried and DNA was denatured by treatment with 0.07 N NaOH in 70% ethanol for 4 minutes. For staining, slides were incubated with a mouse monoclonal antibody against CPD or (6–4) PD for 30 minutes, followed by washing with phosphate-buffered saline. Both antibodies were provided by Dr Tsukasa Matsunaga (Kanazawa University, Kanazawa, Japan). Slides were incubated with FITC-conjugated secondary antibody (sc-2012; Santa Cruz Biotechnology, Santa Cruz, CA), mounted, and then analyzed by fluorescence microscopy (Nikon).

Observation and measurement of cumulative tumor incidence

After chronic UVB exposure, we observed tumor formation until all mice developed skin tumors. The number of tumors with diameters larger than 2 mm was counted, but the tumors in mice that died during the experiment were not included. All mice were killed at the final observation, and all skin tumors were excised and examined histologically with hematoxylin and eosin staining.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by grant from Ministry of Education, Culture, Sports, Science and Technology of Japan (19591296 to YK, 18591232 to FO).

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