Nrf1 CNC-bZIP Protein Promotes Cell Survival and Nucleotide Excision Repair through Maintaining Glutathione Homeostasis

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Background: The regulation of UVB response is not fully understood.

Results: Nrf1 loss increases UVB-induced apoptosis and inhibits nucleotide excision repair in surviving cells by decreasing glutathione levels.

Conclusion: Nrf1 promotes cell survival and DNA repair following UVB irradiation by maintaining glutathione availability.

Significance: These findings identify a new functional role of Nrf1 in the response of keratinocytes to UVB.

Skin cancer is the most common cancer in the United States. Its major environmental risk factor is UVB radiation in sunlight. In response to UVB damage, epidermal keratinocytes activate a specific repair pathway, i.e. nucleotide excision repair, to remove UVB-induced DNA lesions. However, the regulation of UVB response is not fully understood. Here we show that the long isoform of the nuclear factor erythroid 2-related factor 1 (Nrf1, also called NFE2L1), a cytoprotective transcription factor critical for the expression of multiple antioxidant response element-dependent genes, plays an important role in the response of keratinocytes to UVB. Nrf1 loss sensitized keratinocytes to UVB-induced apoptosis by up-regulating the expression of the proapoptotic Bcl-2 family member Bik through reducing glutathione levels. Knocking down Bik reduced UVB-induced apoptosis in Nrf1-inhibited cells. In UVB-irradiated surviving cells, however, disruption of Nrf1 impaired nucleotide excision repair through suppressing the transcription of xeroderma pigmentosum C (XPC), a factor essential for initiating the global genome nucleotide excision repair by recognizing the DNA lesion and recruiting downstream factors. Nrf1 enhanced XPC expression by increasing glutathione availability but was independent of the transcription repressor of XPC. Adding XPC or glutathione restored the DNA repair capacity in Nrf1-inhibited cells. Finally, we demonstrate that Nrf1 levels are significantly reduced by UVB radiation in mouse skin and are lower in human skin tumors than in normal skin. These results indicate a novel role of Nrf1 in UVB-induced DNA damage repair and suggest Nrf1 as a tumor suppressor in the skin.

Proper maintenance of DNA integrity is vital for the viability and the health of organisms. The genome is under constant threats from endogenous metabolic byproducts and environmental factors that can alter its chemical structure and corrupt its encoded message. The resulting DNA lesions must be effectively repaired to prevent loss or incorrect transmission of genetic information as errors can cause developmental abnormalities and tumorigenesis, including skin cancer, the most common cancer in the United States.

As major building blocks of the skin, keratinocytes are constantly challenged by ultraviolet (UV) radiation in sunlight. In particular, UVB (280–315 nm) causes DNA damage and is the major environmental risk factor for skin cancer. Each year, more than one million new cases of skin cancer are diagnosed in the United States alone, accounting for 40% of all newly diagnosed cancer cases (1, 2). Efficient repair of UVB-induced DNA damage lesions, in particular cyclobutane pyrimidine dimers (CPDs), but not the (6-4) photoproduct (6-4PP), is essential to prevent skin tumorigenesis (3).

To remove UVB-induced CPD and 6-4PP, cells are equipped with a delicate DNA repair system, nucleotide excision repair. Defects in nucleotide excision repair by mutations cause the autosomal recessive xeroderma pigmentosum (XP) and Cockayne syndromes (4–7). In particular, XP patients are clinically characterized by cutaneous sensitivity to sunlight and a predisposition to skin cancer. Among the identified genes that correspond to seven XP complementation groups (XPA to XPG), XPC is a key factor in damage recognition in global genome nucleotide excision repair (8–10). Although the function of

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2 The abbreviations used are: CPD, cyclobutane pyrimidine dimer; Bik, Bcl-2-interacting killer; DDB1, DNA damage-binding protein 1; DDB2, DNA damage-binding protein 2; Nrf1, nuclear factor erythroid 2-related factor 1; Scc, squamous cell carcinoma; siRNA, small interfering RNA; XP, xeroderma pigmentosum; XPC, xeroderma pigmentosum group C; PARP, poly(ADP-ribose) polymerase-2; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; 6-4PP, (6-4) photoproduct.
XPC has been investigated in great detail, the regulation of XPC remains largely unknown.

Nuclear factor erythroid 2-related factor 1 (Nrf1, also called NFE2L1) belongs to the Cap’n’ collar basic leucine zipper (CNC-bZIP) family of proteins, which also includes p45 NF-E2, Nrf2, Nrf3, Bach1, and Bach2 (11–17). Nrf1 is widely expressed in mouse and human tissues (11, 18, 19) and regulates cytoprotective genes through antioxidant-response elements in their promoter regions (20–22). Genes transactivated by Nrf1 include those encoding the glutamate-cysteine ligase catalytic and modifier subunits, which together catalyze the rate-limiting step in glutathione (GSH) biosynthesis, as well as (NAD(P)H:quinone oxidoreductase) (NQO1), heme oxygenase 1, ferritin, and metallothionein (23–26). Transcriptional activation of antioxidant-response element-driven genes by Nrf1 prevents oxidative stress in vivo (23). Nrf1 inhibition increased apoptosis in response to arsenic (27).

Although some of the CNC-bZIP factors, particularly Nrf2, have been studied in detail (16, 17), relatively little is known about the biological functions of Nrf1. In particular, the involvement of Nrf1 in skin cancer and UVB response remains unknown. Here we used mouse skin, squamous cell carcinoma (SSC) from human skin, and the human HaCaT keratinocyte cell line to determine the role of Nrf1 in UVB response. We found that Nrf1 plays an important role in cell survival and DNA repair after UVB irradiation and likely acts as a tumor suppressor in skin carcinogenesis.

MATERIALS AND METHODS

Human Normal and Tumor Samples—All human specimens were studied after approval by the University of Chicago Institutional Review Board. Frozen tissues were obtained under consent (Department of Medicine, University of Chicago). Protein lysate was used to determine Nrf1 levels by Western blotting.

Cell Culture—Human HaCaT keratinocytes were obtained from Professor N. Fusenig. HaCaT cells were transduced with MISSION lentiviral particles for short hairpin RNAs targeting Nrf1 to knock down the expression of the long isoform NRF1 (NRF1-KD)3 SHVRS_NM_003204; Sigma) or scrambled nontarget negative control (Scr; SCH002V; Sigma) and then cultured in medium containing 1.0 μg/ml puromycin. The selected cells were cultured in medium without puromycin. Scr and NRF1-KD cells were maintained in a monolayer culture in 95% air, 5% CO₂ at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen).

Animal Treatment—All animal procedures have been approved by the University of Chicago Institutional Animal Care and Use Committee. B6 mice were shaved and 2 days later exposed to UVB (400, 750, and 1000 ml/cm²) dorsally or sham-irradiated, three times a week for 1 week. Mouse skin was snap-frozen at 72 h after the final irradiation for immunoblotting analysis. Mice were housed no more than five animals per cage.

UVB Radiation—For UVB treatment, a Stratagene 2400 equipped with 312-nm UVB bulbs (Stratagene, La Jolla, CA) (UVC 0%, UVB 51%, and UVA 49%) was used. The UVB exposure was performed in PBS after washing the cells with PBS twice to avoid the photosensitization effect of components in culture medium on the cells. Our UVB radiation was monitored every other week to measure the exposure output and dose.

siRNA and XPC Transfection—Cells were transfected with negative control siRNA (siNC) or siRNA (ON-TARGETplus SMARTpool, Dharmacon) targeting Bik (siBik) (5 μg), using an Amaxa Nucleofector according to the manufacturer’s instructions for HaCaT cells. For overexpression of XPC, cells were transfected with a vector expressing human XPC (OriGene, 1 μg) using an Amaxa Nucleofector according to the manufacturer’s instructions for HaCaT cells. Empty vector was used as a transfection control. Briefly, HaCaT cells were resuspended, transfected with siRNAs or plasmids using the Amaxa Nucleofector II device, and then treated with or without UVB at 72 h after transfection.

Western Blotting—Protein concentrations were determined using the BCA assay (Pierce). Equal amounts of protein were subjected to electrophoresis. Western blotting was performed in triplicate using antibodies including Nrf1, DNA damage-binding protein 1 (DDB1) and 2 (DDB2), XPC, HR23B, PARP, β-actin (equal loading control), and GAPDH (Santa Cruz Biotechnology) and Bcl-2 family proteins (Cell Signaling Technology and Epitomics) according to the recommendation of the manufacturer. The blots shown were representative of three replicates. β-Actin blots were done by stripping each blot. In Figs. 2A and 4A, the β-actin shown is a representative of each stripping. Approximate molecular mass is marked in each blot.

MTS Assay, Sub-G₁, Flow Cytometric Analysis, Promoter Reporter Assay, and Real-time PCR Analysis—Twenty-four hours after UVB irradiation, cell viability was measured using the MTS assay (CellTiter 96 AQcious One Solution proliferation assay, Promega) and monitored at 492 nm using a TECAN Infinite M200 plate reader according to the manufacturer’s instructions. Sub-G₁, flow cytometric analysis was performed to determine apoptosis. Briefly, cells were fixed and stained with propidium iodide. The percentage of cells at the sub-G₁ phase was quantified by flow cytometry. For the promoter reporter assay, the plasmid mixtures, containing 1 μg of XPC promoter luciferase construct (XPC-Luc in pGL3 vector, kindly provided by Dr. Pradip Raychaudhuri at the University of Illinois at Chicago) and 0.025 μg of pRL-TK (Promega, used as a transfection efficiency control), were transfected with FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. The luciferase activity was measured with a TD-20/20 luminometer (Turner BioSystems) and normalized with the values of pRL-TK luciferase activity using a Dual-Luciferase Reporter (DLR) assay kit (Promega). Real-time PCR analysis was performed using an ABI7300 (Applied Biosystems, Foster City, CA) with 96-well plates with the SYBR® Green PCR master mix (Applied Biosystems). The primers for XPC (HP207915) and Bik (HP205127) were obtained from OriGene.

3 Throughout this article, the designation NRF1-KD indicates Nrf1 knockdown HaCaT cells, and the designation Scr indicates HaCaT cells transduced with scrambled negative control short hairpin RNA.
Determination of Two Major Forms of UVB-induced DNA Damage in Genomic DNA by Slot Blot Assay—Slot blot assays of CPD and 6-4PP were performed as described previously (28). Briefly, mouse skin or cells were collected at different time points after UVB, and DNA was isolated using a QIAamp DNA mini kit (Qiagen, Valencia, CA). The DNA concentration was calculated from the absorbance at 260 nm using NanoDrop 1000 (Thermofisher). The CPD and 6-4PP in DNA were quantified by slot blot (Bio-Rad) with monoclonal antibodies (TDM-2 for CPD and 64 M-2 for 6-4PP, COSMO BIO Co., Koto-Ku, Tokyo, Japan) (28). The chemiluminescence was detected with a Carestream imaging station (Carestream). For examining repair kinetics, the percentage of repair was calculated by comparing the optical density at the indicated time with that of the corresponding absorbance at time 0, when there was no opportunity for repair and 100% of CPDs (or 6-4PPs) were present after UVB.

Oxidative Stress Assay—Cells were treated with or without UVB radiation and then incubated with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (2′,7′-H2DCFDA, Invitrogen) for 30 min at 37 °C. Cells were washed with PBS three times, and oxidative stress was analyzed by flow cytometry.

Statistical Analyses—Statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, CA). Data were expressed as the mean of three independent experiments and analyzed by Student’s t test. A p value of less than 0.05 was considered statistically significant (supplemental Materials and Methods).

RESULTS

Nrf1 Inhibition Sensitizes Cells to UVB-induced Apoptosis—To determine the role of Nrf1 in apoptosis, we first assessed cell viability in parental and Nrf1-inhibited HaCaT cells after sham or after UVB irradiation. UVB irradiation decreased cell viability in scrambled control (Scr) cells at 30 ml/cm² but not 20 ml/cm² (Fig. 1A). Nrf1 knockdown significantly increased cell death in cells exposed to UVB at both doses (Fig. 1A). Flow cytometric analysis indicated that Nrf1 knockdown significantly increased UVB-induced apoptosis in both serum-starved and nonstarved HaCaT cells (Fig. 1, B–E). Immunoblot analysis showed that Nrf1 knockdown increased PARP cleavage in UVB-irradiated cells (Fig. 1F), further supporting the conclusion that Nrf1 inhibition sensitized cells to UVB-induced apoptosis. These data indicate that Nrf1 is essential for cell survival upon UVB irradiation.

Antiapoptotic Role of Nrf1 Is Mediated by Suppressing Bik Expression—To determine the mechanism of the ant apoptotic function of Nrf1, we analyzed the effect of Nrf1 inhibition on the expression of the key apoptosis-regulating Bcl-2 family members. Nrf1 inhibition had no effect on the antiapoptotic proteins Mcl-1, Bcl-XL, or Bcl-2 or the proapoptotic proteins Bim, Bax, Bad, or Bid (Fig. 2A). However, Nrf1 knockdown increased the expression of the proapoptotic protein Bik (Fig. 2A). Real-time PCR analysis showed that Nrf1 knockdown increased Bik mRNA levels in HaCaT cells (supplemental Fig. S1A), further supporting the conclusion that Nrf1 inhibition sensitized cells to UVB-induced apoptosis. These data indicate that Nrf1 is essential for cell survival upon UVB irradiation.
Nrf1 in Survival and Nucleotide Excision Repair

Nrf1 is Vital for UVB-induced DNA Damage Repair through Regulating XPC—To further determine the role of Nrf1 in the UVB response, we investigated the function of Nrf1 in DNA repair in surviving cells as these cells are the potential sources for cancer formation. As compared with Scr cells, repair of UVB-induced CPDs was significantly reduced in Nrf1 knockdown cells (Fig. 3, A and B; \( p < 0.05 \), Student’s t test). In contrast, it had no effect on the repair of 6-4PPs (Fig. 3, C and D).

To determine the mechanism by which Nrf1 regulates DNA repair, we analyzed the difference in levels of proteins specifically involved in UVB-induced DNA repair, including DDB1, DDB2, HR23B, and XPC (8–10). Nrf1 inhibition had no effect on DDB1, DDB2, or HR23B, whereas it decreased XPC levels (Fig. 4A). Real-time PCR analysis and the promoter reporter assay indicated that Nrf1 knockdown suppressed XPC mRNA levels (Fig. 4B). These data demonstrated that Nrf1 suppresses Bik expression through maintaining glutathione levels.

Nrf1 in Survival and Nucleotide Excision Repair

MAY 25, 2012—VOLUME 287—NUMBER 22
JOURNAL OF BIOLOGICAL CHEMISTRY

FIGURE 2. Nrf1 promotes cell survival through suppressing Bik expression. A, immunoblot analysis of Nrf1, Bik, Bim, Bax, Bad, Bid, Mcl-1, Bcl-XL, Bcl-2, and \( \beta \)-actin in Scr and Nrf1-KD HaCat cells. B, real-time PCR analysis of the Bik mRNA levels. C, immunoblot analysis of Bik, PARP, and GAPDH in Scr and Nrf1-KD HaCat cells transfected with siNC or siBik at 6 h after UVB irradiation (20 mJ/cm\(^2\)). D, sub-G\(_1\) analysis of Scr HaCat cells and Nrf1-KD HaCat cells transfected with siNC or siBik at 24 h after UVB (20 mJ/cm\(^2\)) or after sham. E, flow cytometric analysis of oxidative stress in HaCat cells treated with \( \text{H}_2\text{O}_2 \) (0, 10, and 50 \( \mu \text{M} \)). F, immunoblot analysis of Bik and GAPDH in HaCat cells treated with \( \text{H}_2\text{O}_2 \) (0, 10, and 50 \( \mu \text{M} \)). G, immunoblot analysis of Bik and GAPDH in Scr and Nrf1-KD HaCat cells and Nrf1-KD HaCat cells treated with GSH-monoethyl ester (GSH, 1 and 2 mM). Error bars in panels B and D indicate S.E.

FIGURE 3. Nrf1 is vital for UVB-induced DNA damage repair. A and C, slot blot analysis of the levels of CPD (A) and 6-4PP (C) in Scr and Nrf1-KD HaCat cells at 0, 6, and 24 h after UVB (20 mJ/cm\(^2\)). B and D, quantification of percentage of CPD repair (B) from A and 6-4PP repair (D) from C. \( * \), \( p < 0.05 \), significant differences between Scr and Nrf1-KD groups. Error bars in panels B and D indicate S.E.
levels and its transcription (Fig. 4, B and C). However, it seems that the transcription repressor complex E2F4/p130 had no role in the regulation of XPC by Nrf1 as Nrf1 inhibition decreased nuclear localization of both E2F4 and p130 (supplemental Fig. S3), and mutation of the E2F binding site failed to restore XPC transcription in Nrf1-inhibited cells (Fig. 4D). To determine whether XPC plays an important role, we overexpressed XPC in Nrf1-inhibited cells (Fig. 4E). An increase in XPC levels significantly increased CPD repair (Fig. 4, F and G; p < 0.05, Student’s t test), whereas it did not affect 6-4PP repair (Fig. 4, H and I). Our results demonstrate that Nrf1 promotes UVB-induced DNA damage repair through regulating XPC transcription.

Nrf1 Regulates XPC Expression through Maintaining GSH Levels—To determine the mechanism by which Nrf1 regulates XPC, we investigated the role of redox status and GSH availability. Increasing GSH levels in Nrf1-inhibited cells increased XPC protein levels (Fig. 5A). Furthermore, it also significantly increased CPD repair (Fig. 5, B and C; p < 0.05, Student’s t test), but had no effect on 6-4PP repair (Fig. 5, D and E). Taken together, these data indicate that a proper GSH level is essential for Nrf1-promoted XPC expression.

Down-regulation of Nrf1 Levels in Skin Cancer and by UVB Radiation—To determine the involvement of Nrf1 in skin cancer, we analyzed the difference in Nrf1 protein levels between normal human skin and human skin SCC and between sham and UVB-irradiated mouse skin. Nrf1 was expressed in two of the three normal human skin samples, whereas its expression was lost in all three SCCs (Fig. 6A), suggesting that Nrf1 acts as a tumor suppressor. In mouse skin, repeated UVB radiation down-regulated Nrf1 protein levels (Fig. 6B), implying that Nrf1 is a molecular target of UVB in vivo during skin tumorigenesis.

DISCUSSION

In this study, we have identified the transcription factor Nrf1 as a critical player in UVB response, including apoptosis and DNA repair, in epidermal keratinocytes. As compared with normal skin, Nrf1 was down-regulated in human SCC. UVB radiation reduced Nrf1 levels in mouse skin. Nrf1 inhibition sensitized keratinocytes to UVB-induced apoptosis through oxidative stress-dependent Bik up-regulation. Furthermore, Nrf1 is required for UVB-induced DNA damage repair through GSH-dependent regulation of XPC expression. Our findings indicate that Nrf1 is likely a tumor suppressor in skin cancer development.

In keratinocytes, Nrf1 is a vital factor to promote cell survival. In both untreated and UVB-irradiated cells, Nrf1 knockdown particularly induced the expression of the proapoptotic protein Bik but had no effect on the other members of the Bcl-2 family members. Containing a conserved BH3 domain, Bik binds and regulates the antiapoptotic Bcl-2 proteins Mcl-1 and Bcl-XL to induce apoptosis (29, 30). Bik expression sensitized cancer cells to drug-induced apoptosis, whereas blocking Bik expression reduced apoptosis (31, 32). Inhibiting Bik by siRNA knockdown reduced apoptosis of Nrf1 down-regulated cells, indicating that Nrf1 promotes cell survival in UVB-exposed cells through suppressing Bik expression. As Nrf1 is an important transcription factor for the antioxidant cytoprotective response, Nrf1 inhibition resulted in oxidative stress through depleting GSH, which then induced Bik up-regulation. In addition to Bik up-regulation, Nrf1 may also regulate cell survival.
through proteasome homeostasis as Nrf1 has been shown to regulate proteasome subunit genes (33). The potential contribution of this pathway may explain the significant but incomplete reversal of apoptosis by Bik inhibition.

In addition, we found that Nrf1 is an essential component of the DNA repair network that responds to UVB-induced DNA damage. Nrf1 knockdown compromised DNA repair of CPD but not 6-4PP, suggesting that Nrf1 acts as a tumor suppressor as failure to remove CPD but not 6-4PP is the principal cause of skin cancer (3). At the molecular level, we found that Nrf1 inhibition down-regulated the expression of XPC at the transcriptional level, but had no effect on the other DNA repair proteins including DDB1 and DDB2. Overexpression of XPC restored the DNA repair capacity of Nrf1-inhibited cells, indicating that Nrf1 promotes UVB-induced DNA repair through regulating XPC transcription. Although the E2F4/p130 complex plays an essential role in XPC suppression (34–37), it was not involved in Nrf1-mediated XPC transcription. In contrast, Nrf1-mediated GSH homeostasis seems to be required for XPC expression because adding GSH into Nrf1-inhibited cells restored XPC expression and DNA repair capacity. The relationship between the levels of GSH and DNA synthesis and repair has been noted in previous studies. Glutaredoxin and GSH play important roles in the function of ribonucleotide reductase, an important enzyme that catalyzes the rate-limiting step in deoxyribonucleotide synthesis essential for DNA replication and repair (38). Higher GSH levels may also contribute to increased DNA repair. Furthermore, GSH was found to be recruited into the nucleus in the early phases of cell proliferation (39). Depletion of nuclear GSH impairs cell proliferation of 3T3 fibroblasts (40). Our study further links antioxidant homeostasis with DNA repair ability, in which Nrf1 plays an important role.

Although the antiapoptotic role of Nrf1 implies its role as an oncogene, the role of Nrf1 in UVB-induced DNA repair in surviving cells supports its function as a tumor suppressor in skin carcinogenesis. Furthermore, we found that the Nrf1 level was reduced in human SCCs as compared with normal human skin. In addition, UVB radiation down-regulated Nrf1 levels in mouse skin. These results indicated that although loss of Nrf1 eliminated more cells by apoptosis, the cells that survived did not have the optimal DNA repair ability and thus may still lead to accumulation of genetic mutations. Such a phenotype has been seen in the XP syndrome; XP patients are highly sensitive to sunburn (UV-induced apoptosis) and are susceptible to skin cancer (4–6). Inability to repair DNA damage can also result in increased apoptosis. The role of Nrf1 as a tumor suppressor was further supported by the recent findings that liver-specific Nrf1 inactivation caused liver cancer (41). The dual role of Nrf1 in cell survival and DNA repair suggests that Nrf1 is a vital factor in both antiaging and anticancer processes.

In summary, our findings demonstrate that in response to UVB damage, Nrf1 promotes cell survival through suppressing Bik expression and DNA repair through enhancing XPC transcription. GSH availability is essential for Nrf1-mediated Bik down-regulation and XPC expression. Understanding these modes of regulation by Nrf1 will contribute to a better understanding of skin tumorigenesis in humans and may lead to a better strategy for skin cancer prevention. Furthermore, our results may provide previously unrecognized insights into the precise function of Nrf1 in other types of cancer as impairing nucleotide excision repair capacity plays a key role in increasing cancer susceptibility not only in the skin (4, 5, 7), but also in other organs, including the lung (42–45), bladder (46), and liver (41).

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*Nrf1 in Survival and Nucleotide Excision Repair*
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