Letters

Differential Responses to UVB Irradiation in Human Keratinocytes and Epidermoid Carcinoma Cells*

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Abstract

Objective To examine UVB-induced responses in normal human keratinocytes (HaCaT) and epidermoid carcinoma cells (A431) at the cellular and molecular level, and investigated the protective effect of salidroside.

Methods Cells irradiated by UVB at various dosage and their viability was assessed by MTT assays, cell cycle was analysed by flow cytometry. The expression of NF-κB, BCL-2, and CDK6 after 50 J/m^2 UVB irradiation were detected by RT-PCR and western blotting.

Results Our results confirmed greater tolerance of A341 cells to UVB-induced damage such as cell viability and cell cycle arrest, which was accompanied by differential expression changes in NF-κB, BCL-2, and CDK6. UVB exposure resulted in HaCaT cells undergoing G_1-S phase arrest. When treated with salidroside, HaCaT survival was significantly enhanced following exposure to UVB, suggesting great therapeutic potential for this compound.

Conclusion Taken together, our study suggests that A431 respond differently to UVB than normal HaCaT cells, and supports a role for NF-κB, CDK6, and BCL-2 in UVB-induced cell G_1-S phase arrest. Furthermore, salidroside can effectively protect HaCaT from UVB irradiation.

Key words: UVB; G1-S phase arrest; HaCaT; A431; NF-κB; BCL-2; CDK6; Salidroside

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INTRODUCTION

Ultraviolet (UV) light in the form of solar radiation is known to be responsible for serious harm to human health, including skin cancer[1-2]. Of all the skin cancer types, the majority originate from epidermal keratinocytes[3]. So far, the questions of how cell transformation occurs, and what fundamental properties differentiate cancer cells from normal cells in response to UVB radiation, remain unanswered. It has been shown that following UVB irradiation, human keratinocytes have a higher clonal cell survival rate than that of the fibroblasts

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located in the dermis of the skin[4]. This may be attributed to a more sophisticated network of defense mechanisms against UV-induced damage in the keratinocytes[5-7]. However, the cellular mechanisms leading to UV-induced carcinogenesis are not clear.

Associated with inflammation and cancer, the pleiotropic transcription factor nuclear factor-Kappa B (NF-κB, or p65, nuclear factor kappa-light-chain-enhancer of activated B cells, a protein complex that controls the transcription of DNA) has recently gained considerable attention in skin disease research. It is now widely accepted that NF-κB plays a crucial role in both the physiology and pathology of skin[8]. NF-κB signaling leads to activation of multiple target genes, which in turn induce a variety of cellular responses. Depending on the type of cells and the nature of external stimuli, these responses include inflammation, cell proliferation and differentiation, and apoptosis[9]. In the skin, studies have shown that UV radiation activates molecular pathways regulated by NF-κB, and this acts as a critical step in the development of sunburn reactions[10-11]. It has also been suggested that NF-κB mediates UV-induced cell death, and aberrant NF-κB activities have been shown to increase the sensitivity of HaCaT cells to UVB-induced apoptosis[12-13].

In recent years, many novel agents and approaches with potential protective effects against the deleterious consequences of sun exposure have been proposed and tested. These include antioxidants, alpha-MSH, polyphenol in green teas, genistein, NF-kB decoy oligodeoxynucleotides, pTpT vaccination, and IL-12[14-15]. Salidroside, a phenylpropanoid glycoside isolated from a traditional Chinese medicinal plant, *Rhodiola rosea L.*, has been reported to have potent antioxidative and anti-apoptotic effects by multiple mechanisms[16-17].

In this study, we aimed to gain insights into the molecular mechanisms underlying UVB-induced cell damage, and to improve understanding of the transformation of normal keratinocytes to cancer cells. Therefore, we examined the UVB responses of HaCaT human keratinocytes and A431 epidermoid carcinoma cells in parallel. In particular, we examined and compared changes in expression of NF-κB, BCL-2 and CDK6 (cell division protein kinase 6, which regulated by Cyclin D, and is important for cell cycle G_1 phase progression and G_1/S transition) in the two cell types. Also, to explore novel therapeutic approaches for cellular protection, we studied the effect of salidroside on UVB-induced apoptosis in both keratinocytes and cancer cells.

MATERIALS AND METHODS

Cell Culture

HaCaT human epidermal keratinocytes and A431 human epidermoid carcinoma cells were purchased from the China Center for Type Culture Collection (Wu Han, China). Both cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Beyotime, China). Cells were maintained at 37 °C with 5% CO_2 in a humidified atmosphere, and the culture medium was replaced every other day.

UVB Irradiation

UVB irradiation of cells was performed according to Guo et al., with minor modifications[18]. Briefly, prior to irradiation, the cells were gently washed twice with phosphate saline buffer (PBS), then exposed to varying doses (0, 10, 30, 50, 70, 100, and 150 J/m^2 respectively) of UVB in the presence of 5 mL PBS using a UVB light source (Shanghai Gucun Electro-Optical instrument Factory, China). The dose rate was measured by Shanghai Institute of Measurement and Testing Technology (Shanghai, China). The wavelength peak of the UVB lamp used was 305 nm, and the power density was 16.5 μW/cm^2. After irradiation, fresh culture media was immediately added to the cells, which were harvested 12 h later.

Cell Viability Assays

Cells were grown in a 96-well plate (Corning, USA) with seeding density of 1×10^4 cells/well. For the dosage experiment, the cells were treated with 0, 10, 30, 50, 70, 100, or 150 J/m^2 UVB (irradiated for 0, 1, 3, 5, 7, 10, or 15 min, respectively). For experiments investigating the protective effect of salidroside against UVB-induced cell death, cells were incubated with 1.25 μg/mL – 20 μg/mL salidroside for 24 h, and then irradiated with 50 J/cm^2 UVB. After irradiation, the cells were further cultured in complete culture medium for 12 h until 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. To analyze the effect of

treatment duration, cells were exposed to 50 J/m^2 UVB radiation, and then incubated for 0, 2, 4, 6, 12, 18, 24, 36, or 48 h. After irradiation, MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium to a final concentration of 0.5 mg/mL. The cells were further incubated for 4 h, then collected and dissolved in dimethyl sulfoxide. Colorimetric analysis was performed at 570 nm. Experiments were repeated in triplicate for each dosage and incubation time.

Flow Cytometry

Cells were treated with 50 J/m^2 UVB radiation and further incubated for 12 h. For cell cycle analysis, the cells were then fixed in chilled 70% ethanol for at least 24 h before staining with 100 μg/mL propidium iodide (PI) and 100 μg/mL RNase A (Sigma-Aldrich, St. Louis, MO,USA). The cells were analyzed using FACScan (Coulter, USA) 30 min after the staining procedure. All experiments were repeated in triplicate.

RT-PCR Analysis

Total RNA isolation was performed using Trizol Reagent (Invitrogen, USA), following the manufacturer's instructions. First-strand cDNA synthesis was performed in a total volume of 25 μL, consisting of 3 μg total RNA, 1 μL Random Primer (10 μmol/L), 5 μL 5×Reaction Buffer, 1 μL dNTP mix solution, 1 μL RNase Inhibitor, 1 μL M-MuLV RTase (Promega), and 13 μL RNase-free dH_2O. Reverse transcription was performed at 42 °C for 60 min, and the reaction was terminated by heating at 70 °C for 5 min, then samples were stored at -20 °C until use. The PCR reaction mixture totaled 20 μL, which was composed of 12.5 μL 2.5×Real Master Mix/20×SYBR solution (Promega), 4 μL forward and reverse primers (1:1; Table 1), 1 μL cDNA, 6.5 μL RNase-free ddH_2O. The parameters of the PCR reaction were as follows: initial template denaturing at 95°C for 2 min, cycle template denaturing at 95 °C for 20 sec, annealing for 30 sec, and extension at 68 °C for 30 sec, for 40 cycles. Five microliters of PCR product was resolved by 1.5% agarose gel electrophoresis for 30 min, and observed and photographed under ultraviolet light. A TANON gel image analysis system was used image processing, and analysis was performed using GAPDH to normalize target gene values. The experiment was repeated three times.

Table 1. RT-PCR Primers

Primer	Annealing Temperature (°C)		Sequence (5′-3′)
NF-κB/ p65	51.4	upstream	TCTCAGCAATGTCAACGAC
		downstream	TTTATGCCTACAGCCTCCT
Bcl-2	55.5	upstream	TCCCTCGCTGCACAAATACTC
		downstream	ACGACCCGATGGCCATAGA
CDK6	52.5	upstream	TGAACCAAAATGCCACATACACT
		downstream	TTCGGCCTTTCGCATAGG
GAPDH	55.0	upstream	TTGCCATCAATGACCCCTTCA
		downstream	CGCCCCACTTGATTTTGGA

Western blotting

Cell samples were boiled for 10 min in Laemmli sample buffer, and protein concentrations determined using the Bradford method. Lysate concentrations were adjusted to ensure even protein loading. Aliquots of protein were separated on a 10% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with a buffer solution with 10% nonfat dry milk buffer, and incubated with anti-NF-κB antibody (1:500; sc-7178, Santa Cruz, USA), anti-CDK6 antibody (1:200; BM1616, BOSTER, China), or anti-BCL-2 antibody (1:500 sc-509, Santa Cruz, USA), overnight at 4 °C. After three washes for 5 min in TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:10000; JG1153875, Pierce biotechnology, USA) or goat anti-Rb-IgG/HRP (1:5000; bse-0295G, BIOS, China) for 60 min at room temperature. Protein bands were detected using the ECL Plus kit (GE Healthcare, Buckinghamshire, UK). β-actin was used as control.

Statistical Analysis

All values are presented as mean±SD. Comparisons between groups were analyzed by Student's *t*-test or one-way ANOVA using SPSS 13.0 software. Results were considered statistically significant at $P<0.05$. All data shown are representative of experiments performed in triplicate.

RESULTS

Cell Survival after UVB Irradiation

Cell survival rates were examined after irradiation with varying dosages of UVB or 50 J/m^2 UVB and culture for different durations. The results

indicated that the survival rate of both cell types decreased in a pattern linear to UVB dosage (A431: y=1.1235-0.1398x, r^2=0.9742; HaCaT: y=1.0454-0.1264x, r^2=0.9449) and there was a remarkable difference between the various dosage groups (P<0.05; Figure 1A). There was no difference between both cell types when cultured for different durations after irradiation with 50 J/m^2 UVB (P>0.05; Figure 1B).

Attenuated G1 Checkpoint Induced by UVB in Keratinocytes

To address the question of whether cell cycle progression was differentially affected in the two cell types, cells were exposed to 50 J/m^2 UVB, and the effects on cell cycle were determined 12 h after irradiation. A representative cell cycle distribution at 12 h post-irradiation is displayed in Figure 2. In HaCaT cells, G_1-S phase arrest was observed, whereas the cell cycle distribution was unaltered in A431 cells (P<0.05). Also, a peak characteristic of apoptosis was observed in HaCaT cells, but no apoptotic changes were detected in A431 cells.

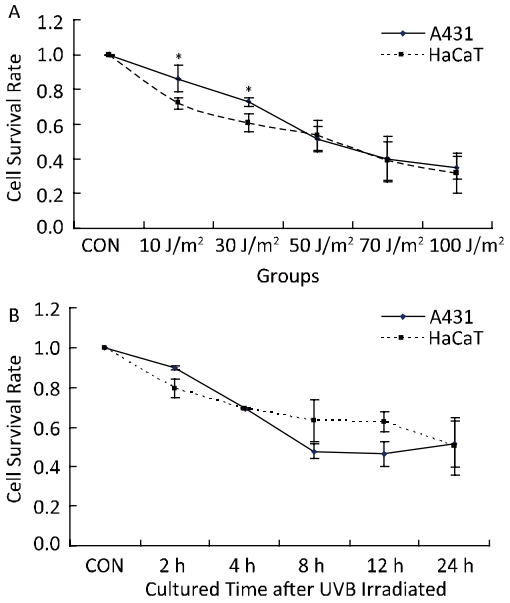

Figure 1. Cell survival rate after UVB irradiation. (A) Cell survival rate with varying dosages of UVB irradiation. *P<0.05 HaCaT *vs*. A431. (B) Cell survival rate at different time points following exposure to 50 J/m^2 UVB.

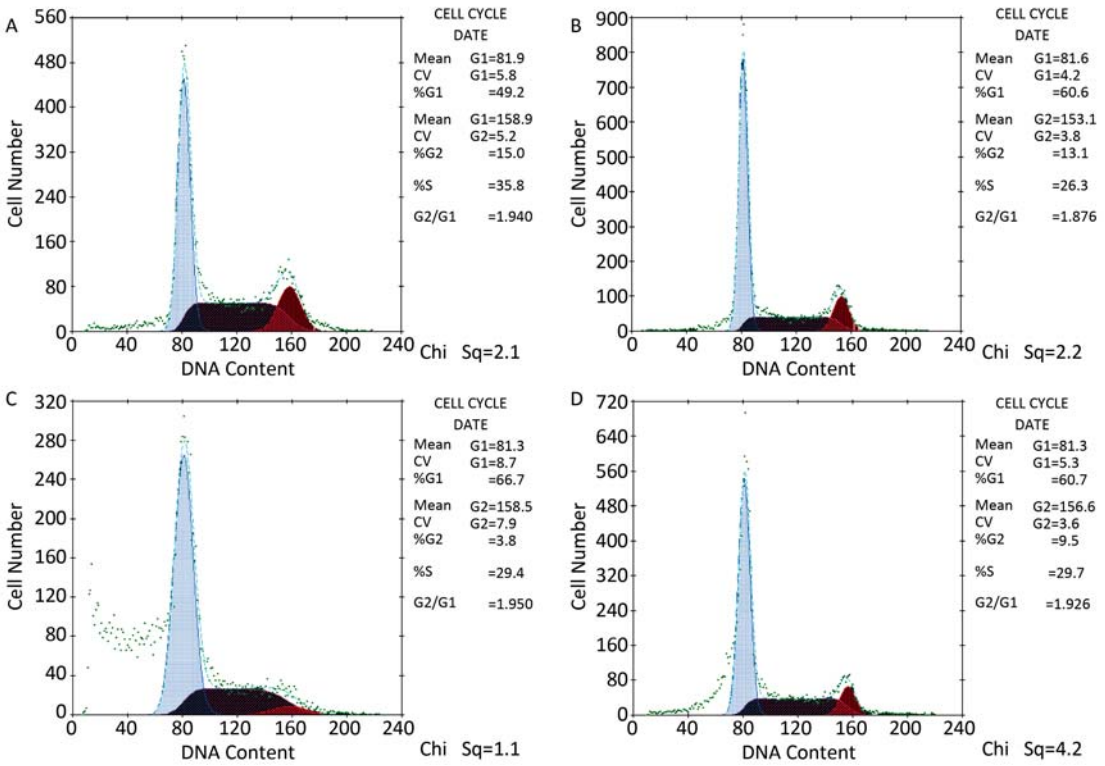

Figure 2. Analysis of cell cycle distribution in HaCaT and A431 cells exposed to 50 J/m^2 UVB. (A) Control HaCaT. (B) HaCaT cells at 12 h following UVB irradiation. (C) Control A431. (D) A431 cells at 12 h following UVB irradiation.

Expression of NF-κB /BCL-2 /CDK6 in Cells after UVB Irradiation

To study the molecular mechanisms that mediate UVB-induced cell death and growth arrest, we next examined gene and protein expression changes of NF-κB, BCL-2, and, CDK6 in both cell types at different time points after exposure to 50 J/m^2 UVB. RT-PCR and western blotting results are shown in Figures 3 and 4, respectively. UVB irradiation resulted in significantly altered expression of NF-κB protein in a time-dependent manner in both cell lines (P<0.05, ANOVA). It started to increase at 2 h, peaked at 8 h and then plateaued until 12 h (protein levels) or 24 h (gene levels). Gene expression levels significantly increased in HaCaT at 4 h and, but at 8 h in A431. BCL-2 protein expression exhibited similar patterns in both cell lines. Expression of CDK6 gene and protein appeared to follow the same pattern as BCL-2 in A431. CDK6 expression in HaCaT at the gene level was not significantly increased until 8 h post-irradiation, and it was reduced at all time points at the protein level, with the most remarkable decrease at 2 h post-irradiation.

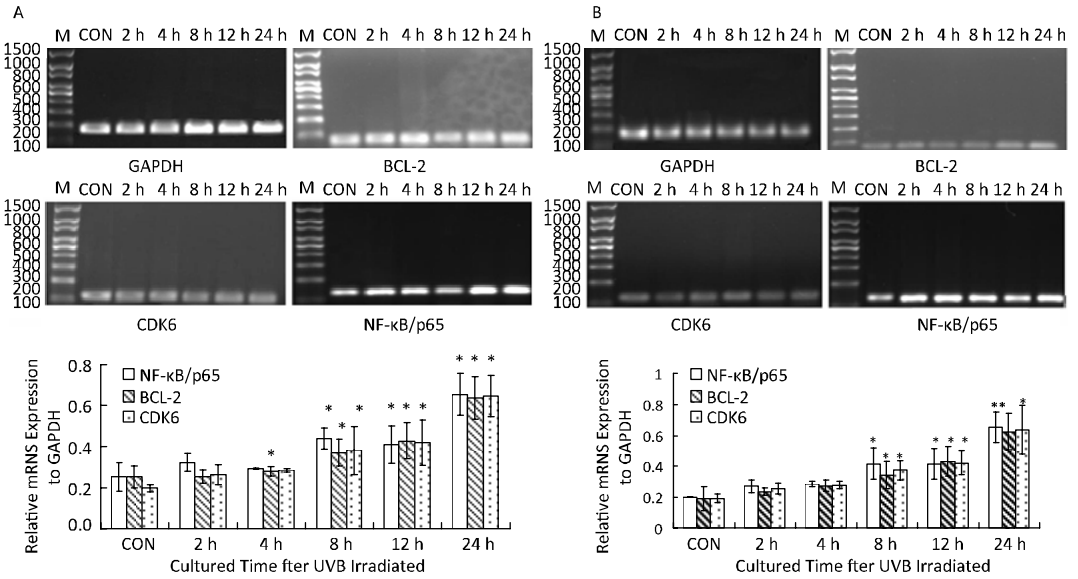

Figure 3. Expression of target genes in HaCaT and A431 cells exposed to 50 J/m^2 UVB as evaluated by RT-PCR. Mean±SD shown in all panels. $^*P$<0.05 *vs.* control. (A) Target gene expression in HaCaT. (B) Target gene expression in A431.

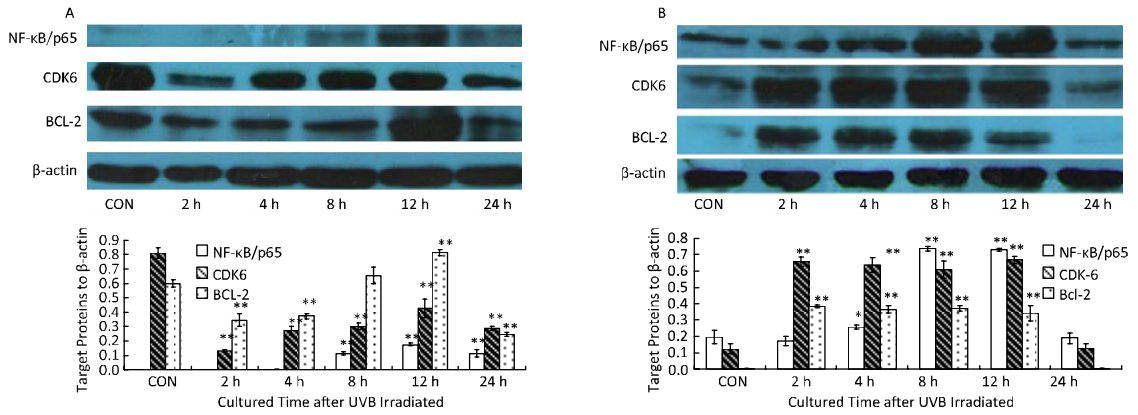

Figure 4. Expression of target proteins in HaCaT (A) and A431 (B) exposed to 50 J/m^2 UVB as evaluated by western blotting. $^*P$<0.05 *vs.* control; $^{**}P$<0.01 *vs.* control. (A) Protein expression in HaCaT. (B) Protein expression in A431.

Protective Effect of Salidroside against UVB-induced Cell Death

Since UVB irradiation at 50 J/cm^2 significantly reduced survival of both cell lines in a culture duration-dependent manner (Figure 1B), we chose this UVB dose when performing experiments examining the effect of salidroside. Cells were incubated with salidroside for 24 h at a series of concentrations, ranging from 1.25 to 20 μg/mL, prior to UVB irradiation, as shown in Figure 5; the survival rate of HaCaT at 1.25, 2.5, 5.0, and 10.0 μg/mL and that of A431 at 1.25, 2.5, and 5.0 μg/mL was substantially enhanced in a dose-dependent manner (P<0.05) The survival rate of HaCaT, on the other hand, was remarkably higher than that of A431 when the concentrations of salidroside were 1.25, 2.5, 5.0, and 10.0 μg/mL (P<0.05), suggesting that salidroside may have a more potential protective effect for HaCaT against UVB irradiation than that for A431.

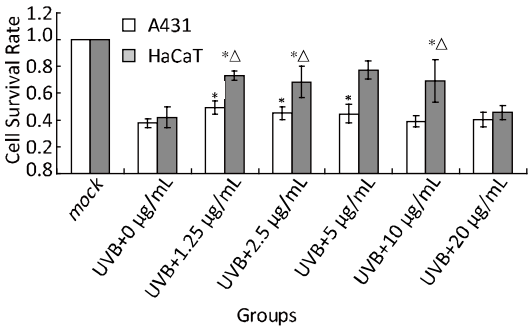

Figure 5. Protective effect of salidroside against UVB-induced cell death in HaCaT(A) and A431 (B) cells. $^*P$<0.05, UVB+salidroside *vs.* UVB+0 μg/mL salidroside; Δ:P<0.05, HaCaT *vs.* A431.

DISCUSSION

UV radiation is a harmful environmental factor that causes skin photoaging, immunosuppression and photocarcinogenesis. In this study, we compared UVB-induced cell cycle arrest and molecular responses in human keratinocyte HaCaT cells and epidermoid carcinoma A431 cells. We first examined cell viability following UVB irradiation in these two cell lines, and found the cell survival rate was associated with dosage of UVB and the duration of culture after irradiation. We also determined whether the inhibitory effect of UVB was accompanied by modulation of cell cycle progression. The results showed that HaCaT cells demonstrated an attenuated G_1 checkpoint following UVB treatment. It has previously been shown that UVB irradiation can lead to same biological effects, such as apoptosis by different mechanisms, and can also cause cell type-specific responses[19]. Different types of UV radiation, on the other hand, can lead to differing levels of sensitivity to death and cell cycle distribution in different cell types[20]. D'Errico et al. found that X-ray exposure induced a strong G_2/M phase arrest in fibroblasts, whereas only a slight effect was detected in keratinocytes under the same exposure regimen[21]. Although different cell cycle position can occur at different doses of irradiation[22], our cell cycle analysis showed G_1-S phase arrest in the HaCaT cells treated with different doses of UVB.

The mechanisms by which UVB causes G_1-S phase arrest in HaCaT cells, and how A431 cells are more tolerant to UVB-induced cell death remain unclear. Different pathways underlying UVB-induced apoptosis have been proposed[23]. In this study, we asked whether UVB activates NF-κB, BCL-2 and CDK6 in HaCaT cells at a lower dose (50 J/m^2), because these molecules are known to play a vital role in cell cycle progression and cell proliferation[24]. UVB is considered to be the main component of the UV solar radiation that activates NF-κB in HaCaT[23,25]. It has been suggested that NF-κB functions as a survival factor in general. However, in HaCaT cells, the sensitivity to UVB-induced apoptosis could be increased due to aberrant NF-κB activity[13]. In our study, NF-κB showed a similar expression pattern in HaCaT and A431 cells, implying that cell death was mostly determined by UVB dose and the role of NF-κB (pro-apoptotic or anti-apoptotic). BCL-2, the founding member of the BCL-2 protein family, regulates both cell cycle progression and apoptosis. The different expression patterns of BCL-2 and CDK6 found in HaCaT and A431 cells in our study are consistent with the differences in UVB-induced cell cycle changes observed in the two cell lines, suggesting that the molecular pathways underlying cell cycle regulation in these cells may be regulated by BCL-2 and CDK6.

Salidroside is a phenylpropanoid glycoside extracted from Rhodiola, a medicinal herb widely found at high altitudes regions in Asia and Eastern Europe. Salidroside is known to have antioxidant, anti-radiation, anti-fatigue, adaptogenic, and anti-inflammation properties. Studies have indicated that salidroside inhibits the proliferation of cancer cells in animal models[17,26-28]. Recently, Hu et al.

have shown the anti-proliferative effect of salidroside on cultured cancer cells[29]. In our study, salidroside increased the survival rate of HaCaT cells under UVB irradiation, consistent with its general cytoprotective effect. In the epidermoid carcinoma A431 cells, however, this effect was not significant, possibly because it was compounded by the general suppressing effect of salidroside on cancer cells.

In summary, our study has identified expression changes of important transcriptional factors, NF-κB, BCL-2, and CDK6, in both human keratinocytes and epidermoid carcinoma cells treated with UVB irradiation. A comparison between the two cell types showed these expression changes were associated with different patterns, suggesting that UVB induces distinct molecular responses in HaCaT and A431 cells. Meanwhile, salidroside, a versatile drug with great therapeutic value, could be optimized as a prophylactic agent to prevent UV-induced injury and eventually protect the natural barrier function of the skin.

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