p53-related apoptosis resistance and tumor suppression activity in UVB-induced premature senescent human skin fibroblasts

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Abstract. Chronic exposure to solar UV irradiation leads to photoaging, immunosuppression, and ultimately carcinogenesis. Cellular senescence is thought to play an important role in tumor suppression and apoptosis resistance. However, the relationships among stress-induced premature senescence (SIPS), tumorigenesis and apoptosis induced by UVB remain unknown. We developed a model of UVB-induced premature senescence in human skin fibroblasts (HSFs). After five repeated subcytotoxic UVB exposures at a dose of 10 mJ/cm^2, the following biomarkers of senescence were markedly present: senescence-associated ß-galactosidase (SA ß-gal) activity, growth arrest, and the overexpression of senescence-associated genes. Firstly, there was an increase in the proportion of cells positive for SA ß-gal activity. Secondly, there was a loss of replicative potential as assessed by MTT assay. FACS analysis showed that UVB-stressed HSFs were blocked mostly in the G1 phase of the cell cycle, and replicative senescence, and protein expression of p53, p21^{WAF-1} and p16^{INK-4a} increased significantly. Thirdly, the mRNA levels of three senescence-associated genes, fibronectin, osteonectin and SM22, also increased. A real time PCR array to investigate the mRNA expression of p53-related genes involved in growth arrest, apoptosis and tumorigenesis indicated that p53, p21, p19, Hdm2, and Bax were up-regulated, and bcl, HIF-1α and VEGF were down-regulated. Collectively, our data suggest that UVB-induced SIPS plays an important role in p53-related apoptosis resistance and tumor suppression activity.

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Introduction

Chronic exposure to UV irradiation from the sun causes photoaging, immunosuppression, and ultimately photocarcinogenesis (1-3). Photocarcinogenesis involves the accumulation of genetic changes, as well as immune system modulation, and ultimately leads to the development of skin cancers. The incidence of skin cancer has been increasing at a marked rate over the past several decades. Long-term and recurrent exposure to sunlight causes the gradual deterioration of cutaneous structure and function. This damage can ultimately lead to the development of skin cancers.

UVA (320-400 nm) and UVB (280-320 nm) radiation reach the earth's surface in amounts sufficient to have important biological consequences for the skin. In contrast to UVA, UVB is more active in inducing mutations in tumor suppressor genes and oncogenes, and in skin carcinogenesis (2). UVB interacts with cellular chromatophores and photosensitizers, resulting in the generation of reactive oxygen species, DNA damage, and the activation of signaling pathways related to senescence and connective tissue degradation (3).

Cellular senescence is a program activated in response to various stress types. These include telomere uncapping, DNA damage, oxidative stress, oncogene activity and UV radiation. Senescence occurs following a period of cellular proliferation or in a rapid manner in response to acute stress [stress-induced premature senescence (SIPS)]. Once cells have entered senescence, they cease to divide, and undergo a series of dramatic morphologic and metabolic changes.

Cell growth takes place more or less continuously as the cell cycle progresses. When new cell growth is called for, the cells re-enter the G1 phase to begin a new cell cycle. Many of the proteins that operate within the G1 phase facilitate

cancer development when they fail to function properly. It is at the G1 checkpoint that a cell can respond in any of several ways, depending on the nature of the damage or environmental triggers such as UV. Two cellular responses, apoptosis and cell senescence, can be viewed as important mechanisms in the suppression of tumorigenesis in mammals (4,5).

Although cellular senescence and apoptosis share many features, the interaction of these two responses and the exact mechanisms by which they regulate each other have not been studied extensively. The hallmark of cellular senescence is the loss of proliferative capacity, whereas the hallmark of apoptosis is sequential cellular events that lead to programmed cell death. These two events are not related and have distinctive biological pathways. Existing studies have indicated that senescent cells are resistant to apoptosis as a mutually exclusive response (4,6-11), but others have shown contradictory evidence (12).

The p53 protein is known to play a key role in growth arrest and apoptosis after cellular stress, primarily through its ability to regulate the transcription of selected downstream target genes in the cell (6). The p53 pathway functions as a central integration point for these various signaling pathways. p53 is one of the most commonly mutated tumor suppressor genes. The significance of a mutated p53 in carcinogenesis is supported by the fact that more than fifty percent of human tumors have cells containing mutations in this unique protein (13).

Published data have demonstrated the feasibility of detecting biomarkers of senescence in skin fibroblasts after subcytotoxic exposures to UVB (14,15). However, the relationships among UVB-induced premature senescence, apoptosis and photocarcinogenesis are still unknown. In this study, we established a model of SIPS by exposing human skin fibroblasts (HSFs) to repetitive subcytotoxic doses of UVB. The scheme induced remarkable senescence-associated features and changes in gene expression profiles. This model will provide insights into elucidating the molecular mechanism of ultraviolet-induced skin cancer.

Materials and methods

Cell culture and UV irradiation. Human skin fibroblasts (HSFs), derived from newborn skin were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA) supplemented with 10% fetal calf serum (FCS) (Invitrogen, AU), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were plated in 60-mm dishes in an incubator at 37˚C with 5% CO_2. An SS-04P lamp (Sigma Corporation, P.R. China) equipped with filters to eliminate wavelengths between 290 and 320 nm was used as the UVB source as previously described (3). The intensity of the radiation from the UVB source was measured prior to each experiment using a UVR radiometer with a UVB sensor (Bioblock Scientific, Belgium). When subconfluent, cells were irradiated with different doses of UVB (5-30 mJ/cm^2). HSFs were washed once with phosphate-buffered saline (PBS) and exposed to UVB irradiation in a thin layer of PBS. The irradiation stress was performed once a day for 5 days. Control cells were kept in the same culture conditions without UVB exposure.

Senescence-associated ß-galactosidase (SA ß-gal) staining. At 48 h after the last stress, cells were trypsinized and seeded in 35-mm culture dishes at a density of 700 cells/cm^2 for 24 h. SA ß-gal activity was assessed with Senescence ß-Galactosidase Staining Kit (CDK, USA) as described by Dimri *et al* (16). The cells were fixed with 1 ml of fixative solution for 15 min at room temperature and incubated at 37˚C overnight with staining solution. The population of SA ß-gal positive cells was determined by counting 400 cells per dish. The proportion of cells positive for SA ß-gal activity is given as a percentage of the total number of cells counted in each dish. The results were expressed as mean ± SD of 3 independent experiments.

MTT cell viability assay. After the last stress, cells were trypsinized and seeded in 96-well plates at a density of 10,000 cells per well as previously described (17). At 24, 48, 72, 96 and 120 h after plating, MTT assay was performed. The volume of culture medium (10% DMEM) in each well was 100 μl. Ten microlitres of MTT solution (5 mg/ml) was added to each well 4 h before detection and incubated at 37˚C. The medium was removed and 200 μl of dimethyl sulfoxide (DMSO) was added into each well to dissolve the formazan by pipetting up and down several times. The absorbance ws measured on an ELISA plate reader at a wavelength of 570 nm. The results were expressed as mean ± SD of 3 independent experiments.

Cell cycle assay. At 72 h after the last stress, cells (1x10^6 cells per sample) were trypsinized and centrifuged. The cells were washed twice at 1000 rpm for 5 min using 2 ml of PBS, and re-suspended in 1 ml of ice-cold 70% ethanol as previously described (18). After being stored at -20˚C overnight, the samples were washed twice again with PBS and re-suspended in 0.5 ml of PBS. The cells were then incubated for 30 min in darkness with 50 μg/ml of propidium iodide and 50 μg/ml of RNaseA before being analyzed with a flow cytometer (FACScalibur, Becton Dickinson, USA). The percentage of cells in G1 phase was determined using the DNAquest program. The results were expressed as mean ± SD of 3 independent experiments.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted with TRIzol reagent (Invitrogen, USA) 72 h after the last stress, according to the manufacturer's instructions as previously described (18,19). Total RNA was reverse transcribed (5 μl of total volume) at 42˚C for 30 min. The total volume of PCR reaction was 30 μl. The PCR conditions were 35 cycles of 94˚C at 30 sec, 57˚C at 40 sec, and 72˚C at 30 sec including a final extension of 7 min at 72˚C. PCR products were then separated by 1.5% agarose gel electrophoresis and photographed. The primers were designed using Primer Premier 5.0 software. The primers are listed in Table I. Semi-quantification was obtained with Quantity One software (Bio-Rad, USA). The results were expressed as mean ± SD of 3 independent experiments.

Western blot analysis. At 72 h after the last stress, the cells were washed once with PBS and lysed on ice. After homogenization of the lysates (20 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 1 mM phenyl-

Table I. Primers used for semi-quantitative RT-PCR.

Gene	Position (bp)	Sequences	Products (bp)
Fibronectin	2041-2060	5'-TGCCAACCTTTACAGACCTA-3'	492
	2532-2551	5'-CTCATCTCCCTCCTCACTCA-3'	
Osteonectin	232-251	5'-GCAGAGGAAACCGAAGAGGA-3'	207
	438-457	5'-GGCAAAGAAGTGGCAGGAAG-3'	
Sm22	294-313	5'-TGAAGGTGCCCGAGAACCCA-3'	367
	690-711	5'-ATCTGCCGAGGTCGTCCGTAGC-3'	
Human ß-actin	487-507	5'-TGCTATCCCTGTACGCCTCTG-3'	678
	1164-1186	5'-ACTCGTCATACTCCTGCTTGCTG-3'	

methylsulfonyl fluoride, 40 mM glycerophosphate, 125 μM Na_3VO_4, 50 mM NaF, 2 μg of leupeptin per ml, 2 μg of aprotinin per ml, 2 μg of pepstatin per ml, and 1 mM dithiothreitol), BCA (Pierce, USA) assay was performed to determine total protein concentrations and 30 μg of proteins were separated by Tris-glycine polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to polyvinylidene fluoride (PVDF) membrane. The antibodies used were anti-p53 (sc-126, Santa Cruz), anti-p21 (sc-6246, Santa Cruz), anti-p16 (sc-1661, Santa Cruz), anti-α-tubulin (AT819, Beyotime, P.R. China) and horseradish peroxidase-linked rabbit or anti-mouse second antibody (DakoCytomation, Denmark). The bands were visualized chemiluminescently (Pierce). Semi-quantification was obtained with Quantity One software (Bio-Rad). The results were expressed as mean ± SD of 3 independent experiments.

Real-time PCR gene array. At 72 h after the last stress, total RNA was extracted from HSFs by using TRIzol (Invitrogen, USA). cDNA was synthesized from the isolated RNA using SuperScript III Reverse Transcriptase (Invitrogen). For PCR array experiments, an RT^2 Profiler Custom PCR Array was used to simultaneously examine the mRNA levels of 32 genes, including three housekeeping genes in 96-well plates according to the protocol of the manufacturer (SuperArray Bioscience, USA). PCR was performed on ABI Prism 7700 Sequence Detector (Applied Biosystems). For data analysis, the ΔΔCt method was used. For each gene, fold-change was calculated as the difference in gene expression between two groups. A positive value indicated gene up-regulation and a negative value indicated gene down-regulation. The results were expressed as mean ± SD of 3 independent experiments.

Statistical analysis. Experiments were repeated three times and results were expressed as means ± SD. Data were statistically analyzed using SPSS 11.0 software. Statistical analysis between the control and treated groups was performed by the Student's t-test. P-values <0.05 were considered significant.

Results

UVB radiation induces cytotoxic stress in cultured human skin fibroblasts. To select a subcytotoxic stress condition, cytotoxicity induced by UVB irradiation was measured preliminarily at 48 h after five repeated exposures to UVB with doses ranging from 0 to 30 mJ/cm^2. Cytotoxicity was determined by MTT assay at 48 h after the last exposure. The results showed that cytotoxicity of cells exhibited after exposure to UV radiation at the dose of 20 mJ/cm^2. Therefore, the subcytotoxic dose of UV radiation used throughout this study was 10 mJ/cm^2 for five repeated exposures (Fig. 1).

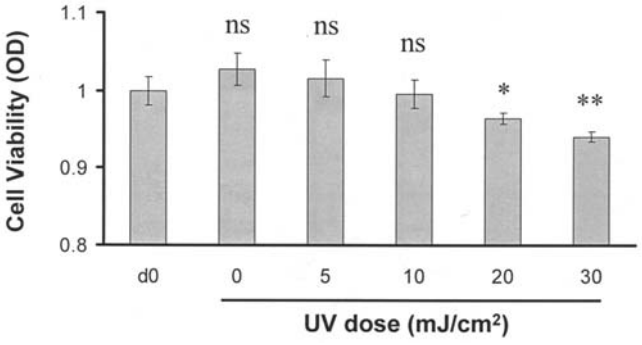

Figure 1. UVB radiation induces cell death in cultured human skin fibroblasts. To select a subcytotoxic stress, cytotoxicity induced by UVB irradiation was measured preliminarily at 48 h after five repeated exposures to UVB doses ranging from 0 to 30 mJ/cm^2. Results are expressed as cell survival compared to day 0 (d0) prior to any stresses. Results are given as mean ± SD of three independent experiments. Statistical analysis was carried out with te Student's t-test. ns, non-significant (P>0.05). *0.05>P>0.01, **P<0.01.

UVB induces cellular senescence in cultured human skin fibroblasts. To further investigate UV-induced cellular senescence, cells were observed under a microscope after 5 repeated exposures to UV radiation. The results showed that the HSFs became enlarged, flattened and irregular after repeated exposures to UVB (Fig. 2B), as compared to control (Fig. 2A). To measure one of the biomarkers of replicative senescence (RS), SA ß-gal assay was performed. The results showed a 2.4-fold increase of positive cells at 72 h after 5 exposures to UVB at the dose of 10 mJ/cm^2 (Fig. 2D), as compared to non-UV radiated cells (Fig. 2C). The percentage of positive cells in UVB-stressed HSFs was 82.0%, while in control cells it was 33.7% (Fig. 2E).

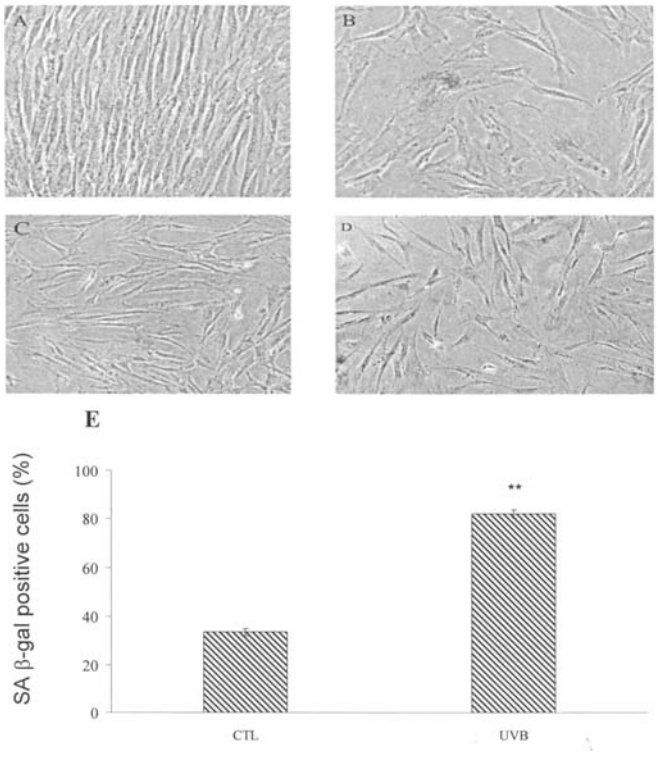

Figure 2. Effect of repeated UVB stress on the proportion of HSFs positive for SA ß-gal activity. HSFs became enlarged, flattened and irregular after five repeated exposures to UVB (10 mJ/cm^2), which was senescence-like. Senescence associated ß-galactosidase (SA ß-gal) activity was another biomarker of senescence. Cells without (CTL) or with UVB exposure (UVB) were examined. (A) CTL and (B) UVB, without staining. (C) CTL and (D) UVB, stained with SA ß-gal. (E) Proportion of cells positive for SA ß-gal activity at 72 h after 5 exposures to UVB at 10 mJ/cm^2 was determined by counting 400 cells per dish under a microscope. The proportions of blue-colored positive cells were given as a percentage of the total number of cells counted in each dish. Results are given as mean ± SD of three independent experiments.

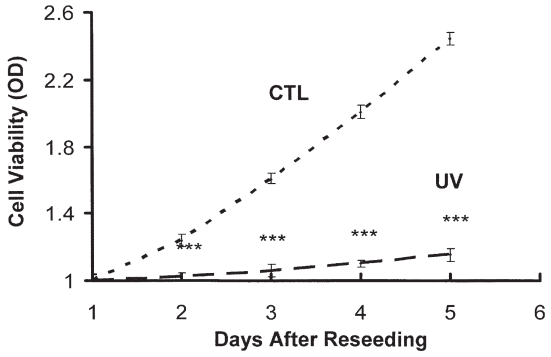

Figure 3. Effect of repeated UVB stress on the proliferation potential of HSFs. HSFs were exposed to 5 repeated UVB (10 mJ/cm^2) stresses or not (CTL). After the last stress, cells were trypsinized and seeded in 96-well plates at a density of 10,000 cells/well. Cells were incubated for 24, 48, 72, 96 and 120 h for detection proliferation potential by MTT assay. Results are given as mean ± SD of three independent experiments. Statistical analysis was carried out with the Student's t-test. ns, non-significant (P>0.05). $^{*}0.05>P>0.01$, $^{**}0.01>P>0.001$, $^{***}P<0.001$.

Repeated subcytotoxic exposure to UVB decreases cell proliferation and induces growth arrest. To further detect whether repeated UV radiation affects cell proliferation or induces cell cycle arrest, MTT dye assay and FACS analysis were performed. The results showed a progressive decrease of the proliferation potential along the population doublings of human diploid fibroblasts (HSFs). MTT assay was performed at 24, 48, 72, 96 and 120 h after 5 UVB exposures at the dose of 10 mJ/cm^2. The absorbance increased very slowly after the last exposure while it increased sharply in the control cells. The results clearly showed that the proliferation potential of UVB-stressed HSFs was inhibited significantly (Fig. 3). To determine whether UVB-stressed HSFs exhibit cell cycle arrest, we examined cell cycle of the cell population by flow cytometry. The results of FACS analysis showed that UVB-stressed HSFs (Fig. 4B) were blocked mostly in G1 phase of the cell cycle with replicative senescence, as compared to the control (Fig. 4A), while the proportion of HSFs in G1 phase in the UVB-stressed group was significantly higher than the control group (Fig. 4C).

Repeated subcytotoxic exposure to UVB induces cell growth arrest-related proteins. Both RS and SIPS are dependent on two major pathways. One is triggered by DNA damage and telomere damage and/or shortening, and involves the activation of the p53 and p21^{WAF-1} proteins. The second pathway results in the accumulation of p16^{INK-4a} with the MAP kinases as possible intermediates. To investigate whether these proteins are up-regulated after UVB stresses, Western blotting analysis was performed using α-tubulin protein level as reference. The results showed that p53, p21^{WAF-1} and p16^{INK-4a} increased by 1.9-, 2.4- and 2.1-fold respectively, when compared to the control cells (Fig. 5).

Senescence-associated genes overexpressed in UVB-stressed HSFs. To further investigate the expression of senescence-associated genes after 5 repeated UV exposures, RT-PCR was performed. The results showed that fibronectin, osteonectin and SM22 mRNA levels increased in senescent HSFs with a fold change of 2.7, 2.0 and 2.3 respectively (Fig. 6). The housekeeping gene coding for human ß-actin was used as reference.

mRNA level of genes involved in p53-related senescence, apoptosis and carcinogenesis after repeated exposures to UVB. Cellular senescence is thought to play an important role in tumor suppression. Studies have shown that replicative senescent fibroblasts are resistant to apoptotic death. UV irradiation leads to apoptosis, premature senescence and carcinogenesis. To determine whether UVB-stressed premature senescence of HSFs inhibits tumorigenesis and apoptosis, a real time PCR array was performed to investigate the mRNA levels of a number of genes involved in growth arrest, apoptosis and tumorigenesis induced by UVB. The results showed that some genes were differentially expressed in UVB-induced SIPS. These genes are listed in Table II. The genes involved in growth arrest, p53, p21^{WAF-1}, p16^{INK-4a} and p19, were overexpressed significantly, and the p53 downstream gene and Hdm2 were up-regulated slightly. Among genes involved in p53-dependent apoptosis, Bax was overexpressed while bcl-2 was down-regulated slightly. Of the genes involved in UVB-induced carcinogensis, which were overexpressed in our previous study (3), HIF-1α and VEGF were down-regulated.

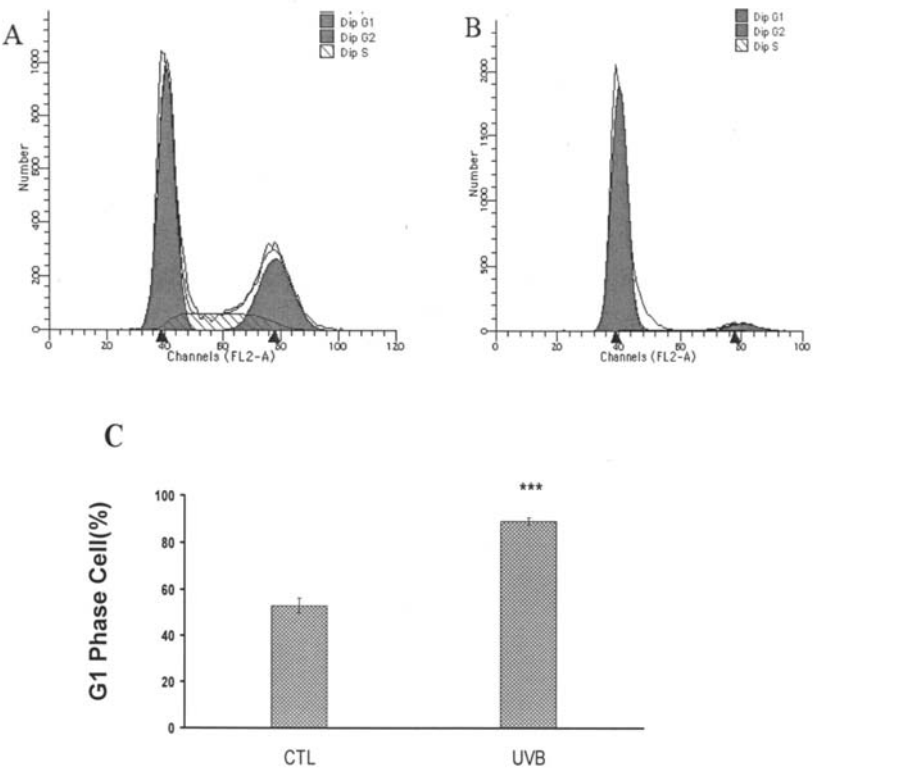

Figure 4. Effect of repeated UVB stress on the cell cycle of HSFs. HSFs were exposed to 5 repeated UVB (10 mJ/cm^2) stresses or not (CTL). At 72 h after the last stress, cells were trypsinized and processed for FACS analysis of cell cycle. (A) CTL, (B) UVB. (C) The proportion of cells at G1 phase out of total cells was shown. Results are given as mean ± SD of three independent experiments. Statistical analysis was carried out with the Student's t-test. ns, non-significant (P>0.05). *0.05>P>0.01, **0.01>P>0.001, ***P<0.001.

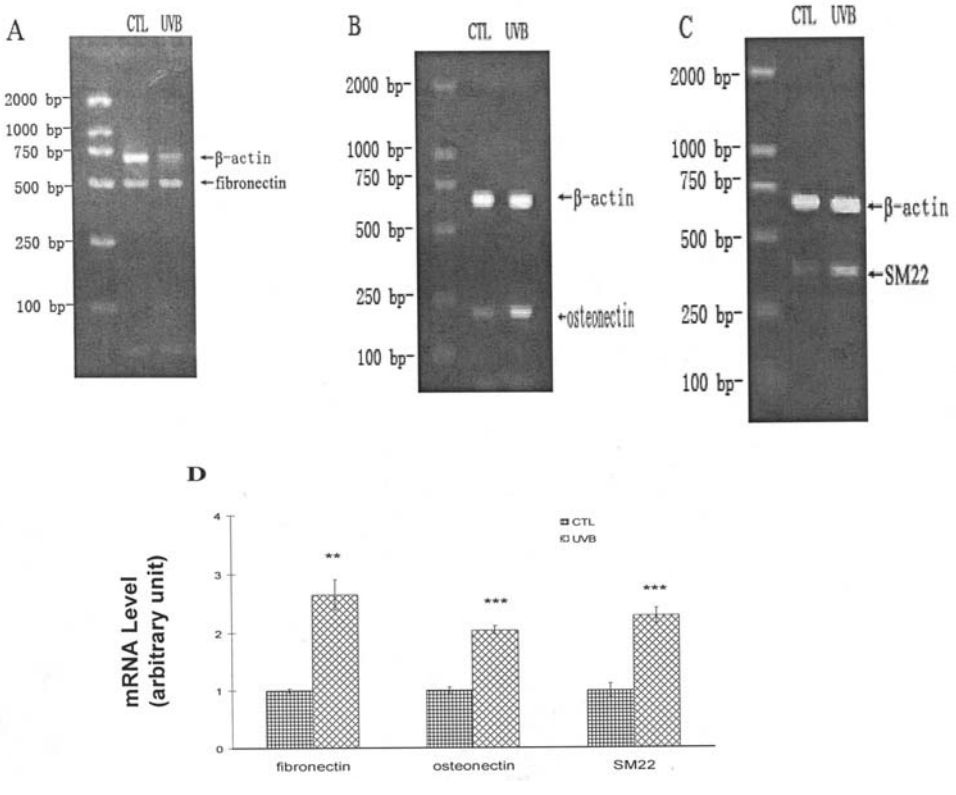

Figure 5. Effect of repeated UVB stress on the mRNA level of senescence-associated genes in HSFs. Total RNA was extracted at 72 h after the last stress. RT-PCR was performed. mRNA levels of (A) fibronectin, (B) osteonectin and (C) SM22. The human ß-actin mRNA level was used as reference in the RT-PCR. (D) The results obtained from the UVB-treated cells (UVB) are expressed as a percentage of the mRNA level of the respective mRNA species in control cells (CTL). Results are given as mean ± SD of three independent experiments. Statistical analysis was carried out with the Student's t-test. ns, non-significant (P>0.05). *0.05>P>0.01, ** 0.01>P>0.001, ***P<0.001.

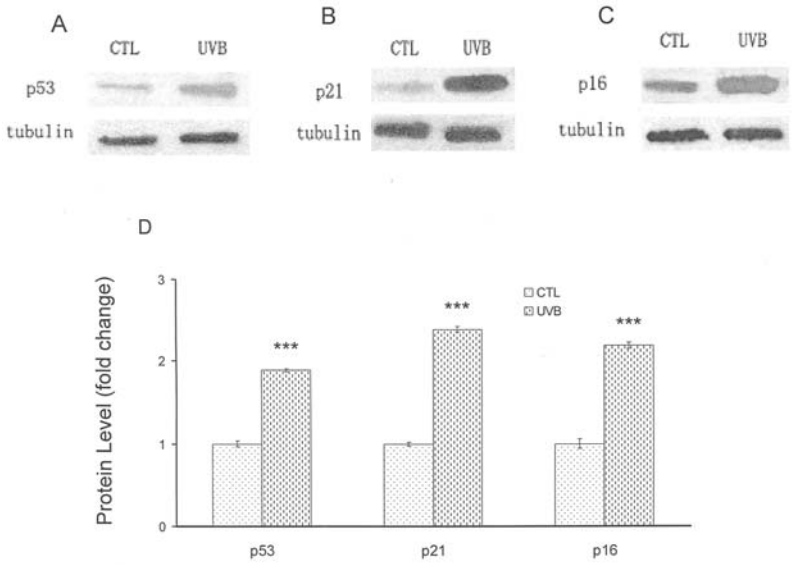

Figure 6. Analysis of p53, p21 and p16 protein levels by Western blotting. Proteins were extracted at 72 h after the last stress. SDS-PAGE Western blot analysis was performed using specific antibodies. (A) p53, (B) p21, (C) p16. (D) Quantification of the protein level when tubulin protein was used as reference. Results are given as mean ± SD of three independent experiments. Statistical analysis was carried out with the Student's t-test. ns, non-significant ($P>0.05$). $^{*}0.05>P>0.01$, $^{**}0.01>P>0.001$, $^{***}P<0.001$.

Table II. Genes differentially expressed in human skin fibroblasts in UVB-induced SIPS.

Gene	Description	UVB/CTL	GenBank
p53	Tumor protein p53	↑1.74	NM_000546
p21	Cyclin-dependent kinase inhibitor 1A	↑2.14	NM_000389
p16	Cyclin-dependent kinase inhibitor 2A	↑2.10	NM_000077
p19	Cyclin-dependent kinase inhibitor 2D	↑2.47	NM_001800
Hdm2	Human double minute-2 oncoprotein	↑1.33	NM_002392
HIF-1α	Hypoxia-inducible factor 1, α subunit	↓2.42	NM_001530
VEGF/VEGF-A	Vascular endothelial growth factor A	↓1.36	NM_003376
Bcl-2	B-cell CLL/lymphoma 2	↓1.26	NM_000633
Bax	BCL2-associated X protein	↓2.41	NM_004324

Discussion

Ultraviolet radiation, in solar or artificial form, is responsible for a number of human skin diseases including skin cancer (1). The International Agency for Research on Cancer has noted that there is sufficient evidence from studies in animals and in humans to establish UV as a human carcinogen. UV radiation damages DNA and thus mutagenizes several genes, including p53, involved in the development of skin cancer. The presence of a typical signature of UV-induced mutations on those genes indicates that the ultraviolet-B component of sunlight is responsible for the evolution of cutaneous carcinogenesis (2).

Uncontrolled cellular growth, which may be a result of defects in cell cycle and apoptotic machinery, is responsible for the development of skin cancer. To maintain the integrity of the cells after DNA damage, several cellular responses are activated that include mechanisms for the removal of DNA damage. One of these mechanisms is growth arrest followed by DNA repair, and the other is cell death by apoptosis. Both of these mechanisms prevent the transmission of mutations to daughter cells that can lead to transformation and carcinogenesis by transcriptional activation of p53-related genes (20). The p53 tumor suppressor gene plays a decisive role in protecting cells from DNA-damage as a consequence of UVB exposure (21).

In addition, cellular senescence or replicative senescence is thought to be involved in the mechanisms preventing such DNA damage leading to carcinogenesis, which can also be activated by the p53-related pathway. Cellular senescence is defined as the irreversible growth arrest of a mitotic cell, first reported in fibroblasts *in vitro* in 1961 (22). It has since been described in a wide variety of cell types *in vitro* and *in vivo*. Stress-induced premature senescence (SIPS) occurs after many different sublethal stresses including UV. Cells in RS share common features with cells in SIPS. Both are dependent on two major pathways. One is triggered by DNA damage, and involves the activation of p53 proteins (23).

Numerous studies have shown that after sublethal stress under UV light, tert-butylhydroperoxide (t-BHP), single H_2O_2, ethanol, mitomycin C and the like, HDFs displayed the phenotype of SIPS (24). In this study we developed a model of stress-induced premature senescence induced by 5 repeated exposures to a subcytotoxic dose of UVB (10 mJ/cm^2). Exposure of HSFs to repeated noncytotoxic UVB stresses triggered the appearance of several biomarkers of cellular senescence: enlarged size, SA ß-gal activity, reduced proliferative capacity, G1 arrest and overexpression of senescence-related genes, beginning 72 h after the end of the stress (Figs. 1-4).

Upon entering the state of senescence, cells undergo a dramatic change in morphology: their volume increases and they lose their original shape with a flattened cytoplasm. The enzyme ß-galactosidase has an abnormal behavior associated with senescent cells, which is termed senescence-associated ß-galactosidase (SA ß-gal) activity (16). We found that after repeated exposures to UVB, a large increase was observed in the proportion of SA ß-gal positive HSFs (Fig. 2). The initial discovery of the RS of human fibroblasts has led to the view that senescence serves as a mechanism whose purpose is to limit the proliferative capacity of normal cells. This limit for proliferation is due to growth arrest. A drastically decreased proliferative potential of HSFs in UVB-induced SIPS was observed after 5 exposures to UVB. UVB-treated HSFs were blocked mainly in the G1 phase of the cell cycle as were senescent ones. H_2O_2 and hyperoxia also leads to a growth-arrest of HSFs mainly in the G1 phase (25,26).

DNA damage triggers a G1 arrest through its effects on the stability of p53 protein. p53 induces the production of negative regulators that inactivate G1-cyclin-Cdk complexes. These Cdk inhibitors fall into two families: the INK4 inhibitors and the Cip/Kip inhibitors, such as $p16^{INK-4a}$ and $p21^{WAF-1}$ (5). These tumor suppressor genes regulate cell cycle and G1 arrest as central signal activating pathways of senescence. The decreased proliferation potential of senescent cells was correlated with an overexpression of p53, $p21^{WAF-1}$ and $p16^{INK-4a}$ (15). In this study, expression of these proteins in UVB-treated HSFs was significantly increased (Fig. 6). However, the signal pathways and complete mechanisms involved in growth arrest and SIPS induced by UVB are unclear, and require further study.

Many senescence-related genes are overexpressed in RS and SIPS. Among others are fibronectin, osteonectin and SM22. Fibronectin is an essential extracellular matrix component involved in cell adhesion, cytoskeletal organisation, mediation of external mitogenic signals, and wound repair. During the RS of fibroblasts, fibronectin may contribute to morphological changes observed in senescent HDFs as well as anchorage of cells to their substrate, thereby decreasing the possibility of apoptosis and explaining in part why senescent cells are resistant to apoptosis. Osteonectin is also a component of the extracellular matrix. Retrovirally mediated stable overexpression of osteonectin reduces the mitogenic effects of platelet-derived growth factor in WI-38 HDFs, which partly explains the sharp decrease of the proliferative potential observed in SIPS (14). SM22 (or transgelin) encodes a putative calcium binding protein involved in senescence-induced morphological changes (27). In this study, the senescence-related genes fibronectin, osteonectin and SM22, of UVB-treated HSFs were over-expressed at 72 h after the last UVB stress (Fig. 5). The steady-state mRNA levels of these genes also increased in RS and in H_2O_2 and t-BHP-induced SIPS (28).

It has recently been demonstrated that senescence is associated with specific post-translational modifications of p53. One study hypothesized that the inability of senescent cells to undergo apoptosis is caused by changes in the state of p53. Senescent fibroblasts were unable to undergo p53-dependent apoptosis, whereas p53-independent apoptosis was only slightly reduced (6). In this study, gene expression involved in p53-dependent apoptosis indicated that anti-apoptotic bcl-2 was not inhibited and pro-apoptotic Bax was down-regulated in senescent HSFs induced by UVB, suggesting that UVB-induced premature senescent HSFs may resist p53-dependent apoptosis.

It is widely believed that cellular senescence is as important an anticancer mechanism as apoptosis. This is supported by the observation that malignant cells become senescent both *in vitro* and *in vivo* (29,30). Cellular senescence limits the replicative capacity of cells, thus preventing the proliferation of cells that are at different stages of malignancy (31). The process of carcinogenesis usually involves one or more events that inhibit senescence, the inactivation of p53 or p16, two of the most commonly disabled tumor suppressors in different types of cancer (32,33). Senescence bypass appears to be an important step in the development of cancer. However, other evidence has indicated that cellular senescence suppresses tumorigenesis early in life by curtailing the development of cancer, but that it may promote cancer in aged organisms by limiting longevity as dysfunctional senescent cells accumulate, as an example of evolutionary antagonistic pleiotropy.

It has also been proposed that senescence may promote tumor progression by secreting certain matrix metallo-proteases, growth factors, and cytokines. In particular, senescent fibroblasts were shown to facilitate tumorigenesis by immortal premalignant epithelial cells (34,35). In addition, therapy-mediated premature senescence has been identified as a drug-responsive program that impacts the outcome of cancer therapy. In a niche that has fixed a mutation, deletion of mutant stem cells is not efficient since they will be replaced by replication of other mutant stem cells. Cellular senescence can provide an efficient solution to this problem (36). Treatment-induced senescence, was shown to be one of the key determinants of tumor response to therapy in *in vitro* and *in vivo* studies (37,38).

We found that UVB radiation induces HIF-1α and VEGF expression via the EGFR/PI3K/DEC1 signaling pathway (3). HIF-1α activates the transcription of many genes including VEGF (39), many of which play important roles in tumor angiogenesis and growth (40-42). It is possible that the induction of HIF-1 and VEGF play an important role in UV-induced carcinogenesis. Activation of EGFR is also involved in the UV signal transduction pathway leading to skin cancer (43). The role of UVB-induced premature senescence of HSFs in photocarcinogenesis remains unclear. The expression of HIF-1α and VEGF in UVB-induced premature senescence has not been extensively studied. Our results

indicated that expression of HIF-1α was down-regulated in senescent HSFs while p53 and Hdm2 were up-regulated. Our results are consistent with previous studies which indicate that low p53 expression attenuates HIF-1α transactivation by competing for p300, whereas high p53 expression destroys HIF-1α protein and thereby eliminates HIF-1 reporter activity (44,45).

Other studies have found that p53 promotes Mdm2-mediated ubiquitination and proteasomal degradation of the HIF-1α (46). Mdm2 may act as a bridge and mediate the indirect interaction between HIF-1α and p53 in cells (47). Some investigators have demonstrated that apigenin inhibits HIF-1 and VEGF expression through two distinctive signaling pathways: PI3K/AKT/p70S6K1 and Hdm2/p53 (48,49). We propose that overexpression of p53 in UVB-induced senescent HSFs may inhibit HIF-1α and VEGF, and then facilitate tumor suppression, but further study is required.

In conclusion, human skin fibroblasts exposed to repeated subcytotoxic doses of UVB presented a phenotype of stress-induced premature senescence, which shared many similarities with the fibroblasts obtained in replicative senescence. Expression of some p53-related genes involved in apoptosis and tumorigenesis suggests that UVB-induced premature senescent HSFs may play a role in p53-dependent apoptosis resistance and tumor suppression.

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