Translocation of p53 Protein in Melanocytes and Malignant Melanoma Cells After UVB Irradiation

Kyoung-Hee Choi, M.D., Hye-Jin Kim, M.D., Yong Choi, M.D., Kyu Han Kim, M.D., Kyoung-Chan Park, M.D.,

Department of Dermatology, Seoul National University College of Medicine, Seoul, Korea

Background: UVB is responsible for most of the carcinogenic effects of sun exposure.
Objective: Aim of this study was to investigate the regulation and intracellular redistribution of p53 protein after UVB irradiation.
Methods: Cultured normal human melanocytes and G361 melanoma cells were used for western blotting and confocal microscopic examination for determining expression and distribution of p53.
Results: UVB irradiation increased p53 expression in cultured normal human melanocytes and G361 malignant melanoma cells according to increasing doses of UVB. Furthermore, p53 moved from cytosol to nucleus after UVB irradiation.
Conclusions: UVB irradiation induced overexpression and redistribution of p53 in cultured normal human melanocytes and G361 malignant melanoma cells.

Key Words: p53/UVB/translocation

The majority of human skin carcinomas are associated with exposure to ultraviolet (UV) radiation and UV is one of the major causes of stress, which can induce DNA damage. p53 is a tumor suppressor gene that has been implicated in a number of important cellular processes, including DNA repair and apoptosis. After DNA damage, p53 level increases and halts the cell cycle by upregulating expression of the p21WAF1/CIP1 protein, which in turn inhibits cyclin-dependent kinases required for cell-cycle progression. UV irradiation is known to induce the expression of the tumor suppressor gene p53. In addition, it is reported that p53 expression is predominantly perinuclear in cultured normal human keratinocytes and exposure to UVB irradiation induces a major shift of p53 expression to the nucleus. However, effects of UVB irradiation on the distribution and movement of p53 in melanocytes are not clearly studied yet. In this study we have investigated the effects of UVB irradiation on the expression and translocation of p53 in normal human melanocytes and malignant melanoma cells.

MATERIALS AND METHODS

Cell line
Normal human melanocytes were isolated from the foreskins of healthy males (18-20 years of age) as described. Melanocytes were inoculated in a 25cm² tissue culture flask and were maintained at 37°C in 5% CO₂ with a growth medium previously described by Medrano and Nordlund, which consisted of the following: MCDB 153 (Sigma, St Louis, MO, USA), 5% fetal bovine serum (FBS; Hyclone, Logan, Utah), 13 ng/ml bovine pituitary...
Fig. 1. Effects of UVB irradiation on the viability of melanocytes (open circle) and G361 cells (closed circle). MTT assay was done 24 hours after irradiation.

![Graph showing the effects of UVB irradiation on cell viability](image)

extract (Sigma), 8 nM 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma), 5 0 µg/ml insulin (Sigma), 1 µg/ml transferrin (Sigma), 1 µg/ml tocopherol (Sigma), 0.6 ng/ml human recombinant basic fibroblast growth factor (bFGF; Gibco BRL, Gaithersburg, MD, USA), and 1% penicillin-streptomycin (10,000 U/ml and 10,000 µg/ml, respectively, Gibco BRL). A lightly pigmented melanoma cell line, G361 human melanoma cells (ATCC, Rockville, MD, USA) were grown in RPMI supplemented with 10% FBS and 1% penicillin-streptomycin (10,000 U/ml and 10,000 µg/ml, respectively) with 5% CO₂ at 37°C.

**UVB irradiation**

The cultured cells were irradiated with a UVB source (BLE-1T158, Spectronics Corp., Westbury, NY, USA). A Kodacel filter (TA401/407 Kodak, Rochester, USA) was used to remove wavelengths < 290 nm (ultraviolet C). The applied energy was measured with a Waldmann UV meter (model No. 585100; Waldmann Co., VS-Schwenningen, Germany). Immediately after irradiation, the transferred medium was returned to each well. The irradiated cells were maintained for 24 hrs at 37°C in a 5% CO₂ atmosphere.

**Cell viability assay**

Cell viability was determined by the MTT assay. After UVB irradiation, the cells were cultured for 24 hrs and 20 µl of MTT (5 mg/ml) was added to each well of the 96-well plates. Four hrs later, the supernatant was removed and 200 µl of dimethylsulfoxide (DMSO) was added to dissolve the formazan products. The absorbance was determined at 540 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

**Cellular fractionation and protein extraction**

G361 melanoma cells were collected and washed with cold PBS. After centrifugation, cells were resuspended in 400 µl of buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.5 mM PMSF) by gentle pipetting. After swelling on ice for 15s, 25 µl of 10% NP-40 (Sigma) was added and cells were vortexed vigorously. To separate cytoplasm and nucleus, samples were centrifuged for 30s and the resulting pellet was used as the nuclear fraction. The supernatant was centrifuged at 320,000 Xg for 30 min and the resulting pellet was used as the cytoplasmic fraction. Both fractions were lyzed in cell lysis buffer (0.0625 M Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 2 mM PMSF, 2% SDS, 10 mM EDTA).

**Western blot analysis**

Five to ten micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and was blotted onto nitrocellulose paper. Blots were incubated with antibody to p53 (1:500, Santa Cruz Biotech Inc., Santa Cruz, CA, USA) followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amerham International, Little Chalfont, U.K.). In most cases, blots were also restained using actin.
Fig. 3. Translocation of p53 in normal human melanocytes after UVB irradiation. A, control, B, 1 min, C, 30 min, D, 1 hr, E, 6 hr, and F, 24 hr after UVB irradiation.
for 20 min, and permeabilized in 0.5% Triton-X 100 in PBS for 5 min at room temperature. The cells were incubated with primary antibody in blocking buffer (5% BSA in PBS). The primary antibody was detected with fluorescein-conjugated anti-mouse IgG. Cells were observed with a BioRad confocal microscope (MRC 1024, Hercules, CA, USA).

RESULTS

Cell viability after UVB irradiation
Cultured normal human melanocytes and G361 human melanoma cells were irradiated with increasing doses of UVB ranging from 0 to 75 mJ/cm². A dose-dependent decrease of cell viability was observed (Fig. 1).

Increased expression of p53 protein according to increasing doses of UVB
Various doses of UVB were used to irradiate cultured normal human melanocytes and G361 malignant melanoma cells. Twenty-four hrs after UVB irradiation, cells were harvested and protein was extracted. Western blotting disclosed that p53 protein was elevated at a dose dependent manner in normal human melanocytes (0-50 mJ/cm²) and G361 malignant melanoma cells (0-50 mJ/cm²) (Fig. 2).

Translocation of intracellular p53 protein after UVB irradiation
After UVB (50 mJ/cm²) irradiation of normal human melanocytes, the staining pattern of p53 was observed at various time intervals using confocal microscopy. Before UVB irradiation, p53 was distributed diffusely in cytoplasm and nucleus. However, p53 moved to perinuclear area of melanocytes 30 min and 1 hr after UVB irradiation. Six to twenty four hrs after UVB irradiation, p53 protein was found to localize in nucleus (Fig. 3). We also evaluated the changes of amounts of p53 protein in cytoplasmic and nuclear fractions extracted from G361 human melanoma cells. As it is shown, accumulation of p53 in nucleus was observed at 6 hrs after irradiation (Fig. 4).

DISCUSSION
DNA damage caused by ultraviolet B (UVB) ir-
radiation has been shown to induce both apoptosis and p53 expression\(^1\). The tumor suppressor p53 protein is a transcription factor\(^7\). The tumor suppressor p53 protein has been implicated in cell differentiation, cell contact inhibition of growth, protection of the cells from the acquisition of genomic abnormalities, and cell senescence\(^8\). After activation, p53 can bind directly single-stranded DNA, interact with DNA replication machinery and regulate the transcription of genes involved in both cell cycle arrest and apoptosis. UV irradiation is a known mutagen and is implicated in the genesis of human cutaneous malignancies\(^9\). Stress such as UV leads to the stabilization of p53 protein and its translocation to the nucleus, resulting in activation or suppression of p53-responsive genes. Study on the expression of p53 protein has been performed using various kinds of cell type. However, expression and distribution of p53 in melanocytes is not studied well. In this study, we evaluated the expression and distribution of p53 after UVB irradiation using normal human melanocytes and G361 malignant melanoma cells. Levels of p53 protein increased after UVB irradiation in normal human melanocytes and G361 malignant melanoma cells. Furthermore, p53 of cytoplasmic fraction increase slightly at 6hrs after UVB irradiation and decreased abruptly 24 hrs after UVB irradiation. P53 of nuclear fraction increase dramatically at 6hrs after UVB irradiation and decrease thereafter. These results showed that the amount of p53 protein seemed to be maximal at 6hrs after UVB irradiation both in cytoplasmic and nuclear fractions. Increased amount of p53 protein might be the result of increased translation of the genes but at least in part by the stabilization of the normally rapidly degraded wild-type p53 protein. In addition, increase of p53 protein in nuclear fraction might result from the translocation of cytoplasmic p53 protein into the nucleus\(^5\). We also observed a dramatic difference in p53 distribution after UVB irradiation (Fig. 3). Distribution of p53 was diffuse before UVB irradiation. However, confocal microscopic examination revealed that p53 moved to perinuclear area after 30 min and 1hr after UVB irradiation. It is reported that nuclear envelope binding provides an optimal site for rapid entry of p53 into the nucleus\(^9\). Our results suggest that perinuclear collection of p53 is the preceding process for entry of p53 protein into the nucleus after UVB irradiation (Fig. 3). These finding support the reason why the nuclear fraction of p53 is dramatically increased 6hrs after UVB irradiation.

UVB inhibits the proliferation of cultured normal human melanocytes by inducing cell cycle arrest in G1 after one UVB dose or in G2 after multiple doses\(^3\). The mechanism by which p53 carries out these functions seems to be related to its ability to induce G1 or G2/M cell cycle arrest and/or apoptosis\(^9\).

As we showed in our study, p53 move from cytoplasm into nucleus after UVB irradiation. After translocation, p53 may induce both growth arrest and apoptosis of melanocytes or melanoma cells.

ACKNOWLEDGEMENT

This study was supported by grant no 04-1999-040-0 from the Seoul National University Hospital Research Fund. The authors also wish to acknowledge the support of the Pacific Corporation.

REFERENCES

7. Medrano EE, Im sungbin, Yang F, Abdel-Malek ZA: Ultraviolet B light induces G1 arrest in human melanocytes by prolonged inhibition of retinoblastoma protein phosphorylation associated with long-

