Caspases Activation in Ultraviolet B-induced Apoptosis of G361 Human Melanoma Cell Line

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Background **: Ultraviolet B (UVB) irradiation can induce apoptosis of melanocytes and melanoma cells. However, mechanism of UVB-induced apoptosis of melanoma cells is not clarified yet.**

Objective **: Our purpose was to study the molecular mechanism of UVB-induced apoptosis of melanoma cells.**

Methods **: G361 lightly pigmented melanoma cells were analyzed for apoptotic mechanism by flow cytometry and western blotting.**

Results **: G361 melanoma cells showed apoptotic features with gradual increment of UVB doses by MTT and flow cytometry. Western blotting disclosed activation of caspase-3 and poly (ADP-ribose) polymerase (PARP) after UVB irradiation.**

Conclusion **: In this study, we showed that UVB-induced apoptosis of melanoma cells is mediated by PARP activation which is induced by caspase cascade.**

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Key Words : Melanoma cells, Ultraviolet B, Apoptosis, Caspase

The skin serves as a barrier between human and injurious environmental influences, including ultraviolet (UV) light. Most of the damaging UV light is absorbed in the epidermis and leads to apoptosis of keratinocytes.[1] Apoptosis, a distinct form of cell death, is implemented by a death machinery whose executionary arm is a family of cysteine proteases. A family of cysteine proteases called caspases is activated by diverse apoptotic stimuli from outside and inside of the cell[2,3]. In this study, we analyzed whether apoptosis of human melanoma cells is induced by ultraviolet B irradiation and whether activation of downstream-caspases is involved in UVB-induced apoptosis of human melanoma cells[3].

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MATERIALS AND METHODS

Cell culture

Lightly pigmented melanoma cell line, G361 (ATCC, Rockville, MD, USA) was grown in RPMI supplemented with 5% FBS and 1% penicillin-streptomycin (10,000 U/ml and 10,000μg/ml, respectively) with 5% CO_2 at 37℃.

UVB irradiation

The source of UVB was BLE-1T158 (Spectronics, Westbury, NY, USA). A Kodacel filter (TA401/407, Kodak, Rochester, NY) was used to remove wavelength of less than 290 nm (ultraviolet C). The energy was measured with a Waldmann UV meter (model No. 585100; Waldmann Co.). The medium was replaced by 1 ml of phosphate-buffered saline (PBS), and cells were exposed to UVB (0-40mJ/cm^2) light. After irradiation, the cells were placed immediately in the same medium.

MTT

Cell viability was determined by the MTT assay, which is based on reduction of soluble yellow MTT tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to a blue MTT formazan product by mitochondrial succinic dehydrogenase. After UVB irradiation, the cells were cultured for 24 hours; 20μl of MTT(5 mg/ml) was added to each well, and the cells were incubated for 4 hours at 37℃. The supernatant was removed, and 200μl of dimethylsulfoxide was added to each well to dissolve formazan products. The absorbance was determined spectrophotometrically at 570 nm with an ELISA reader. The results were expressed as a percentage of control in triplicate cultures.

Flow cytometric analysis

Twentyfour hours after irradiation, the supernatant of cultures, which contained floating dying cells and apoptotic cells, were collected. Then, adherent cells were harvested by brief trypsinization. Two fractions were put together and washed with PBS. Propidium iodide(PI, 40μg/ml) was added to the cell suspension. Flow cytometric analysis were performed on a FACSCalibur™ (Becton Dockinson, San Jose, CA). Data was submitted for computerized analysis using special software (ModFit LT™ version 2.0, Verity software House, Topsham, ME).

Western blot analysis

Cells were collected and lyzed in cell lysis buffer[0.0625 M Tris-HCl (pH 6.8), 5% ß-mercaptomethanol, 2 mM phenylmethylsulfonyl fluoride, 2% SDS, 10 mM EDTA]. Extracted protein(5-10μg) was separated by SDS-polyacrylamide gel electrophoresis and was blotted onto a nitrocellulose paper. Blots were incubated with primary antibodies followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, U.K.). Antibodies used in this experiment were as follows: CPP32/caspase-3 (Transduction Lab, Lexington, KY), mouse anti-PARP (Pharmingen International, San Diego, CA).

RESULTS

Cell viability after UVB irradiation

The number of viable cells measured 24 hours after UVB irradiation showed a dose dependent decrease. A significant decrease of O.D. was observed from 10 mJ/cm2 of UVB irradiation (Fig. 1).

UVB irradiation induces apoptosis in melanoma cell line

To determine whether UVB-induced melanoma cell death is due to apoptosis, duplicate cultures were exposed to 0-40 mJ/cm^2 of UVB. To confirm the apoptotic death of UVB-irradiated melanoma cell line, flow cytometric analysis was performed. Changes were analyzed by a combination of forward light scatter(FSC) and PI staining. UVB irradiated cells were categorized into two populations (Fig. 2). Results showed that the majority of UVB irradiated melanoma cells went into apoptotic status (Fig. 3).

UVB induces activation of caspase-3 and PARP

Changes of caspase-3 and PARP were analyzed and the results are shown in Fig 3. Twenty four hours after irradiation, the level of precursor form of

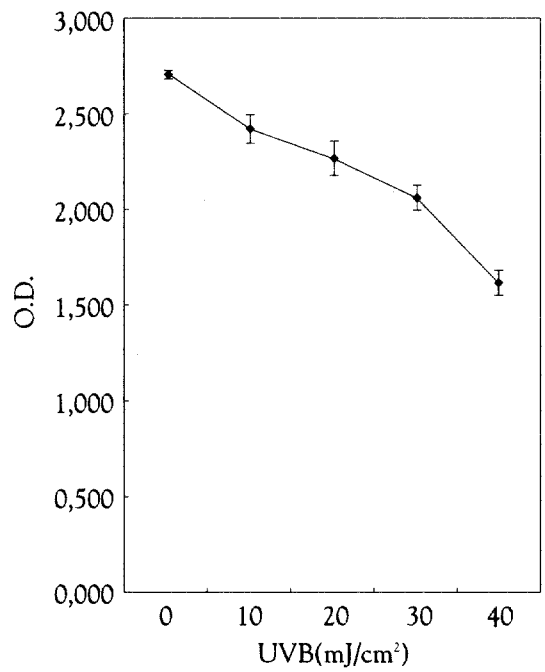

Fig. 1. The effects of UVB irradiation on viability of G361 melanoma cells. G361 cells were irradiated with UVB and cultured for 24 hours (Mean±SD).

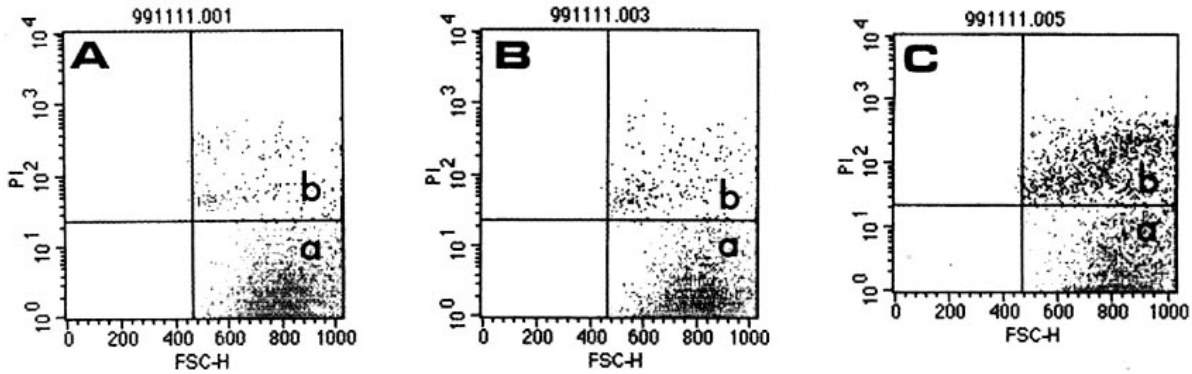

Fig. 2. Flow cytometric analysis of UVB irradiated G361 melanoma cell line. Cell populations were gated into two groups: a(normal cells), b(apoptotic cells). A. sham-irradiated melanoma cells. B. UVB(20mJ/cm^2)-irradiated melanoma cells. C. UVB(40mJ/cm^2)-irradiated melanoma cells.

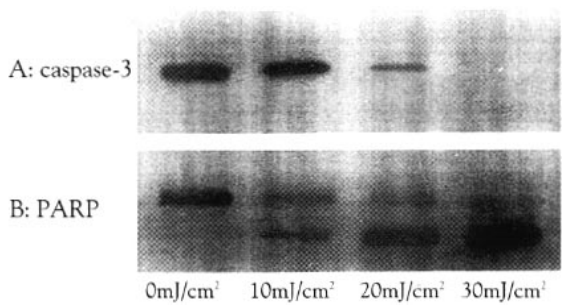

Fig. 3. Western blot analysis of caspase-3 and PARP after UVB irradiation. Subconfluent cultures of G361 cells were irradiated with UVB(0-30 mJ/cm^2). Cells were harvested 16-24 hours after UVB irradiation. Levels of caspase-3 and PARP were analzyed. Proteins recognized by each antibody are indicated on the left hand side (A: caspase-3 B: PARP).

caspase-3 gradually decreased with increasing doses of UVB. Almost all precursor form of caspase-3 disappear with 20 mJ/cm^2 of UVB irradiation and this finding means that almost all precursor form of caspase-3 is activated. Levels of PARP were also analyzed. With increasing doses of UVB, 116 kDa form of PARP gradually decreased and 84 kDa form of PARP gradually increased. These findings suggest that caspase-3 is activated by UVB irradiation and activated caspase-3 finally cleaved PARP.

DISCUSSION

Apoptosis, a process of cell suicide critical for development and tissue homeostasis, is controlled by an evolutionarily conserved program. The uniform morphological features observed in apoptotic cells suggest that a common mechanism may operate to trigger apoptosis. It is reported that a family of cysteine proteases called caspases are involved in apoptotic process.[3] Previously, we reported that apoptosis of normal human melanocytes can be induced by UVB irradiation and the resistance to UVB of in vivo melanocytes may originate from not only their constitutive high expression of Bcl-2 but also the assistance of neighboring environment[4].

In order to investigate the apoptotic pathway of melanoma cell line, UVB was irradiated to the cultured human melanoma cell line and activation of downstream-caspases was examined. As already reported, viability of melanoma cell line decreased according to UVB doses[4]. In addition, flow cytometry analysis clearly showed that majority of irradiated melanoma cell line went into apoptotic status. After these findings were confirmed, levels of caspase-3 were studied after UVB irradiation. Caspase-3 (CPP32-like protease) is a member of the family of cysteine proteases which includes interleukin-1-beta-converting enzyme (ICE) and C. *elegans* protein, Ced-3[5]. An apoptotic signal such as granzyme B of cytotoxic T-cells induces the intracellular cleavage of caspase-3 from the inactive proform (32 kDa) to the active form[6]. Our experiments showed that proform of caspase-3 disappeared with increasing doses of UVB. This finding showed that UVB irradiation activate caspase-3[7]. The active form of caspase-3 cleaves several other apoptotic proteins such as PARP. PARP is a 116

kDa nuclear chromatin-associated enzyme that catalyzes the transfer of ADP-ribose units from NAD^+ to a variety of nuclear proteins including topoisomerases, histones, and PARP itself. PARP is also a target of the caspase protease activity associated with apoptosis[8]. During apoptosis, PARP is cleaved from 116 kDa intact form into 85kDa and 25 kDa fragments[8]. In our experiment, we observed fragmentation of PARP according to increasing doses of UVB. Therefore, UVB-induced apoptotic death of melanoma cell line may be induced by activation of PARP mediated by activation of caspase-3.

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