Ultraviolet B-Induced Apoptosis of Normal Human Melanocytes and G361 Cells

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Background **: Ultraviolet(UV) light is one of the injurious environmental agents which is known to lead to apoptosis of cells. However, studies on UVB-induced apoptosis of melanocytes are still lacking and there are some discrepancies between researchers.**

Objective **: Our purpose was to evaluate the characteristics of UVB-induced apoptosis of melanocytes and G361 cells.**

Methods **: Cultured normal human melanocytes and malignant melanoma cell lines (G361 cells) were analyzed by several detection methods including morphological examination of propidium iodide(PI) stained cells under fluorescence microscopy, quantitation of fragmented DNA, and flow cytometric analysis.**

Results **: Both melanocytes and G361 cells showed similar rate of apoptosis with gradual increment of UVB doses by the quantitation of fragmented DNA. However, flow cytometric analysis using scatter properties and PI stainability revealed that the melanocytes were more resistant to UVB than G361 cells.**

Conclusion **: We suggest that melanocytes seem to be more resistant to UVB-induced injury than G361 cells. In addition, various methods for the detection of apoptosis might be necessary for its study. (Ann Dermatol 10:(3) 147~152, 1998).**

Key Words : Melanocyte, G361 cell, Ultraviolet B, Apoptosis

The term 'apoptosis' was introduced in 1972 by Kerr et al. to describe a distinct form of cell death which is morphologically different from necrosis[1]. Studies of apoptosis have mainly focused on thymocytes/lymphocytes or haematopoietic cells. Apoptosis in these cells has been shown to be induced by a variety of agents including UV irradiation[2].

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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[3]. Although apoptosis has been recognized as an important control mechanism in the maintenance of tissue homeostasis and in the elimination of cells with damaged DNA, information on the induction and characteristics of apoptosis in melanocytes are rather scarce. Zhai et al[4]. reported that physiological doses of UV irradiation induced characteristic apoptotic death in melanocytes. The UVB doses of 5 to 25 mJ/cm^2 are proposed to be the physiological range of UVB expected to reach cells in irradiated human skin[5]. On the contrary, it was reported that melanocytes have innate resistance to the in-

duction of apoptosis by various injuries including UV radiation and a high level of expression of bcl-2 in melanocytes is a major contributor to their resistance[6]. This discrepancy may partially depend on the differences in the melanocyte culture conditions and detection methods of apoptosis.

Although increasing evidence suggests that apoptosis can be induced in melanocytes by UVB irradiation[4], the mechanisms and characteristics of apoptosis in melanocytes are still unknown. The present study was performed to investigate the characteristics of UVB-induced apoptosis of cultured melanocytes and G361 melanoma cell lines.

MATERIALS AND METHODS

Cell Culture

Human epidermal melanocytes were isolated from adolescent foreskin according to the method of Eisinger and Marko[7]. Briefly, the skin was processed by trimming off the subcutaneous fat and placing the tissue in 0.25% trypsin solution overnight at 4℃. The epidermis was separated from the underlying dermis and both tissues were transferred to a centrifuge tube containing growth medium and vortexed vigorously. The resulting cell suspension was placed in a 25cm^2 tissue culture flask. Stock cultures of epidermal melanocytes were maintained in a growth medium previously described by Medrano and Nordlund[8], which consisted of the following : MCDB 153(Sigma, St Louis, MO), 5% FBS(Hyclone, Logan, Utah), 13ng/ml bovine pituitary extract(BPE)(Gibco BRL, Gaithersburg, MD), 8nM 12-O-tetradecanoylphorbol 13-acetate(TPA)(Sigma), 5μg/ml insulin(Sigma), 1μg/ml transferrin(Sigma), 1μg/ml tocopherol(Sigma), 0.6ng/ml human recombinant basic fibroblast growth factor(Gibco BRL), and 1% penicillin-streptomycin(10,000 U/ml and 10,000μg/ml, respectively) (Gibco BRL). The cells were maintained in a humidified incubator with 5% CO_2 at 37℃.

Second-passage melanocytes were used in the experiments. The cells were liberated from culture dishes with brief treatment with trypsin-EDTA (0.025-0.01%) and subcultured at 4-5 × 10^4 cells per 35mm culture dishes. The experiments were performed after 48 hrs of subculture.

Lightly pigmented melanoma cell lines, G361[9](ATCC, Rockville, MD, USA) were grown in RPMI supplemented with 5% FBS and 1% penicillin-streptomycin(10,000 U/ml and 10,000 μg/ml, respectively) with 5% CO_2 at 37℃.

UVB Irradiation

The source of UVB was Waldmann UV-800 (Waldmann Co., VS-Schwenningen, Germany), which used a fluorescent lamp (Philips type TL 20W/12) that emitted 2.5mW/cm^2 of UV light between 285 and 350nm (peak 310-315nm). The energy was measured with a Waldmann UV meter (model No. 585100; Waldmann Co.). The medium was replaced by 1ml of phosphate-buffered saline(PBS), and cells were exposed to UVB (0-100mJ/cm^2) light. Sham-irradiated control cultures were handled identically. Immediately after irradiation cells were placed in the same medium.

Nuclear Staining

Propidium iodide (PI, 40μg/ml, Sigma) was added to cultures 24 hrs after irradiation, and incubated for 10 min at room temperature. Then, the culture media were discarded and adherent cells were washed with PBS. The morphology of the nuclei were examined under a fluorescent microscope. The cells with fragmented nuclei were regarded as apoptotic.

Quantitation of Fragmented DNA[10]

The tritiated thymidine (5μCi/ml) was added 24 hrs before irradiation. Before UV irradiation, the unincorporated isotope was gently washed out with PBS. After irradiation, cells were cultured for 24hrs. Cells were detached with a cell scraper and dispersed by repetitive pipetting. The cells were spun down and the culture supernatant was transferred to another tube(A). One ml of cell lysis buffer (10mM Tris, 1mM EDTA, 0.2% Triton X-100) was added. After vigorous vortexing, the cell debris was pelleted by centrifuge for 10 min, at 13,000rpm, at 4℃. The supernatant was carefully transferred to the other tube(B). The pellet of tube(C) was solubilized with 0.5ml sample buffer(1% sodium dodecyl sulfate, 10mM Tris, 1mM EDTA) and sonicated for 10 sec. The radioactivity in each fraction was measured and the percentage of fragmented DNA was calculated as follows.

per cent fragmented DNA=(A+B)/(A+B+C) × 100

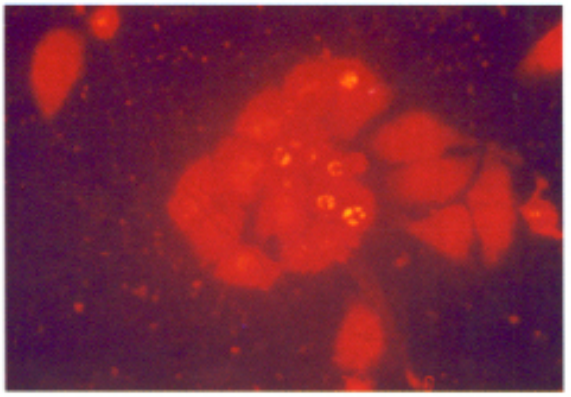

Fig. 1. PI-stained, fragmented nuclei are shown in some G361 cells which were irradiated with 30 mJ/cm^2 UVB.(×400, fluorescence microscopy).

Flow Cytometric Analysis[11]

Twenty-four hours after irradiation, the culture supernatants, which contained floating dying cells and apoptotic cells, were collected. Then, adherent cells were trypsinized briefly. Two fractions were put together and washed with PBS. PI (40μg/ml) was added to the cell suspension and incubated for 10 min at room temperature. Flow cytometry was performed using the FACStar apparatus (Becton-Dickinson, San Jose, CA). Changes in light scatter properties were analyzed by a combination of forward light scatter(FSC) and right angle scatter(SSC). We also performed flow cytometric measurements on the PI-stained cells.

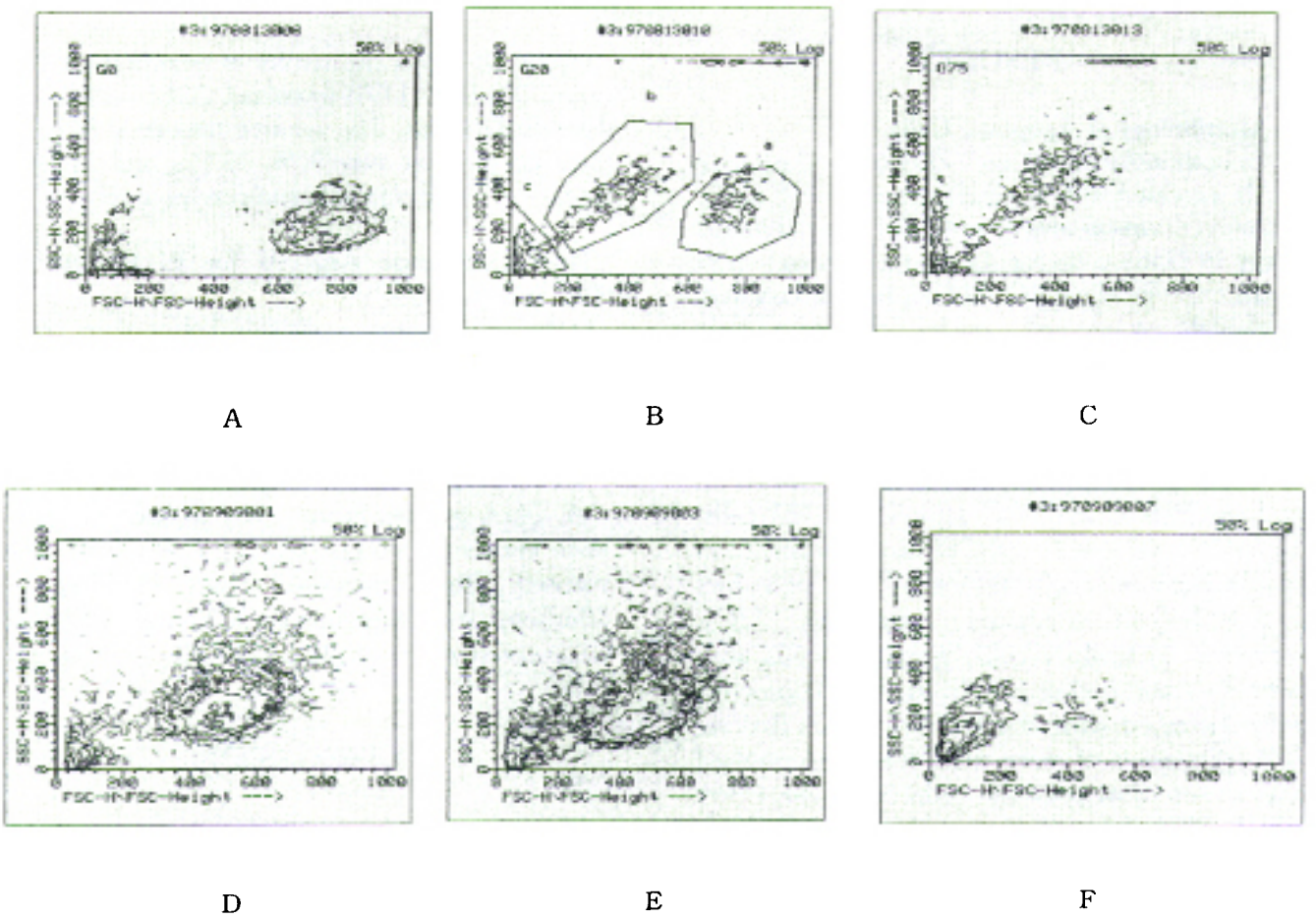

Fig. 2. Flow cytometric analysis of UVB irradiated melanocytes and G361 cells. A. Sham-irradiated G361 cells. B. UVB(20mJ/cm^2)-irradiated G361 cells. Cell populations were gated into A(normal cells), B(apoptotic cells), and C(necrotic cells or cell debris). C. 75mJ/cm^2 UVB-irradiated G361 cells. D. Sham-irradiated melanocytes. E. 20mJ/cm^2 UVB-irradiated melanocytes. F. 75mJ/cm^2 UVB-irradiated melanocytes.

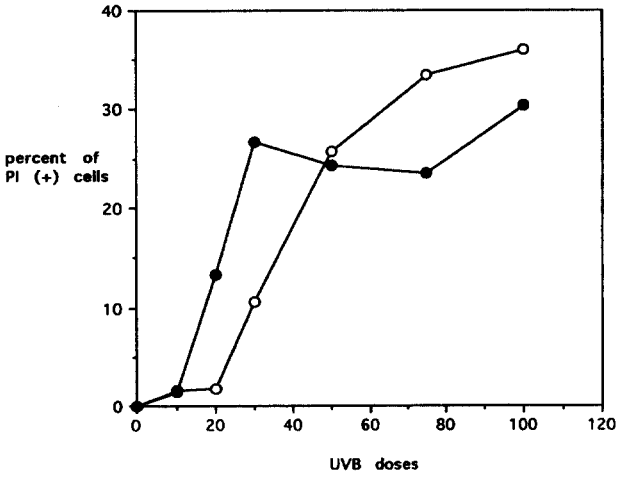

Fig. 3. Flow cytometric analysis of PI stained cells. The open and closed circles designate melanocytes and G361 cells, respectively. Percents of PI stained cells increase according to UV doses.

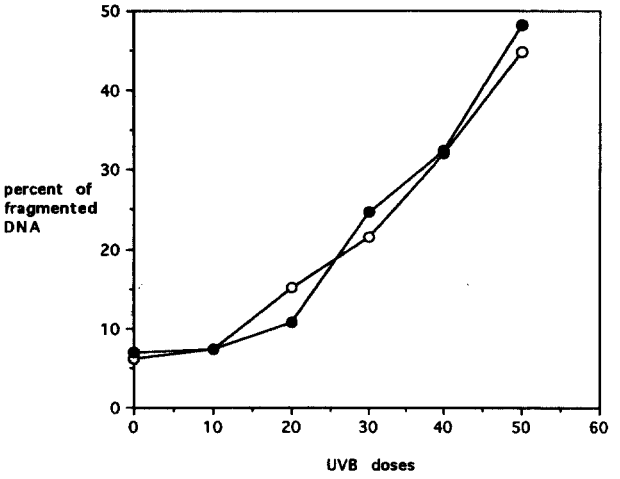

Fig. 4. Quantitation of fragmented DNA. Percents of fragmented DNA increase according to UV doses. The open and closed circles designate melanocytes and G361 cells, respectively.

RESULTS

Morphology of Apoptotic Cells

In both melanocytes and G361 cells, PI-positive cells increased with the increments of UVB doses. However, typical apoptotic nuclei were observed only in G361 cells(Fig. 1). In melanocytes, cells with fragmented nuclei could not be demonstrated, although condensed nuclei, homogeneously stained with PI, were seen with more than 20mJ/cm^2 irradiation of UVB.

Flow Cytometric Aanalysis (Fig. 2)

Three populations can be easily distinguished on the basis of FSC and SSC signals in G361 cells. The healthy cells with high FSC and low SSC(a in Fig.2B) became nearly absent in the high dose UVB(>20mJ/cm^2)-irradiated group(Fig. 2C). Compared to healthy cells, apoptotic cells with lower FSC and higher SSC(b in Fig.2B) progressively increased with the increase of UVB dose. UVB irradiation of more than 30mJ/cm^2 resulted in apoptosis of most of the cells. However, G361 cells hardly changed into necrotic cells(c in Fig.2B) which are characterized by low FSC and low SSC. Contrary to G361 cells, most of the melanocytes remained healthy at the dose of 20mJ/cm2(Fig. 2E). Above 30mJ/cm^2, many melanocytes abruptly showed the scattering features of necrotic cells or cell debris(Fig. 2F).

Figure 2B shows the results of quantitation by gating 20mJ/cm^2 UVB-irradiated G361 cells into three populations. The relative frequency of the three populations were 30%, 35.5%, and 34.5% for each of the groups a, b, and c, respectively.

Flow Cytometric Analysis for PI Positivity (Fig. 3)

G361 cells stained positively better than the melanocytes at a low dose of UVB (20-30mJ/cm^2). However both cells stained well with higher doses of UVB (>50mJ/cm^2).

DNA Fragmentation (Fig. 4)

A DNA fragmentation assay showed no difference between melanocytes and G361 cells. Fragmented DNA increased from the dose of 20mJ/cm^2 of UVB and about 50% of the DNA was fragmented at the UVB dose of 50mJ/cm^2 in both cells.

DISCUSSION

UVB irradiation induced DNA damage in melanocytes and G361 cells and they were detected by a DNA fragmentation assay. The amount of fragmented DNA also increased according to the increasing dose of UVB. Furthermore, the membrane-permeabilized melanocytes and G361 cells, positively stained with PI, gradually increased

with increasing doses of UVB. These results suggest that UVB irradiation induced features of apoptosis in melanocytes and G361 cells. However, our data showed discrepancies between melanocytes and G361 cells, when both cells were irradiated with UVB.

There are numerous methods for the detection of apoptosis and they have various sensitivity and specificity in detecting apoptosis[12].

In cultured cells, apoptosis can be readily detected by fluorescence microscopic examination after staining with a DNA binding dye like PI. Hallmark changes of apoptosis are chromatin condensation and nuclear fragmentation, and these changes are readily visible by microscopic examination[13]. We could demonstrate apoptotic changes in G361 cells, but not in melanocytes.

Flow cytometry, a method used increasingly for studying cells undergoing apoptosis, seems to be one of the most popular methodologies to analyze apoptosis. Apoptosis is accompanied by water loss, cell shrinkage, and nuclear fragmentation, whereas necrosis is not. Changes in the morphology of cells affect their light scattering properties[11,14]. Apoptotic cells give lower forward scatter and higher side scatter values than viable cells, reflecting the smaller size and different nucleus/cytoplasm consistency[12]. Cell death by necrosis results in an immediate decrease of the forward and right angle scatter signals[14]. Flow cytometric analysis of UVB-irradiated G361 cells showed cell fractions with lower FSC and higher SSC, which are characteristic features of apoptotic cells. However, we were not able to observe an apoptotic population in UVB-irradiated melanocytes by scattering analysis of flow cytometry.

It is known that discrimination between apoptotic cells and debris is difficult, because apoptosis results in the complete fragmentation of the nucleus and cytoplasm, ultimately producing cell debris[11]. Although the scattering discrimination between apoptotic and non-apoptotic cells is dependent on the type of cell studied[11], UVB irradiation on melanocytes demonstrated no definite apoptotic population showing lower FSC and higher SSC. Healthy cells with high FSC and low SSC directly entered into the ranges of debris or necrotic cells with low FSC and low SSC. This population may have included both necrotic and apoptotic cells.

In our experiment, we could not demonstrate any specific evidence of apoptosis in UVB-irradiated melanocytes by morphological examination of PI-stained nuclei and flow cytometry based on scattering properties.

PI positivity is not specific for apoptosis but related to membrane permeability for PI staining. Flow cytometric analysis for PI positivity, another useful method to study apoptosis, showed that G361 cells were better stained than melanocytes at low doses of UVB. In other words, melanocytes still maintained intact membranes at low doses of UVB. These results are well correlated with those of the flow cytometric analysis of light scattering properties and examination of PI-stained cells under fluorescence microscopy. Overall, these results suggest that melanocytes are more resistant to UVB irradiation than G361 cells.

In order to quantify the amount of fragmented DNA, a DNA fragmentation assay was performed. Interestingly, the DNA fragmentation assay showed a similar rate of fragmentation in both cells. It can be said that these differences can be explained by basic principles of this assay. It has an inherent limitation that small DNA fragments resulting from UV irradiation or necrosis cannot be differentiated from those by apoptosis. The DNA fragmentation assay may be one of the most sensitive methods to study apoptosis but is not always specific for analysis of apoptosis[13].

Accordingly, to confirm the UVB-induced apoptosis of melanocytes, alternative techniques with high specificity are needed, for example, a DNA ladder assay, which was demonstrated by one group with combination of UVB irradiation and serum deprivation[4].

In conclusion, melanocytes seemed to be more resistant to apoptotic injury than G361 cells and various methods for the detection of apoptosis need to be performed for the analysis of apoptosis in experimental conditions.

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