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PI3-kinase/Akt Pathway Mediates Expression of p53 after UVB Irradiation

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The cells having unrepaired DNA damage after ultraviolet (UV) exposure must go to apoptotic cell death to avoid mutation and transformation leading to cancer, whereas they must be protected against excess apoptotic cell death. The phosphatidylinositol 3-kinase (PI3-kinase)/Akt is a well-known survival pathway which suppresses apoptosis. The p53 tumor suppressor is a universal sensor of genotoxic stress that also regulates apoptosis. Until now, there are many reports of p53 expression after UV irradiation; however, the detailed time-course study has not been performed, especially the relationship with PI3-kinase/Akt pathway. In this study, PI3-kinase/Akt pathway-mediated time-dependent expressions of p53 and its related molecules after UVB irradiation were examined involving induction of apoptosis. Inhibition of PI3-kinase/Akt pathway by wortmannin and siRNA for Akt1 augmented apoptosis induced by UVB irradiation. p53 was time-dependently phosphorylated and stabilized after UVB irradiation, consistent with the expression of p21. Inhibition of PI3-kinase/Akt pathway clearly suppressed the expressions of p53 and p21. On the other hand, the expression levels of p53 in the presence of proteasome inhibitor were same, whether PI3-kinase/Akt pathway was inhibited or not, indicating that the activation of PI3-kinase/Akt pathway after UVB exposure suppressed proteasome-dependent degradation of p53. These results suggested that PI3-kinase/Akt pathway has important roles to mediate p53 expression and induction of apoptosis after UVB irradiation although the p53 revealed no positive correlation with the induction of apoptosis.

Key words: Akt, p53, UVB, apoptosis

Introduction

Ultraviolet (UV) radiation from the sun is among ubiquitous damaging environmental factors from which human skin must protect itself. The UV spectrum can be divided into three based on wavelength-UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm). As UVC could not reach to surface of the earth due to the effective absorbance in the stratospheric ozone layer, UVB directly absorbed by DNA mainly accounts for damaging biological effect of UV irradiation leading to

skin cancer.

UVB-induced DNA damage is usually repaired through nucleotide excision repair (NER) system; however, with increasing the damage, NER will ultimately fail and replication of damaged DNA leads to an error-prone incorporation of nucleotides at the site of a photolesion, increasing the risk of malignant transformation. To avoid this process, cells possess a fail-safe mechanism. This is the formation of sunburn cells, which is undergoing apoptosis in skin keratinocytes. This means that dysregulation of the apoptosis is a factor of development of skin cancer (1).

In apoptosis, p53 tumor suppressor is considered to be a key protein (2). p53 mutation and deletion attenuate apoptosis of abnormal cells and permit undesirable cell proliferation, leading to cancer. The critical role of p53 in the prevention of cancer development is demonstrated by p53 mutation in approximately 50% of human cancer cases. The action of p53 in the case of UV-exposure has been well examined. In response to UVB radiation, p53 is phosphorylated by MAP kinases, ERKs and p38 (3). Ziegler *et al.* (4) showed a reduction of sunburn cells in the skin of *p53*^{-/-} mice, indicating that the process whereby precancerous keratinocytes are eliminated may not work well in cells that harbor p53 inactivated by mutations. This was followed with the report by Jiang *et al.* (5) that UVB-induced skin tumors develop more in *p53*^{-/-} and *p53*^{+/-} mice than in the wild type mice. Activation of p53 by polyphenolic flavonoid enhanced UVB-induced apoptosis (6). Although some studies supported a contribution of p53 to the promotion of UV-induced apoptosis as described above, several studies have claimed that UV-induced apoptosis is not necessarily mediated by p53. Knockdown of p53 and inhibition of the activity enhanced UV-inducing apoptosis (7–9). Ikehata *et al.* (10) showed that p53 protected UVB-induced genotoxicity by facilitating NER

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and that its contribution to the UVB-induced apoptosis was limited. At present, the contribution of p53 to apoptosis induction in the response to UV-induced damage is controversial.

Several signal pathways activated by UV irradiation have been reported, in which phosphatidylinositol 3-kinase (PI3-kinase)/Akt is a noted survival pathway (11,12). Exposure to UVB induces clustering and internalization of cell surface receptors like epidermal growth factor (EGF) receptor (13), resulting in activation of the enzyme PI3-kinase leading to the formation of 3-phosphorylated phosphatidylinositols, which in turn activate the serine/threonine kinase Akt/PKB (11). Akt promotes cell survival by phosphorylating the Bcl-2 family member Bad (14) and activation of mitochondrial Raf-1 (15). Many studies have reported that UVB-induced apoptosis could be suppressed by treatment with insulin-like growth factor (IGF), demonstrating that the PI3-kinase/Akt pathway was essential for protecting cells against UV-induced apoptosis (16–19). We have previously shown that UVB irradiation inhibited apoptotic cell death induced by cell detachment and serum starvation (anoikis), which was mediated by activation of PI3-kinase/Akt pathway (20). On the other hand, the relationship between activation of the PI3-kinase/Akt pathway and p53 expression after UV irradiation has not been well investigated. A few reports have shown that the activation of the PI3-kinase/Akt pathway by exposure to UVB was observed in keratinocytes both *in vivo* and *in vitro*, which might erase the excess production of sunburn cells in the skin, indicating an enhancement of the survival of mutated cells leading to the promotion of skin cancer (11,12). If p53 contributes to induction of sunburn cells after UVB irradiation, PI3-kinase/Akt pathway might mediate the expression of p53 by UVB irradiation. Boehme *et al.* (21) reported that Akt stabilized p53 in response to DNA damage by ionizing radiation. However, time-dependent Akt-mediated changes of expression of p53 after UV irradiation have not yet been clarified.

In this study, time-dependent p53 expression after UVB irradiation and contribution of PI3-kinase/Akt pathway were examined involving induction of apoptosis. PI3-kinase inhibitors, wortmannin and LY294002, and knockdown method using siRNA for Akt1 were applied for inhibition of PI3-kinase/Akt pathway.

Materials and Methods

Cell culture and siRNA transfection: NIH3T3 cells were used for experiments because of its reproducibility of knockdown efficiency for Akt1. They were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum (CS) at 37°C in a humidified atmosphere containing 5% CO₂ in air. For the siRNA experiments, the cells were seeded at 3×10^6

cells/100 mm tissue culture dish. Control non-targeting siRNA (control siRNA) was purchased from Dharmacon, Inc. (Chicago, IL.). Akt1 siRNA was synthesized using the sequence which showed the most effective gene silencing of the four Akt1 siRNA which were in the duplex oligonucleotide cocktail purchased from Dharmacon, Inc. (20). After 24 h of culture, the cells were transfected with 1.2 nmol of annealed siRNA per 100 mm dish using Lipofectamine 2000 (Invitrogen, Co., Carlsbad, CA) as recommended by the manufacturer. Following further culture for 48 h, the cells were plated in 24 well plates or 35 mm dish for experiments and cultured for 24 h.

UVB irradiation: After culture for 3 h in serum-free DMEM, cells were irradiated with UVB. The wavelength characteristics of the UVB lamp (HP-30LM; Atto, Co., Tokyo, Japan) with a 280–320 nm emission and a max peak of 312 nm were as described in the previous paper (22). The approximate irradiance of UVB at the sample level was 0.8 mW/cm². The duration of UVB irradiation to obtain a dose of 0.1 J/cm² was about 2.5 min.

Viability assay: UVB-irradiated cells were cultured for 48 h and trypsinized to obtain single cell suspension. They were incubated in DMEM containing fluorescein diacetate (FDA) (0.1 µg/ml) for 10 min at 37°C, and viability was determined using a flowcytometer (FCM) (Epics XL; Beckman Coulter, Inc., Hialeah, FL).

Determination of apoptotic cells: Apoptosis was examined by detecting chromatin-condensed cells. UVB-irradiated cells were stained with the DNA-binding fluorochrome Hoechst33342 as described previously (22). Briefly, trypsinized cells were fixed in 2% glutaraldehyde for 4 h, and then suspended in PBS containing 1 mM Hoechst33342. Following incubation for 10 min at room temperature, chromatin-condensed cells were scored microscopically.

Western blotting: Cells were lysed in lysis sample buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM PMSF). When cell fractionation was needed, cells were lysed in buffer (10 mM Hepes-KOH, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, and 1 mM PMSF) and centrifuged at 12000 rpm for 5 min (supernatant: cytoplasm fraction). The precipitation was lysed in lysis sample buffer (nucleus fraction). The lysates were separated by SDS-PAGE followed by electroblotting onto PVDF membranes (Immobilon-P; Millipore Co., Bedford, MA). After the blocking of nonspecific binding with 1% skim milk, the membranes were incubated with primary antibody followed by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Lab., Inc., West Grove, PA). The primary antibodies against p53 (Oncogene Research Product), p21, Bcl-2, Bax, Actin (Santa Cruz Biotech. Inc., Santa

Cruz, CA) and Akt1 (Sigma-Aldrich Co., St. Louis, MO) were diluted 1:1000 to 1:8000 for use and secondary antibodies were diluted 1:2500. Signals were detected with the ECL chemiluminescence detection system (GE Healthcare Bio-Sciences, Co., Piscataway, NJ). Brightness of protein bands was calculated using NIH-image (version 1.61).

Statistics: All experiments were repeated two or three times. ANOVA was used to test the significance between groups ($n=3\sim5$). P values <0.05 were considered statistically significant.

Results

Akt1 knockdown enhances UVB-induced cell death: Figure 1A shows Akt1 expression 48 h after transfection of siRNA. The expression decreased below 30% of control. UVB exposure induced dose-dependent cell death, which was enhanced by the Akt1 knockdown (Fig. 1B). The enhanced cell death was apoptosis because typical apoptotic marker, chromatin condensation increased in Akt1-knockdown cells (Fig. 1C). Treatment with PI3-kinase inhibitor, wortmannin also increased the chromatin condensation after UVB irradiation (Fig. 1D).

Inhibition of PI3-kinase/Akt pathway suppresses expressions of p53 and p21 after UVB exposure:

Figure 2A shows the expression changes of p53, p21 and Bcl-2 family proteins after UVB exposure. After UVB exposure (0.05 J/cm^2), expression of p53 and phosphorylated p53 (upper bands) increased up to 8 h, then gradually decreased to 24 h. The pattern was different from the case of 0.1 J/cm^2 of UVB exposure, in which p53 expression continuously increased up to 24 h. The expression patterns determined using image analyzer are shown in Fig. 3A. Akt1 knockdown attenuated the increase of p53 expression. PI3-kinase inhibitors, wortmannin and LY294002, also suppressed the increase of p53 expression (Figs. 2B and C). Behavior of p21 was consistent with p53 (Figs. 2 and 3B). On the other hand, the expressions of Bcl-2 family, Bcl-2 and Bax were not much changed by UVB irradiation comparing with p53 and p21. Slight decrease of Bcl-2 was observed 16 to 24 h after UVB irradiation only in the condition of Akt1 knockdown.

Localization of p53 and its stabilization via PI3-kinase/Akt pathway: To clarify where the accumulation of p53 was caused and attenuated by inhibition of PI3-kinase/Akt pathway, the cells irradiated with UVB

Fig. 1 Akt1 knockdown and apoptosis induction after UVB exposure. (A) Nontargeting siRNA and Akt1 siRNA (1.2 nmol) were transfected into NIH3T3 cells (3×10^6 cells/100 mm dish) and cultured for 48 h. Akt1 protein expression was analyzed by western blotting. (B) Survival 48 h after UVB exposure was determined by FDA assay. Akt1-knockdown cells (C) or cells treated with 100 nM wortmannin for 1 h (D) were irradiated with UVB and further cultured for 48 h. The cells were stained by Hoechst33342 and chromatin-condensed cells were scored microscopically. Values are means $\pm$ SD. * $P < 0.05$.

Fig. 2 Expression changes of p53, p21 and Bcl-2 family proteins after UVB exposure. Akt1-knocked down cells (A) and the cells treated with 100 nM wortmannin (B) and 10 μ M LY294002 (C) for 1 h were irradiated with UVB (0.05 and 0.1 J/cm²) and cultured for indicated time. They were harvested, and subjected to western blotting with anti-p53, anti-p21, anti-Bcl-2, anti-Bax and anti-Actin antibodies.

were fractionated to cytoplasm and nucleus. Nuclear import of p53 is essential for its normal function (23). p53 was accumulated in nucleus, not in cytoplasm (Fig. 4). In the case of Akt1 knockdown and treatment with

inhibitors of PI3-kinase, p53 in nucleus decreased similar to in whole cells (Fig. 2), which was not due to the translocation to cytoplasm.

As the localization was not changed by Akt1 knock-

Fig. 3 Expression changes of p53 and p21 after UVB exposure. The bands of p53 (A) and p21 (B) in Fig. 2A were determined by NIH image (ver.1.61).

Fig. 4 Expression of p53 in subcellular fractions prepared from UVB irradiated cells. Akt1-knocked down cells (A) and the cells treated with 100 nM wortmannin (B) and 10 μ M LY294002 (C) for 1 h were irradiated with UVB (0.05 and 0.1 J/cm²) and cultured for indicated time. They were fractionated into nucleus and cytoplasmic fractions and subjected to western blotting with anti-p53 antibody.

down and treatment with PI3-kinase inhibitors, the attenuation of p53 was considered to be due to proteolysis. Proteasome inhibitor, MG132 was added to the cell culture immediately after UVB irradiation and time-dependent expression of p53 was examined (Fig. 5). In the presence of MG132, p53 expression enhanced by UVB exposure was not degraded. Furthermore, attenuation of p53 by Akt1 knockdown and inhibitors of PI3-kinase as shown in Fig. 2 also were not observed, suggesting that PI3-kinase/Akt would stabilize p53 after UVB irradiation via inhibition of proteolysis.

Discussion

Many researchers have been studied p53 phosphorylation, stabilization and degradation after UV irradiation. However, the detailed time-dependent change has not been reported. In this study, we demonstrated time-dependent expressions of p53 and the related molecules, and contribution of PI3-kinase/Akt survival pathway involving induction of apoptosis.

UVB irradiation phosphorylated and stabilized p53 in a time-dependent manner (Fig. 2). The pattern was consistent with expression of p21. The patterns of induction of p53 and p21 were different dependent on UVB doses. In 0.05 J/cm² of UVB, p53 increased up to 8 h and

Fig. 5 Effect of proteasome inhibitor on the change of p53 expression after UVB irradiation. Akt1-knocked down cells (A) and the cells treated with 100 nM wortmannin or 10 μ M LY294002 (B) for 1 h were irradiated with UVB (0.05 J/cm²) and cultured for indicated time in the presence of 10 μ M of MG132. The bands of p53 in Akt1-knocked down cells (A) were determined by NIH image (ver.1.61).

decreased. p21 increased up to 8 h and kept the expression. In 0.1 J/cm² of UVB, both p53 and p21 continuously increased up to 24 h. The exact reason of the different patterns was not clarified; however, unrepaired DNA damages by exposure to excess doses of UVB might contribute to the continuous expression of these proteins.

The expressions of p53 and p21 after UVB irradiation were clearly attenuated by inhibitors of PI3-kinase and Akt1 knockdown. PI3-kinase/Akt is reported to facilitate the stabilization of p53 by reducing the interaction between negative regulator of p53, Mdm2 and p53 (24). In response to ionizing radiation, activation of Akt was resulted in hypophosphorylation of Mdm2 and incapability of mediating degradation of p53 (21). From our data that p53 was expressed at the same level in the presence of proteasome inhibitor even if Akt was knocked down (Fig. 5), PI3-kinase/Akt pathway would mediate proteasome-dependent degradation of p53 after UVB exposure.

Growth factors, IGF-1 and EGF, are well known to activate PI3-kinase/Akt pathway and suppress UV-induced cell death (15–17). Murray (25) showed that IGF-1 enhanced p21 expression, which inhibited UV-induced cell death. Downstream of p53, p21 is expressed and participates in DNA repair and apoptotic process (26). Suppression of p21 expression using PI3-kinase in-

hibitors and Akt1 siRNA after UVB irradiation might contribute to the enhanced formation of apoptotic cells.

Although there are some reports that p53 induction after UV irradiation enhanced apoptosis (4,5), the positive correlation of p53 and induction of apoptosis was not observed in this study. On the other hand, it was clear that PI3-kinase/Akt pathway mediated UVB-induced apoptosis. Ogawa *et al.* reported that the p53 family member p51/p63 inhibits UVB-induced apoptosis via PI3-kinase/Akt pathway (27). Furthermore, PI3-kinase/Akt pathway can have multiple pro-survival effects, one of which is suppression of mitochondrial-mediated apoptosis via phosphorylation and translocation of Bcl-2 family molecules (28). In this study, the expressions of pro- and anti-apoptotic Bcl-2 family were not so much changed after UVB irradiation. In addition, the expressions were kept even if PI3-kinase inhibitors and siRNA for Akt1 were treated. We checked it until 24 h after irradiation, but culture for longer time might be needed to observe the change. Bcl-2 family proteins were reported to be degraded 48 to 96 h after UV irradiation (29). Furthermore, only amounts of expressions of Bcl-2 family proteins were examined in this study, but phosphorylation and distribution on mitochondria membrane might be changed after UVB irradiation. UV irradiation translocated Bax on mitochondria membrane and initiated apoptosis (18,30,31).

In conclusion, the PI3-kinase/Akt pathway has important roles in apoptosis, and phosphorylation and stabilization of p53 after UVB irradiation. However, the pathway-mediated induction of apoptosis and change of expression of p53 did not have a positive correlation. Attenuation of UVB-induced apoptosis via activation of the pathway would have a role to suppress the formation of sunburn cells, leading to survival of mutated and transformed cells in skin. To understand the mechanism of UV-induced skin cancer, the pathway-mediated cellular responses would be required to examine in more detail.

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