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World J Gastroenterol 2007 February 7; 13(5): 692-698
World Journal of Gastroenterology ISSN 1007-9327

COLORECTAL CANCER

Early apoptosis and cell death induced by ATX-S10Na (Ⅱ)-mediated photodynamic therapy are Bax- and p53-dependent in human colon cancer cells

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Received: 2006-10-20 Accepted: 2006-12-20

Abstract

AIM: To investigate the roles of Bax and p53 proteins in photosensitivity of human colon cancer cells by using lysosome-localizing photosensitizer, ATX-S10Na (Ⅱ).

METHODS: HCT116 human colon cancer cells and Bax-null or p53-null isogenic derivatives were irradiated with a diode laser. Early apoptosis and cell death in response to photodynamic therapy were determined by MTT assays, annexin V assays, transmission electron microscopy assays, caspase assays and western blotting.

RESULTS: Induction of early apoptosis and cell death was Bax- and p53-dependent. Bax and p53 were required for caspase-dependent apoptosis. The levels of anti-apoptotic Bcl-2 family proteins, Bcl-2 and Bcl-x_L, were decreased in Bax- and p53-independent manner.

CONCLUSION: Our results indicate that early apoptosis and cell death of human colon cancer cells induced by photodynamic therapy with lysosome-localizing photosensitizer ATX-S10Na (Ⅱ) are mediated by p53-Bax network and low levels of Bcl-2 and Bcl-x_L proteins. Our results might help in formulating new therapeutic approaches in photodynamic therapy.

Key words: Photodynamic therapy; ATX-S10Na (Ⅱ); Apoptosis; Bax; p53

Mitsunaga M, Tsubota A, Nariai K, Namiki Y, Sumi M, Yoshikawa T, Fujise K. Early apoptosis and cell death induced by ATX-S10Na (Ⅱ)-mediated photodynamic therapy are Bax- and p53-dependent in human colon cancer cells. *World J Gastroenterol* 2007; 13(5): 692-698

http://www.wjgnet.com/1007-9327/13/692.asp

INTRODUCTION

Photodynamic therapy (PDT) is a therapeutic procedure involving the use of tissue-penetrating laser light after the administration of tumor-localizing photosensitizers, and is used for the efficient treatment of a variety of solid and superficial cancers[1]. Tumor cell death in response to PDT is induced *via* apoptosis and/or necrosis, and depends on various conditions, such as tumor cell type, intensity of laser irradiation, and subcellular localization and concentration of the photosensitizer[2-4]. Localizing photosensitizers in cytoplasmic organelles generate reactive oxygen species by photochemical reactions, resulting in induction of cell damage[1,5,6]. Cell damage modes and the initial subcellular targets are related to the localization sites of the photosensitizers, specifically, mitochondria and lysosomes[7]. Mitochondria-localizing photosensitizers, such as silicon phthalocyanine (Pc) 4, cause rapid dissipation of the mitochondrial membrane potential and result in the release of cytochrome *c*[8]. In contrast, the hydrophilic chlorine photosensitizer ATX-S10Na (Ⅱ), localizes mainly in lysosomes[9,10] and activates apoptotic pathways *via* mitochondrial destabilization following the photodamage of lysosomes[11]. Lysosomal proteases released by lysosomal photodamage, in turn activate caspases directly and/or indirectly subsequent to mitochondrial damage[12]. Nonetheless, the mechanisms by which lysosome-localizing photosensitizers activate apoptotic pathways are not fully understood.

Members of the p53 tumor suppressor gene family play various roles in response to DNA damage, such as cell cycle regulation, DNA repair, and induction of apoptosis[13,14]. Expression of the wild-type p53 induced by chemotherapy or radiation increases the sensitivity to apoptosis, whereas a mutated or deleted p53 alters the sensitivity[15]. Furthermore, p53 regulates pro-apoptotic Bcl-2 family proteins[16,17], and these proteins localize to mitochondria and heterodimerize through a BH3 domain with anti-apoptotic Bcl-2 family members, such as

Bcl-xL[18,19]. Bax modulates the mitochondrial pathway of apoptosis by allowing the efflux of apoptogenic proteins. The shift in the balance of the pro-apoptotic and anti-apoptotic Bcl-2 family members regulates the translocation of cytochrome *c* from mitochondria to cytosol[20]. Although various studies indicate the involvement of Bax and p53 in PDT-mediated apoptosis[21-24], there are conflicting reports about whether the induction of apoptosis correlates with cell death.

The aim of the present study was to elucidate the roles of Bax and p53 in response to lysosomal photodamage induced by ATX-S10Na (Ⅱ)-PDT, which might contribute to more effective clinical use of PDT in cancer therapy. We used an established human colon cancer cell line, HCT116, which expresses wild-type Bax and p53, and derivative lines of HCT116 that differ from the parental line by virtue of a selective knockout of either Bax or p53[25,26].

MATERIALS AND METHODS

Reagents

ATX-S10Na (Ⅱ), 13, 17-bis (1-carboxypropionyl) carbamoylethyl-8-ethenyl-2-hydroxy-3-hydroxyiminoethylidene-2, 7, 12, 18-tetramethylporphyrin sodium salt was provided by Photochemical Co. (Okayama, Japan), and was dissolved in phosphate-buffered saline (PBS).

Cell cultures

Bax-null or p53-null derivatives of the wild-type HCT116 cell line, generated by targeted homologous recombination to create homozygous deletion, were a generous gift from B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD, USA)[27,28]. Cells were maintained in McCoy's 5A medium (GIBCO-BRL, Bethesda, MD, USA) supplemented with 10% fetal calf serum (Thermo Trace, Melbourne, Australia) and 1% penicillin/streptomycin (GIBCO-BRL) in a 37℃, 5% CO_2, fully humidified incubator and passaged twice weekly.

PDT protocols

Exponentially growing cells were seeded in 96-well microplates or 35-mm dishes to approximately 30% confluence 48 h before PDT. ATX-S10Na (Ⅱ) was added to the culture medium to a final concentration of 20 μg/mL 24 h before PDT. Medium was replaced with fresh medium, and the cells were irradiated with a diode laser (Hamamatsu Photonics, Hamamatsu, Japan) at a wavelength of 670 nm. The energy fluence rate was 0.167 W/m^2 as measured using LaserMate power meter (Coherent, Auburn, CA, USA). Exposure for 5 min resulted in an incident energy fluence of 5 J/cm^2.

MTT assays

Cytotoxicity of PDT was determined by colorimetric assay with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt reagent (Cell Counting Kit-8, Wako Pure Chemical Industries, Osaka, Japan). Following PDT, cells were incubated in 96-well microplates for 24 h. Ten microliters of Cell Counting Kit-8 reagent[27] were added to each well, and cells were incubated at 37℃ for 4 h. After thorough mixing, the absorbance of each well was measured at 450 nm with an Ultramark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Annexin V assays

Cells were collected by trypsinization and washed with ice-cold PBS, suspended in 500 μL annexin V binding buffer containing 5 μL of propidium iodide (PI) and 5 μL of annexin V-FITC (BioVision, Mountain View, CA, USA), and incubated for 15 min at room temperature in the dark. Fluorescence was measured on a BD LSR flow cytometer (Becton Dickinson, NY, USA) and processed with Cell Quest software (Becton Dickinson) for analysis.

Cell cycle analysis

Cells were collected and washed in cold PBS, fixed in 70% ethanol pre-chilled at -20℃, washed, resuspended in 25 μg/mL of PI with 100 μg/mL RNase A, and incubated for 30 min at 37℃. Fluorescence was measured on a BD LSR flow cytometer. Data were analyzed using the MODFIT 2.0 program (Verity Software).

Transmission electron microscopy assays

Ultrastructural appearances of apoptotic cells were confirmed by electron microscopy. Following PDT, cells were prefixed with 2% glutaraldehyde, post-fixed with 1% osmic acid, dehydrated in graded ethanol, embedded in resin, and cut into sections on an ultramicrotome. The cells were examined by a transmission electron microscope (TEM) (H-7500, Hitachi, Tokyo, Japan).

Quantification of caspases 3, 8 and 9 activity

Activities of caspases 3, 8 and 9 were measured by Caspase Fluorometric Assay kits (R&D Systems Inc., Minneapolis, MN, USA) according to the instructions provided by the manufacturer. Briefly, cells in 35-mm dishes were washed twice with ice-cold PBS and lysed in 100 μL of lysis buffer. Next, 50 μL of cell lysates were transferred to a 96-well plate containing reaction buffer, and were incubated for 2 h at 37℃ with 5 μL of caspase 3-, 8- or 9-specific fluorescent substrate DEVD-AFC, IETD-AFC and LEHD-AFC, respectively. Plates were read with an ARVOsx-2 fluorescence microplate reader (Wallac, Turku, Finland) using an excitation light of 400 nm and an emission light of 505 nm.

Preparation of protein extracts and immunoblotting

Cells in 35-mm dishes were washed twice with ice-cold PBS, lysed in 100 μL of lysis buffer [50 mmol/L HEPES (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 150 mmol/L sodium chloride, 5 mmol/L EDTA with protease inhibitors pepstatin A (2 μg/mL), aprotinin (10 μg/mL), leupeptin (10 μg/mL) and phenylmethylsulfonyl fluoride (100 μg/mL)] and sonicated. The lysates were centrifuged at 10000 × *g* at 4℃ for 10 min. The supernatant was recovered and protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL, USA). Twenty microgram of proteins were loaded on an SDS-polyacrylamide gel. After electrophoresis, proteins were electrotransferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% nonfat milk

in Tris-buffered saline with 0.05% Tween-20 (TBS-T) for 2 h at room temperature, and then probed with primary antibodies for 1 h at room temperature or overnight at 4℃. Horseradish peroxidase (HRP)-labeled secondary antibodies were used for signal detection and blots were visualized with Enhanced Chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and recorded on an X-ray film. For cytosolic fractionation, cells were harvested in digitonin lysis buffer (75 mmol/L NaCl, 1 mmol/L NaH_2PO_4, 8 mmol/L Na_2HPO_4, 250 mmol/L sucrose and 190 μg/mL of digitonin), supplemented with protease inhibitors, and incubated on ice for 5 min. Samples were centrifuged at 10000 × *g* at 4℃ for 30 min and the resulting supernatant was used for Western blotting. Antibodies were as follows: mouse monoclonal p53 (DO-1) (Calbiochem, San Diego, CA, USA), mouse monoclonal Bcl-2 (100) and rabbit polyclonal Bax (N-20), Bcl-x$_{S/L}$ (S-18) and β-tubulin (H-235) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal cytochrome *c* (7H8.2C12) (BD Pharmingen, San Diego, CA, USA), and HRP-labeled secondary anti-mouse and anti-rabbit antibodies (Santa Cruz). The image of the specific protein band on the membrane was scanned, and the intensity of the image was analyzed by an NIH Image J program version 1.31.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were evaluated by *t*-test. *P* values < 0.05 were considered statistically significant.

RESULTS

Bax- and p53-dependent cell death and apoptosis in response to ATX-S10Na (Ⅱ)-PDT

To determine the role of Bax and p53 in ATX-S10Na (Ⅱ)-PDT, we first examined the cells' phototoxicity by MTT assays 24 h after laser irradiation. The extent of ATX-S10Na (Ⅱ) phototoxicity was dependent on the intensity of laser irradiation (Figure 1A) and the concentration of ATX-S10Na (Ⅱ) (data not shown). Wild-type HCT116 cells were significantly more sensitive to ATX-S10Na (Ⅱ)-PDT than Bax-null or p53-null cells at 5 J/cm^2 (Figure 1A). Laser irradiation greater than 10 J/cm^2 resulted in global phototoxic cell death, thereby preventing the accumulation of cells with PDT-mediated regulatory responses. Thus, laser irradiation at 5 J/cm^2 was chosen for further studies. Next, to determine the role of early apoptosis in Bax and p53 dependence of phototoxicity, we examined ATX-S10Na (Ⅱ)-PDT-mediated apoptosis by flow cytometry with annexin V and cell cycle analysis that determines a population of cells with sub-G_1 DNA content 3 or 6 h after laser irradiation. The percentage of early apoptotic, annexin V-positive and PI-negative, cells was significantly reduced in Bax-null or p53-null cells compared with wild-type HCT116 cells (Figures 1B and D). In contrast, the percentage of late apoptotic or necrotic, annexin V-positive and PI-positive, cells was not significantly different (Figure 1C). These results indicate that Bax and p53 play a central role in early apoptosis induced by ATX-S10Na (Ⅱ)-PDT. TEM studies revealed that untreated cells have thickened mitochondria and numerous cytoplasmic vesicles. In contrast, when treated with PDT, the sections contained many apoptotic cells with condensed chromatin, apoptotic bodies in the cytoplasm and cell membrane budding (Figure 1E). The percentage of apoptotic cells was higher in the wild-type than Bax-null or p53-null cells 6 h after laser irradiation.

Roles of Bax and p53 in caspase-dependent ATX-S10Na (Ⅱ)-PDT-induced apoptosis

Since caspases are early effectors for triggering PDT-mediated apoptosis[28,29], we examined the roles of Bax and p53 in caspase activation by ATX-S10Na (Ⅱ)-PDT. Caspase-3 activity increased in intensity in laser irradiation- and time-dependent manners within 24 h following PDT in wild-type HCT116 cells (data not shown). As shown in Figure 2, activities of caspase-3 and -9 apparently increased in wild-type HCT116 cells 6 h after laser irradiation. In contrast, it was significantly inhibited in Bax-null or p53-null cells. These results indicate that the caspase-dependent apoptotic process induced by ATX-S10Na (Ⅱ)-PDT was Bax- and p53-dependent. Activation of caspase-8 was slightly increased compared with caspase-9, indicating that the mitochondrial pathway of apoptosis is the major process in response to ATX-S10Na (Ⅱ)-PDT. In Bax-null HCT116 cells, caspase-9 activity was significantly but not completely inhibited, and the protein level of cytochrome *c* released from mitochondria was reduced but not absent (Figure 3), indicating that the mitochondrial pathway of apoptosis is induced by ATX-S10Na (Ⅱ)-PDT, even though Bax was absent. In p53-null cells, caspase-9 activity was slightly inhibited, and the protein level of cytochrome *c* released from mitochondria was not reduced (Figure 3), indicating that p53 is required for ATX-S10Na (Ⅱ)-PDT-mediated apoptosis.

Decreased levels of anti-apoptotic proteins Bcl-2 and Bcl-x$_L$ in response to ATX-S10Na (Ⅱ)-PDT play a role in early apoptosis, and are Bax- and p53-independent

Previous studies indicated that the levels of anti-apoptotic proteins Bcl-2 and Bcl-x$_L$ were reduced and pro-apoptotic proteins Bcl-x$_S$, Bak and Bad, but not Bid, were up-regulated in response to PDT[30-33]. Therefore, we determined the association of Bax and p53 with anti-apoptotic Bcl-2 family proteins in ATX-S10Na (Ⅱ)-PDT. Immunoblots demonstrated no significant changes in Bax and p53 expression (Figure 3). In contrast, Bcl-2 and Bcl-x$_L$ expression were decreased to similar levels in each cell type, indicating that Bax- and p53-independent downregulation of Bcl-2 and Bcl-x$_L$ plays a role in early apoptosis induced by ATX-S10Na (Ⅱ)-PDT.

DISCUSSION

Many studies have shown that PDT kills tumor cells *via* apoptosis and/or necrosis *in vivo* and *in vitro*. Although cell death in response to PDT depends on various conditions, the role of Bax or p53 in PDT remains controversial. This study was designed to determine the role of these proteins in ATX-S10Na (Ⅱ)-PDT by using an isogenic set

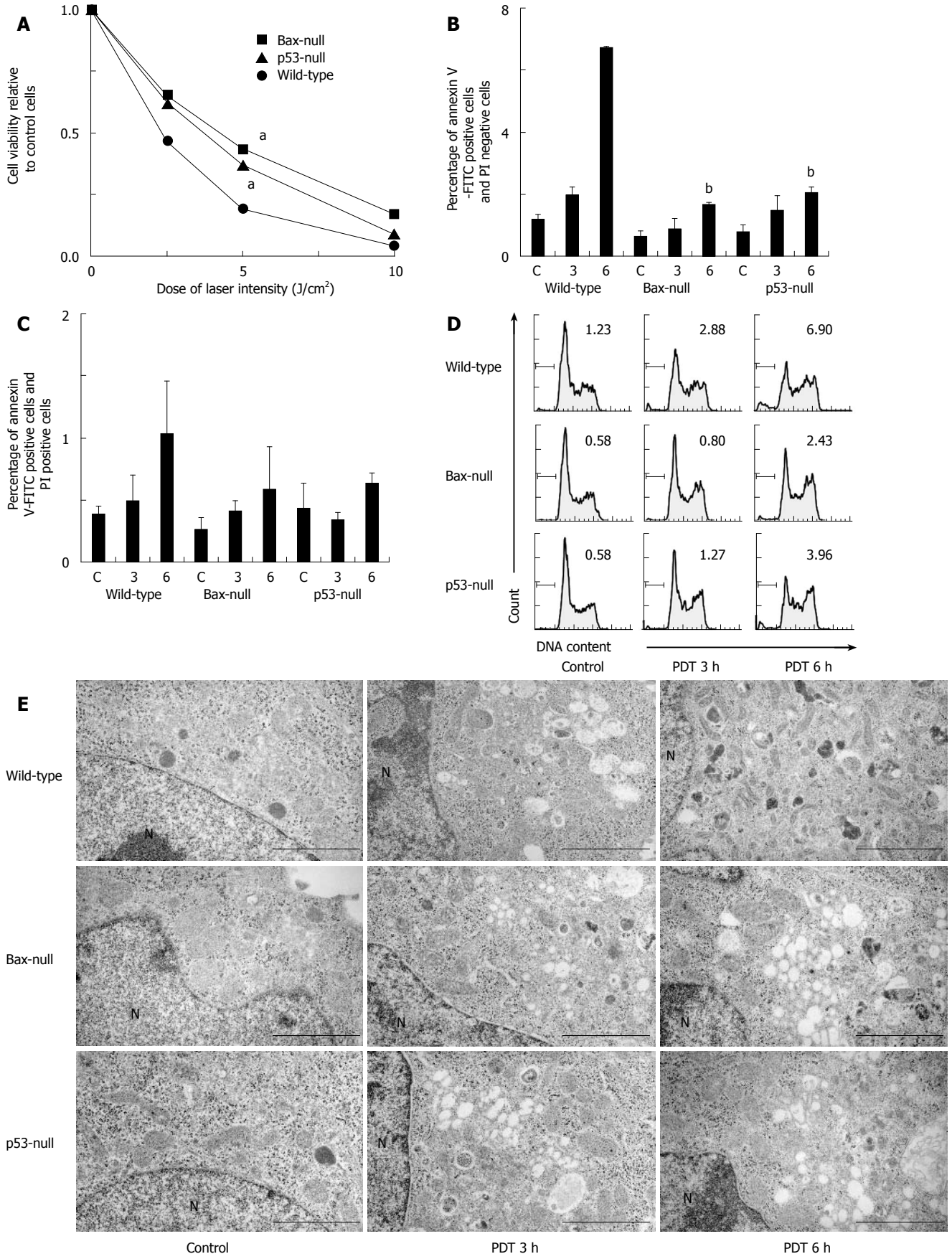

Figure 1 ATX-S10Na(Ⅱ)-PDT-mediated cell death and early apoptosis were Bax- and p53-dependent. Cells were treated with, or without, ATX-S10Na(Ⅱ)-PDT and harvested at each indicated time point following irradiation. **A**: Phototoxicity was determined by MTT assay 24 h after laser irradiation. Data are expressed as a ratio of cell viability relative to untreated control cells. Data represent the mean ± SD of three independent experiments ($^aP < 0.05$ *vs* wild-type cells). **B**, **C**: Early apoptotic changes (**B**) and late apoptotic or necrotic changes (**C**) were determined by annexin V apoptosis assays at 3 or 6 h after laser irradiation. Data are expressed as a percentage of annexin V-positive and PI-negative (**B**) or annexin V-positive and PI-positive cells (**C**). Data represent the mean ± SD of three independent experiments ($^bP < 0.05$ *vs* wild-type cells). **D**: Cell cycle distributions were determined by flow cytometry at 3 or 6 h after laser irradiation. Data are expressed as a percentage of sub-G_1 fraction. **E**: Morphological changes in response to ATX-S10Na(Ⅱ)-PDT were determined by transmission electron microscopy assays at 3 or 6 h following laser irradiation. Typical subcellular changes in response to PDT are shown (original magnification × 12 000). Scale bar: 15 μm, N: nuclei.

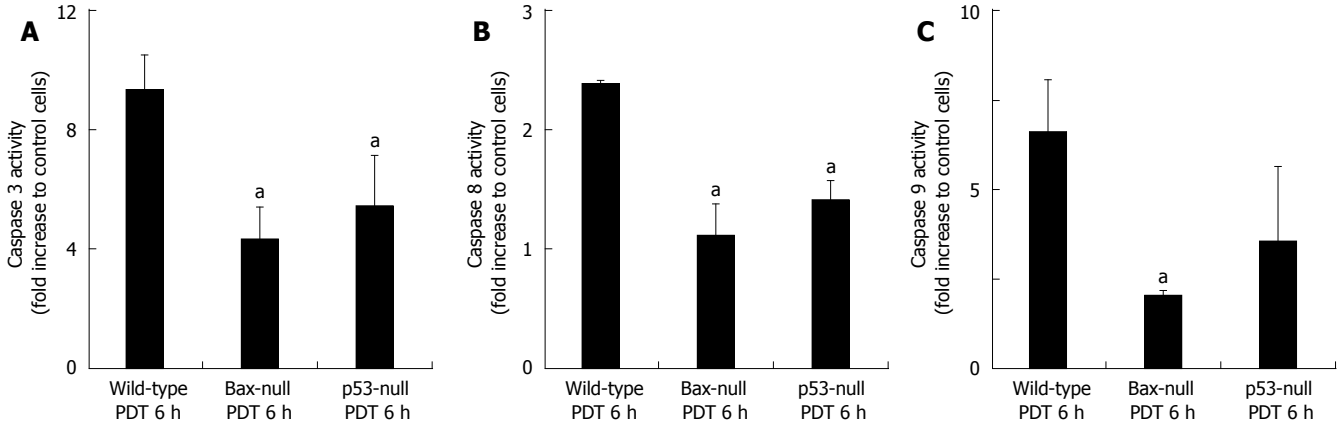

Figure 2 Induction of apoptosis by ATX-S10Na (Ⅱ)-PDT was caspase-dependent. Cells were treated with, or without, ATX-S10Na (Ⅱ)-PDT, harvested and lysed at 6 h after laser irradiation. Induction of caspase activity was assayed. **A**: caspase-3; **B**: caspase-8; **C**: caspase-9. Data represent the mean ± SD of three independent experiments ($^aP < 0.05$ *vs* wild-type cells).

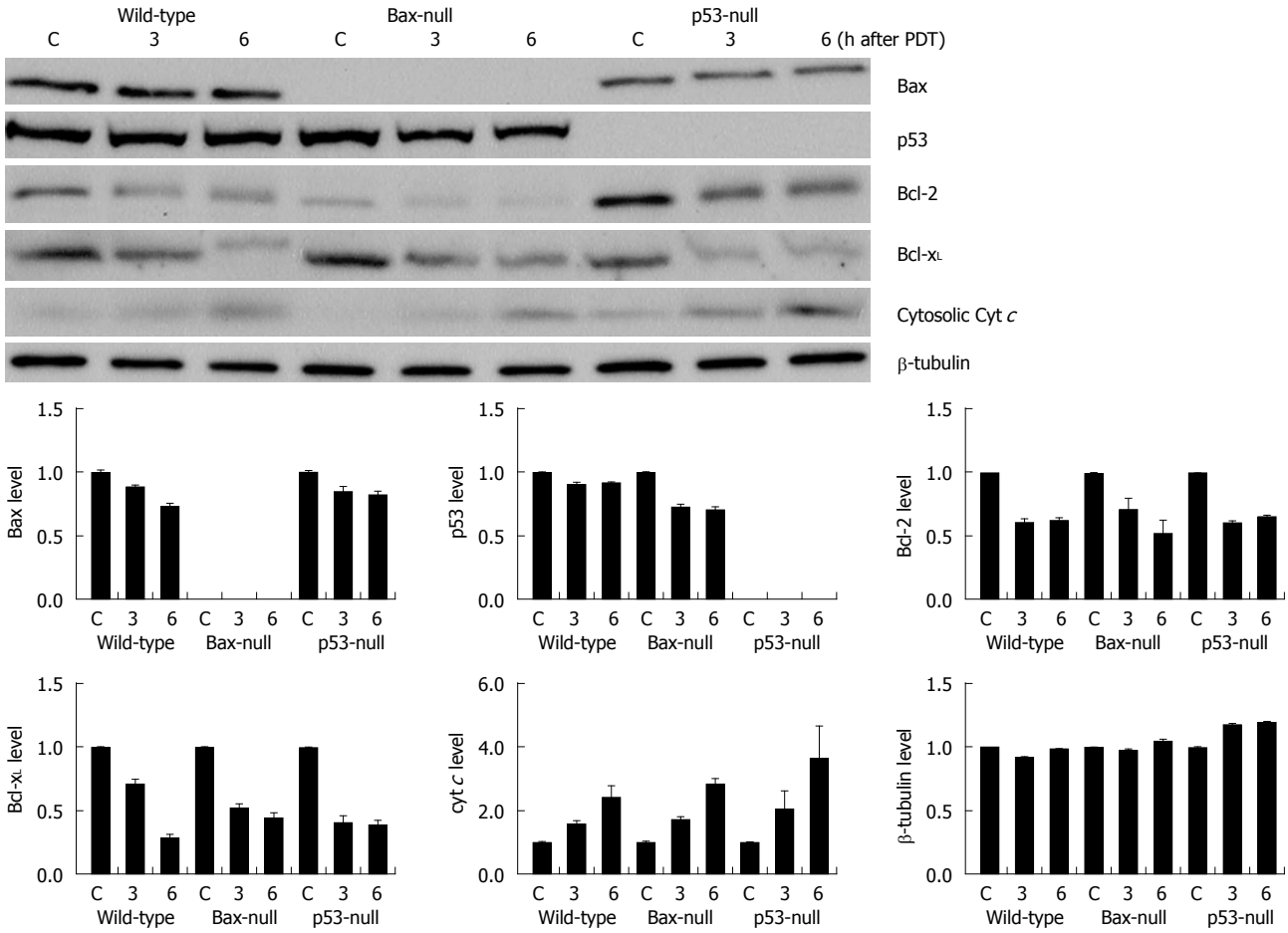

Figure 3 Low levels of Bcl-2 and Bcl-xL played a role in ATX-S10Na(Ⅱ)-PDT. Cells were treated with, or without, ATX-S10Na(Ⅱ)-PDT, harvested and lysed at 3 or 6 h after laser irradiation. Western blot analysis was performed. Each protein level was quantified by using NIH Image J program. Data represent the mean ± SD of three independent experiments.

of human colon cancer cell lines derived from HCT116. Our results indicated that ATX-S10Na (Ⅱ)-PDT induced Bax- and p53-dependent cell death and early apoptosis of human colon cancer cells. ATX-S10Na (Ⅱ)-PDT induced caspase-dependent apoptosis and reduced the levels of anti-apoptotic proteins Bcl-2 and Bcl-xL. Apoptosis was mediated mainly through a Bax-regulated mitochondrial pathway. Taken together, these results suggest that Bax and p53 play a central role in inducing early apoptosis and cell death by ATX-S10Na (Ⅱ)-PDT.

Various experiments have shown that the pro-apoptotic Bcl-2 family protein Bax plays a role in the mitochondrial pathway of apoptosis by translocating from the cytosol to the mitochondria[20]. Our present experiments revealed that

wild-type HCT116 cells were significantly more sensitive to ATX-S10Na (Ⅱ)-PDT than Bax-null cells, as determined by MTT assays (Figure 1). Induction of early apoptosis by ATX-S10Na (Ⅱ)-PDT was also Bax-dependent, as determined by the annexin V assay and cell cycle analysis (Figure 1). Bax was required for caspase activation and cytochrome *c* release, but the mitochondrial pathway of apoptosis was not completely inhibited in the absence of Bax (Figures 2 and 3). The protein level of Bax did not apparently change in the wild-type or p53-null cells (Figure 3). These findings suggest that Bax plays a central role in PDT-mediated cell death and the mitochondrial pathway of apoptosis. As Bax was a major mediator of p53-dependent apoptosis, early apoptosis was apparently processed without Bax activation. Contrary to our findings, earlier reports showed that DU-145 human prostate cancer cells that lack Bax expression do not show release of cytochrome *c* from mitochondria, loss of mitochondrial potential, caspase activation or apoptosis, but do have an altered sensitivity to overall cell death[24]. In contrast, a comparison of the PDT response of wild-type and Bax-null HCT116 human colon cancer cells showed reduced release of cytochrome *c* but it was not completely blocked in Bax-null cells. In both cell lines, caspase-dependent apoptosis was triggered and cell killing was equally sensitive; however, no significant differences in the activation of caspase-3 were found[34]. The authors concluded that the commitment to cell death after PDT occurs at a step prior to and irrespective of Bax activation. Differences between our results and the above findings may be due to cellular differences unrelated to Bax expression, intensity of laser irradiation or type of photosensitizer.

The tumor suppressor protein p53 plays an important role in response to various stress conditions[13,14]. Our present experiments revealed that wild-type HCT116 cells were significantly more sensitive to ATX-S10Na (Ⅱ)-PDT (Figure 1). In addition, induction of early apoptosis by ATX-S10Na (Ⅱ)-PDT was p53-dependent (Figure 1). p53 was required for caspase-3 activation (Figure 2). The mitochondrial pathway of apoptosis was not significantly inhibited in the absence of p53 (Figures 2 and 3). These findings suggest that p53 might play a role in PDT-mediated early apoptosis and cell death. Consistent with our findings, HL60 human promyelocytic leukemia cells that express wild-type p53 are more photosensitive than p53-deleted or mutated HL60 cells[21]. Introduction of the wild-type p53 gene into the HT29 colon cancer cells induced growth arrest but not cell death by PDT[23]. In non-isogenic colon carcinoma cell lines, increased photosensitivity of a wild-type p53 phenotype was observed compared with a mutated p53 phenotype[22]. Taken together, it is conceivable that Bax and p53 play central roles both, in cell death and in early apoptosis induced by ATX-S10Na (Ⅱ)-PDT.

The ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members helps determine the threshold for inducing mitochondrial-related apoptosis[20]. Overexpression of Bcl-2 in Chinese hamster ovary (CHO) cells inhibited apoptosis and partly protected against cell death induced by PDT[35]. Reduction of Bcl-2 protein levels by Bcl-2 antisense oligonucleotides in radiation-induced fibrosarcoma (RIF-1) cells resulted in sensitization to PDT-mediated apoptotic death[31]. Our experiments revealed that decreased levels of Bcl-2 and Bcl-xL were Bax- or p53-independent in response to ATX-S10Na (Ⅱ)-PDT. Cytochrome *c* release from mitochondria was not completely inhibited in the absence of Bax or p53 (Figure 3). These findings suggest that reduced levels of anti-apoptotic Bcl-2 family proteins, that are Bax- or p53-independent, played a role in cytochrome *c* release and apoptosis induced by ATX-S10Na (Ⅱ)-PDT. Low levels of these proteins could be the result of caspase activation or photochemical targets of PDT. PDT using mitochondrial photosensitizer phthalocyanine Pc 4, directly damaged Bcl-2 and Bcl-xL, and contributed in the induction of apoptosis[33,36].

In conclusion, Bax and p53 play a central role in the apoptotic process and cell death induced by ATX-S10Na (Ⅱ)-PDT in human colon cancer cells. Low levels of anti-apoptotic Bcl-2 family proteins Bcl-2 and Bcl-xL, which are Bax- and/or p53-independent, play a role in the early apoptotic process. Bax and p53 can induce apoptosis and cell death in response to ATX-S10Na (Ⅱ)-PDT, which might help in the design of new therapeutic approaches.

ACKNOWLEDGMENTS

We thank Yoko Yumoto, Yuko Arai and Eiko Uga for technical help, Hisako Arai and Hiroyuki Sasaki for TEM analysis, Hisao Tajiri for critical reading of the manuscript, Bert Vogelstein for providing the HCT116 cell lines and Photochemical Co. for providing ATX-S10Na (Ⅱ).

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