Involvement of Bcl-2 and Bax in Photodynamic Therapy-mediated Apoptosis

ANTISENSE Bcl-2 OLIGONUCLEOTIDE SENSITIZES RIF 1 CELLS TO PHOTODYNAMIC THERAPY APOPTOSIS

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Photodynamic therapy (PDT), a promising treatment modality, is an oxidative stress that induces apoptosis in many cancer cells in vitro and tumors in vivo. Understanding the mechanism(s) involved in PDT-mediated apoptosis may improve its therapeutic efficacy. Although studies suggest the involvement of multiple pathways, the triggering event(s) responsible for PDT-mediated apoptotic response is(are) not clear. To investigate the role of Bcl-2 in PDT-mediated apoptosis, we employed Bcl-2-antisense and overexpression approaches in two cell types differing in their responses toward PDT apoptosis. In the first approach, we treated radiation-induced fibrosarcoma (RIF 1) cells, which are resistant to silicon phthalocyanine (Pc 4)-PDT apoptosis, with Bcl-2-antisense oligonucleotide. This treatment resulted in sensitization of RIF 1 cells to PDT-mediated apoptosis as demonstrated by i) cleavage of poly(ADP-ribose) polymerase, ii) DNA ladder formation, iii) terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells, and iv) DEVDase activity. This treatment also resulted in oligonucleotide concentration-dependent decrease in cell viability and down-regulation of Bcl-2 protein with a concomitant increase in apoptosis. However, the level of Bax, a pro-apoptotic member of Bcl-2 family, remained unaltered. In the second approach, an overexpression of Bcl-2 in PDT apoptosis-sensitive human epidermoid carcinoma (A431) cells resulted in enhanced apoptosis and up-regulation of Bax following PDT. In both the approaches, the increased Bax/Bcl-2 ratio was associated with an increased apoptotic response of PDT. Our data also demonstrated that PDT results in modulation of other Bcl-2 family members in a way that the overall ratio of pro-apoptotic and anti-apoptotic member proteins favors apoptosis.

Photodynamic therapy (PDT) with the phthalocyanine photosensitizer Pc 4 (HOSiPcOSi(CH3)2(CH2)3N(CH3)2) is an oxidative stress that induces cell death, mainly through apoptosis, in many tumor cells in vitro and during tumor shrinkage in vivo (1–6). PDT, a United States FDA-approved modality for the treatment of esophageal and lung cancer and actinic keratosis, is currently undergoing clinical trials for the treatment of many other solid cancers as well as many non-malignant conditions (1–6). PDT relies on a bimodal protocol in which visible light of an appropriate wavelength activates tumor cell-associated photosensitizer to produce reactive oxygen species. The involvement of apoptosis has been shown as an early response of PDT, both during in vitro tumor cell killing and in vivo situations during tumor ablation (7–11). Studies from this laboratory have shown the involvement of i) an increased generation of nitric oxide (12), ii) a WAF1/p21-mediated inhibition of the cyclin-cdk network (10), and iii) a deregulation of pRb/E2F-DP machinery (13) during Pc 4-PDT-mediated apoptosis. Studies have shown the involvement of phospholipases A2 and C (8), intracellular Ca2+ (8, 14), ceramide (15, 16), caspases (17, 18), c-Jun N-terminal kinase (JNK)/p38 MAPK (19, 20), and cytochrome c release (17, 18) during PDT-mediated apoptotic cell death. Thus, it has become clear that multiple pathways are involved in PDT-mediated apoptosis. This offers exciting opportunities to take advantage of these pathways in improving PDT treatment protocol. The understanding of the mechanism(s) of triggering event(s) of PDT-mediated apoptosis is far from complete. Mitochondrial damage has been suggested as an early event in PDT-mediated apoptosis, which could result in the release of apoptotic factors like cytochrome c, which in turn, activates the downstream targets in the apoptotic pathway (21). Members of Bcl-2 family of proteins are critical regulators of the apoptotic pathway. Bcl-2, an antiapoptotic member of Bcl-2 family inhibits the release of cytochrome c from mitochondria whereas Bax and Bid proteins, pro-apoptotic members of Bcl-2 family has been shown to release cytochrome c from the mitochondria thereby enhancing apoptotic response (22–24). The role of Bcl-2 protein in PDT-mediated apoptosis is not convincingly established (4, 25–28). In the present study, employing two cell lines differing in their response to Pc 4-PDT-induced apoptosis and antisense treatment and overexpression approaches we show that Bcl-2 plays an important role in PDT-mediated apoptosis. Furthermore, the ratio of Bax

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The abbreviations used are: PDT, photodynamic therapy; Pc 4, phthalocyanine photosensitizer (HOSiPcOSi(CH3)2(CH2)3N(CH3)2); JNK, c-Jun NH2-terminal kinase; RIF 1, radiation-induced fibrosarcoma cells; HBSS, Hanks’ balanced salt solution; PARP, poly(ADP-ribose) polymerase; DEVD, N-acetyl-Asp-Glu-Val-Asp; AFC, 7-amino-4-trifluoromethyl coumarin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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(pro-apoptotic) and Bcl-2 (anti-apoptotic) proteins appears to determine the response to PDT-induced apoptosis.

MATERIALS AND METHODS

Cells—Radiation-induced fibrosarcoma (RIF 1) and human epidermoid carcinoma (A431) cells were maintained in Eagle’s minimal essential medium supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin and Dulbecco’s modified Eagle’s medium (10% fetal bovine serum, 1% penicillin/streptomycin) and kept in an atmosphere of 95% air/5% CO2 in a 37 °C humidified incubator. In all experiments, 70–80% confluent cells were used.

Antisense Treatment—Antisense oligonucleotides directed against the coding region of Bcl-2 protein were custom-synthesized from Operon Biotechnologies Inc. (Alameda, CA). Phosphorothioate-modified 20-mer antisense (ATTCTCCCTCCACGTTCACC) and scrambled mismatched control oligo (CTATTTCCTACCAGCACCC) were used in this study. Oligonucleotides were diluted with HEPES-buffered saline to a final concentration of 5.0 μM or unless otherwise mentioned. Lipofectin reagent (Sigma Chemical Co., St. Louis, MO) was mixed with diluted DNA (at a concentration of 10 μg/μg of DNA) and incubated at room temperature for 30 min. This mixture was further diluted in 5 ml of Eagle’s minimal essential medium and added to cells in 100-mm plates followed by incubation at 37 °C for 12 h.

Transfection of A431 Cells—The A431 cells were transfected with a eukaryotic expression vector (pSFFV/neo) containing an EcoRI fragment of human Bcl-2 cDNA. The cells were seeded at 2 × 105 cells in 100-mm plates and were allowed to attach overnight. On the following day, cells were transfected with 1.0 μg of vector using Lipofectin transfection reagents according to the manufacturer’s protocol (Sigma Chemical Co.). The transfectants were selected on neomycin-containing media and were allowed to grow on the selection media for 48 h and then subjected to PDT as described below.

PDT—Cells were washed with Hanks’ balanced salt solution (HBSS, with Ca2+ and Mg2+), and treated with Pc 4 (0.5 μM in complete media) overnight in 100-mm disposable culture plates. Next morning, the cells were washed with HBSS, irradiated with 15 kJ/m2 light, as measured by a digital photometer (Tektronix, Beaverton, OR), using a 300-watt halogen lamp. The light was filtered through a Lee primary red filter (no. 106, Vincent Lighting, Cleveland, OH) to remove light with wavelengths of <600 nm. Following irradiation, the cells were incubated in darkness for selected time in a humidified incubator at 37 °C. Appropriate controls, as specified at appropriate places, were also included. After the specified times, the medium was aspirated, the cells were washed with cold PBS (10 mM, pH 7.4), and processed as desired.

Preparation of Cell Lysate—Following the treatments, the cells were washed with PBS (10 mM, pH 7.4) and incubated in lysis buffer (150 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 100 mM Na2VO4, 0.5% Nonidet P-40, 1% Triton X-100, 1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate) on ice for 60 min. The plates were scrapped, and cell lysates were collected in a centrifuge tube and passed through a 21-gauge needle to break the cell aggregates. Cell lysates were centrifuged at 14,000 × g at 4 °C, and the supernatants (total cell lysate) were collected and stored at –80 °C. The protein of cell lysate was determined by DC Bio-Rad assay using manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA).

Immunoblot Analysis—50–100 μg of protein was resolved over SDS/12% polyacrylamide gels and electrophoresed to nitrocellulose membrane. The blot was blocked in blocking buffer (5% nonfat dry milk, 1% Tween-20; in 20 mM Tris-buffered saline, pH 7.5) for 1 h at room temperature, incubated with appropriate monoclonal or polyclonal primary antibody (Bcl-2, Bcl-xL, Bcl-xS, and Bak from Oncogene Research Products Cambridge, MA; PARP and DNA fragmentation factor from Upstate Biotechnology, Lake Placid, NY; Bad from Calbiochem-Novabiochem Corp., San Diego, CA; Bid from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in blocking buffer for 1 h to overnight at 4 °C. Blots were then incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody and detected by chemiluminescence and autoradiography using ECL Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ).

DNA Isolation, Electrophoresis, and Quantification of Apoptosis by Flow Cytometry—After treatment, the cells were washed with PBS and suspended in cytoplasm extraction buffer (10 mM Tris, 150 mM NaCl/5 mM MgCl2/0.5% Triton-X), left over ice for 30 min, and then centrifuged at 14,000 × g at 4 °C. The resultant pellet was discarded, and the supernatant was incubated with RNase H (0.2 mg/ml) overnight at 4 °C and then with Proteinase K (0.1 mg/ml) at 37 °C for 2 h. DNA was extracted by treatment of the supernatant with phenol:chloroform (1:1) and then precipitated with 95% ethanol for 2 h at −80 °C. The DNA pellet was centrifuged at 14,000 × g at 4 °C for 15 min. The DNA was air-dried and resuspended in 40 μl of TE buffer (10 mM Tris-HCl, pH 8.0/0.1 mM EDTA). Total DNA was resolved on 1.5% agarose gel containing 0.3 μg of ethidium bromide in Tris buffer EDTA (1× TBE). Bands were visualized under a UV transilluminator followed by Polaroid photography. The quantification of apoptosis was performed using an APODIRECT flow cytometry kit (Phoenix Flow Systems, San Diego, CA) as per the manufacturer’s protocol.

DEVdase Assay—Caspace activity was measured by DEVdase assay. In brief, the cell lysates were collected at different time points and measured for their enzymatic activity at 540 nm. The data was expressed as percent viable cells. Each assay was performed in triplicate.

Cell Viability Assay—The cytotoxic effect of antisense oligonucleotide was assessed by the 9-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. The cells were seeded at a density of 1 × 105 cells/well in a 96-well microtiter plate and allowed to attach overnight. Cells were grown to 70% confluency and then treated with different concentrations of antisense oligonucleotide (1–5 μg of DNA) overnight. Scrambled oligonucleotide was used as positive control. Next day, the media containing DNA was aspirated and washed with HBSS and then subjected to Pc 4-PDT. Cells were incubated for 6 h in complete media with 50 μl of MTT (1 mg/ml) reagent at 37 °C for 6 h. MTT reagent was aspirated and washed with PBS, 150 μl of Me2SO was added, and the mixture was placed on a shaker for 10 min. Optical density was read at 540 nm in ELISA plate reader, and the data was expressed as percent viable cells. Each assay was performed in triplicate.

Effect of Antisense Bcl-2 Oligonucleotide on RIF 1 Cell Survival after PDT—The radiation-induced fibrosarcoma cells (RIF 1) are resistant to photodynamic therapy (PDT)-mediated apoptosis (12). To determine whether treatment of antisense oligonucleotide, directed against the coding region of Bcl-2 protein, induces cell death in RIF 1 cells following PDT, we treated the cells with different concentrations of antisense oligonucleotide. As shown in Fig. 1A, treatment of cells with antisense oligonucleotide resulted in a significant decrease in the viability of cells in a concentration-dependent manner as compared with cells treated with scrambled nucleotide. Cells treated with scrambled control oligonucleotide did not show any significant reduction in the cell survival.

Induction of Apoptosis with Antisense Bcl-2 in RIF 1 Cells—Because our data showed that the antisense oligonucleotide treatment resulted in oligonucleotide concentration-dependent decrease in cell survival in RIF 1 cells, we investigated whether this decreased cell survival is due to apoptosis. As shown by DNA ladder assay (Fig. 1B), treatment of PDT apoptosis-resistant RIF 1 cells with antisense Bcl-2 oligonucleotide resulted in a significant induction of apoptosis, 6 h following PDT. Next, we quantified the extent of apoptosis by flow cytometric analysis of the cells labeled with dUTP and propidium iodide. The PDT of RIF 1 cells treated with antisense resulted in 39.2% TUNEL-positive cells at 6 h post-PDT compared with only 3.0% TUNEL-positive cells for similarly treated cells without antisense. Consistent with our previous observation, the untreated control cells or cells treated with light alone or Pc 4 alone did not result in any appreciable apoptosis (Fig. 1C). These data clearly establish that the effects are photodynamic treatment-mediated.

Caspace activation is an important event in the apoptotic pathway and can be used as a marker of apoptosis. Therefore,
Effect of Antisense Bcl-2 Oligonucleotide on the Protein Expression of Bcl-2 in RIF 1 Cells—To assess the effect of antisense Bcl-2 oligonucleotide on the protein expression of Bcl-2, we performed immunoblot analysis. As shown by Fig. 2A, the immunoblot analysis revealed a down-regulation of Bcl-2 protein in RIF 1 cells treated with antisense oligonucleotide followed by PDT whereas the level of Bcl-2 remains unaltered in cells without antisense treatment. Treatment of cells with scrambled control oligonucleotide showed no effect on the Bcl-2 level in RIF 1 cells after PDT (data not shown). The densitometric analysis showed almost 2-fold decrease in the protein expression of Bcl-2 in cells treated with antisense followed by PDT (Fig. 2B). This observation suggested that constitutively high levels of Bcl-2 might be a reason for the known PDT apoptosis-resistant property of RIF 1 cells.

To further establish the role of Bcl-2 in PDT-mediated apoptosis, we assessed the level of Bcl-2 by ELISA and compared it with the extent of apoptosis as assessed by TUNEL assay, in the cells treated with antisense followed by PDT. The estimation of Bcl-2 by ELISA demonstrated a concentration-dependent decrease in the level of Bcl-2 with a concomitant increase in the extent of apoptosis, as evident from flow cytometric determination of TUNEL-positive cells (Fig. 3A). Therefore, our data demonstrated that the decrease in Bcl-2 levels is correlated with the increased sensitivity of RIF 1 cells toward PDT apoptosis, following antisense oligonucleotide treatment (Fig. 3A). We also assessed the effect of antisense oligonucleotide treatment on PDT-mediated modulation in the relative ratio of Bax/Bcl-2 proteins by immunoblot analysis (Fig. 3B). The antisense oligonucleotide treatment of RIF 1 cells followed by PDT resulted in a down-modulation of Bcl-2 protein whereas the levels of Bax protein remained unaltered (Fig. 3B). However, the densitometric analysis showed that with the increase
in the Bax/Bcl-2 ratio there is increase in the apoptotic response (Fig. 3C).

Effect of PDT on the Protein Expression of Bcl-2 in A431 Cells—Human epidermoid carcinoma cells are sensitive to PDT-mediated apoptosis (10). Therefore, we were interested in evaluating the effect of Bcl-2 during PDT-mediated apoptosis in these cells. We assessed the levels of Bcl-2 protein following PDT in these cells. As shown in Fig. 4A, PDT of these cells resulted in a significant time-dependent down-regulation of Bcl-2 protein.

Effect of Bcl-2 Overexpression on Apoptotic Response of A431 Cells—The data in Fig. 1 cells suggested that the down-regulation of Bcl-2 induces apoptosis in PDT apoptosis-resistant cells. In another approach, to investigate the role of Bcl-2 in PDT-mediated apoptosis, we overexpressed the Bcl-2 protein in the A431 cells. In these Bcl-2-transfected A431 cells, as expected, the Bcl-2 protein was overexpressed (Fig. 4B) and PDT resulted in a down-regulation of Bcl-2 protein levels (Fig. 4B). Interestingly, as shown by TUNEL assay (Fig. 5A), the Bcl-2 overexpression resulted in an increase in apoptosis by PDT as compared with normal wild type A431 cells. To evaluate the effect of overexpression of Bcl-2 on caspase activity, we performed DEVDase assay in both wild type and Bcl-2-overexpressing cells. As shown by data in Fig. 5B, the overexpression of Bcl-2 was found to enhance the PDT-mediated caspase activity indicative of increased apoptosis. The increase in caspase activity was highest at 3 h post-PDT and declined thereafter.

Effect of Bcl-2 Overexpression on Bax Protein in A431 Cells—Bax is a pro-apoptotic member of the Bcl-2 protein family. The relative amounts or equilibrium between the pro- and anti-apoptotic proteins influences the susceptibility of cells to apoptosis. Therefore, in the next set of experiments, we assessed the effect of PDT on the status of Bax protein in Bcl-2-overexpressing A431 cells. The immunoblot analysis showed that PDT resulted in an increased level of Bax protein in Bcl-2-overexpressing cells as compared with normal A431 cells (Fig. 6A). The densitometric analysis demonstrated that Bcl-2-overexpressing cells have high Bax/Bcl-2 ratio as compared with their wild type counterparts (Fig. 6B).

Modulations in Other Bcl-2 Family Members in RIF 1 and A431 Cells Subjected to PDT—The Bcl-2 family of proteins consists of apoptosis regulators with both anti- and pro-apoptotic effect. The anti-apoptotic group includes Bcl-2 and Bcl-xL, whereas the pro-apoptotic group includes Bad, Bak, Bik, Bak, and Bcl-xB. Therefore, we assessed the effect of PDT on these family members in both the systems, viz. RIF 1 cells (without and with antisense Bcl-2 oligonucleotide treatment) and A431 cells (wild type and transiently transfected with Bcl-2). As shown by data in Fig. 7, PDT was found to result in an increase in Bcl-xB protein levels in RIF 1 cells subjected to oligonucleotide antisense treatment at all the time points studied, as compared with untreated cells. An increasing trend in the protein levels of Bid was also observed in the cells treated with oligonucleotide. Another pro-apoptotic protein Bak was not affected at early times (1 h and 3 h) following PDT, but its level was found to be elevated at 6 h following PDT. On the other hand, the protein expression of Bad was found to be up-regulated at all the time points following PDT (Fig. 7). The interesting observation of this experiment, however, was the observed increasing trend in the protein expression of anti-apoptotic protein Bcl-xL in cells treated with oligonucleotide, compared with the cells without oligonucleotide treatment (Fig. 7).

We also assessed the effect of PDT on modulations in other Bcl-2 family members in A431 cells overexpressing Bcl-2. As shown by data in Fig. 7, in Bcl-2-overexpressing A431 cells, PDT was found to result in a significant increase in the levels of Bcl-xB at 1 h post-PDT and was found to diminish at later time points. The level of Bid was not found to be significantly affected following PDT. PDT was also found to result in significant up-regulation of Bak (at 1 h) and Bad (at 1 h, 3 h, and 6 h post-PDT) in Bcl-2-overexpressing cells compared with their wild type counterparts (Fig. 7).

DISCUSSION

PDT, a new treatment modality for many cancer types and for certain non-malignant diseases, has been shown to induce apoptosis of cancer cells both in vitro during tumor cell killing and in vivo during tumor ablation. Understanding of the mechanisms involved in PDT-mediated apoptosis is far from complete. This study was designed to investigate the hypothesis that the Bcl-2 family of proteins plays a critical role in PDT-mediated apoptosis. The Bcl-2 gene, initially recognized as a proto-oncogene in human follicular B-cell lymphoma, is the prototype of a novel class of oncoproteins that contribute to neoplastic progression by enhancing tumor cell survival through inhibition of apoptotic cell death (22, 23). The product of Bcl-2 is known to play a role in promoting cell survival and inhibiting apoptosis following variety of stimuli, including γ-radiation, glucocorticoid, hypothermia, growth factor withdrawal, and chemotherapeutic agents (23, 29, 30).

In the present study, we evaluated the role of Bcl-2 protein in PDT-mediated apoptosis of cancer cells. For this reason, we employed two independent unique approaches. First, we used radiation-induced fibrosarcoma cells (RIF 1), which are resistant to PDT-mediated apoptosis under the conditions employed in this study. As assessed by multiple methods, the treatment of RIF 1 cells with antisense oligonucleotides directed against the coding region of Bcl-2 protein was found to sensitize these PDT apoptosis-resistant cells to PDT apoptosis. Because Pc 4
Bcl-2 is an upstream effector molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis. Bcl-2 is found at inappropriately high levels in more than half of all human tumors (23). Several mechanisms are proposed for the antiapoptotic function of Bcl-2. Bcl-2 might act as a regulator of Ca$^{2+}$ homeostasis (31) or as an antioxidants (32). Bcl-2 has been show to form a heterodimer with the pro-apoptotic member Bax and might thereby neutralize its pro-apoptotic effects (33, 34). In addition, Bcl-2 is also known to prevent the release of potent mitochondrial activators of the cytosolic death effector proteases, the caspase family, which mediates the intracellular proteolysis characteristics of apoptosis (35). The antisense treatment of RIF 1 cells followed by PDT resulted in down-regulation of Bcl-2 in RIF 1 cells, which might initiate the apoptotic pathway sensitizing the RIF 1 cells to PDT-mediated apoptosis. Caspases are known to cleave many proteins, including the inhibitor of nuclear DNase, the DNA fragmentation factor, the ICAD, the DNA repair enzyme poly(ADP-ribose polymerase (PARP), and DNA-dependent protein kinase subunit and structural proteins such as α-fodrin. These proteins are required by cells for protection against apoptosis (36, 37). Our results demonstrate high caspase activity and cleavage of PARP with antisense treatment of RIF 1 cells followed by PDT. Our study also showed unaltered levels of Bax (a pro-apoptotic member of the Bcl-2 family) with the antisense treatment. Antisense treatment resulted in decreased levels of Bcl-2 protein in RIF 1 cells, thus shifting the ratio in favor of Bax protein that initiates the apoptotic pathway sensitizing the RIF 1 cells to PDT-mediated apoptosis.

To further substantiate our findings, in the second approach, we overexpressed Bcl-2 in A431, which readily undergoes apoptosis with PDT (10). Immunoblot analysis revealed a decrease in Bcl-2 levels following PDT in a time-dependent manner in A431 cells. Interestingly, the overexpression of Bcl-2 resulted in an increased apoptotic response in A431 cells compared with their normal wild type cells as evident by TUNEL assay and caspase activity assay. Immunoblot analysis revealed the high level of Bax (a pro-apoptotic member of Bcl-2 family) in Bcl-2-overexpressing cells. Earlier reports have suggested that the overexpression of Bcl-2 may stabilize Bax protein (28), which,
shown here are representative of two independent experiments with similar results. Cells were harvested at specified time points post-PDT and labeled with dUTP using terminal deoxynucleotide transferase and propidium iodide and analyzed by flow cytometry. The data were transiently transfected with Bcl-2 as described under “Materials and Methods” followed by PDT. Cells were harvested at specified time points following PDT and labeled with dUTP using terminal deoxynucleotide transferase and propidium iodide and analyzed by flow cytometry. The data shown here are representative of three independent experiments. Statistical analysis was performed by Student’s t test, and p < 0.005 was considered significant. NT = untreated control.

FIG. 5. Overexpression of Bcl-2 enhances PDT-mediated apoptosis in A431 cells. A, quantification of apoptosis by flow cytometry. Cells were transiently transfected with Bcl-2 as described under “Materials and Methods” followed by PDT. The data shown here are representative of two independent experiments with similar results. n = untreated control. B, DEVDase activity assay. The caspase activity was measured in both wild type and Bcl-2-transfected cells at different time points following PDT. Caspase activity was measured by AFC-DEVDase assay as described under “Materials and Methods.” Data represents means ± S.D. of three independent experiments. Statistical analysis was performed by Student’s t test, and p < 0.005 was considered significant. NT = untreated control.

FIG. 6. Overexpression of Bcl-2 stabilizes Bax protein following Pc 4-PDT in A431 cells. A, effect of PDT on Bax protein levels in wild type and Bcl-2-overexpressing A431 cells. A431 cells were transiently transfected with Bcl-2 followed by PDT. Cell lysates were prepared at specified times following PDT and 50 μg of protein was subjected to 12% Tris-glycine gel electrophoresis followed by immunoblot analysis and chemiluminescence detection as detailed under “Materials and Methods.” The data shown here are representative of three independent experiments with similar results. n = untreated control. B, effect of PDT on Bax/Bcl-2 ratio in wild type and Bcl-2-transfected cells. The ratio of Bax/Bcl-2 was determined by densitometric analysis of bands in immunoblots for Bax (A) and Bcl-2 (Fig. 4A). The data shown here are means ± S.E. from three independent experiments. NT = untreated control.

in turn, may promote apoptosis by triggering the pore-forming activity in the mitochondrial membrane (38) and can promote release of cytochrome c from mitochondria (35). It was reported previously that caspases mediated conversion of Bcl-2 in a Bax-like molecule can also increase the apoptotic response (39, 40). High Bax level (stabilization effects of Bcl-2) shifting the ratio between Bax/Bcl-2 toward Bax, and high caspase activity may be responsible for the enhanced PDT-mediated apoptotic response in Bcl-2-overexpressing cells.

Earlier reports have revealed a dual identity for Bcl-2 protein (Bax) in PDT-mediated cell death. The first report on PDT-mediated apoptosis (8) suggested that mitochondrial damage could result in degradation of Bcl-2 and related proteins that normally function as apoptotic suppressors. He et al. (25) reported that Bcl-2 transfection of Chinese hamster ovary cells leads to partial resistance to PDT-mediated apoptosis. Consistent with this finding, Granville et al. (26) have reported that overexpression of Bcl-2 protein blocks the activation of caspases and downstream events instigated by PDT. Meanwhile using antisense retroviral vector-mediated reduction of Bcl-2, Zhang et al. (27) have shown increased phototoxicity and sensitivity to apoptosis induced by 2-BA-2-DHIA PDT in human gastric adenocarcinoma, further supporting the role of Bcl-2 in PDT-mediated apoptosis. However, in a recent report Kim et al. (28) have reported contradictory findings. In this study, the overexpression of Bcl-2 in a human breast cell line resulted in enhanced apoptosis by PDT.

Based on our findings, the controversy regarding the role of Bcl-2 protein in PDT-mediated apoptosis can be put in proper context. Down-regulation of Bcl-2 with antisense oligonucleotide resulted in sensitization of PDT apoptosis-resistant cells to apoptosis; however, the overexpression of Bcl-2 in a PDT apoptosis-sensitive cell line further enhances the response. This study clearly suggested that the shift in the ratio of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) in favor of pro-apoptotic protein (Bax) is an important factor in determining the response toward PDT.

Our data also demonstrated that PDT results in modulation of other Bcl-2 family members in a way that the overall ratio of pro-apoptotic and anti-apoptotic member proteins remains in favor of pro-apoptotic proteins. We found that PDT of RIF 1 cells (treated with Bcl-2 antisense oligonucleotide) caused an up-regulation of Bcl-xL, Bid, Bak, and Bad, the pro-apoptotic members of the Bcl-2 family. The up-regulation of these pro-apoptotic proteins by PDT in RIF 1 cells (treated with Bcl-2 antisense oligonucleotide) was found to be an early response for Bcl-xL and Bad (occurring as early as 1 h post-PDT), whereas it was a late response for Bak (occurring at 6 h post-PDT). An interesting observation of this experiment was the observed increasing trend in the levels of anti-apoptotic Bcl-xL as a result of PDT in RIF 1 cells treated with Bcl-2 antisense oligonucleotide. It is likely that, despite this increasing trend in the
levels of anti-apoptotic Bcl-x<sub>L</sub> protein, the high levels of pro-apoptotic members were able to overcome its anti-apoptotic activity that ultimately results in apoptotic death of RIF 1 cells following PDT.

Like RIF 1 cells treated with Bcl-2 antisense oligonucleotide, we also found that PDT of Bcl-2-overexpressing A431 cells caused an up-regulation of protein expression in pro-apoptotic members, viz. Bcl-x<sub>L</sub>, Bak, and Bad, but not of Bid. The protein expression of anti-apoptotic Bcl-x<sub>L</sub> was found to decrease as a result of PDT in these cells. Thus, it seems that in these cells PDT results in a general up-regulation in the protein expression of the pro-apoptotic members while down-modulating the anti-apoptotic proteins, shifting the balance in favor of apoptosis. Another interesting observation was the decreased protein expression of some of the molecules studied as a result of antisense or overexpression treatments. The reason for this decrease is not clear at present, however, it is clear that alterations of Bcl-2 levels in our experimental setup modulates expression of some of the molecules studied as a result of antisense or overexpression treatments. The reason for this decrease is not clear at present, however, it is clear that alterations of Bcl-2 levels in our experimental setup modulates expression of some of the molecules studied as a result of antisense or overexpression treatments. The reason for this decrease is not clear at present, however, it is clear that alterations of Bcl-2 levels in our experimental setup modulates expression of some of the molecules studied as a result of antisense or overexpression treatments.

Extensive experimental evidence suggest that antisense Bcl-2 oligonucleotides induce apoptotic cell death in various types of malignant cell lines in vitro, including leukemia, lymphoma, small cell lung carcinoma, and cholangiocarcinoma. Furthermore, combined use of antisense Bcl-2 oligonucleotide with chemotherapeutic agents resulted in additive inhibition of small cell lung cancer cells in vitro and melanoma cells in vitro and in vivo. Our results demonstrate that Bcl-2 plays an important role in PDT-mediated apoptosis and the shift of balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 protein family in favor of pro-apoptotic members decides the susceptibility of cells to PDT-mediated apoptosis.

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