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Association Between the Photodynamic Loss of Bcl-2 and the Sensitivity to Apoptosis Caused by Phthalocyanine Photodynamic Therapy¶

Jitsuo Usuda[1], Kashif Azizuddin[1], Song-mao Chiu[1] and Nancy L. Oleinick*[1,2]

[1]Department of Radiation Oncology, School of Medicine, Case Western Reserve University, Cleveland, OH and
[2]Ireland Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH

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ABSTRACT

We have reported that photodynamic therapy (PDT) using the photosensitizer phthalocyanine (Pc) 4 and red light damages the antiapoptotic protein Bcl-2. Recently, using transient transfection of Bcl-2 deletion mutants, we identified the membrane anchorage domains of Bcl-2 as necessary to form the photosensitive target. However, it is not clear how Bcl-2 photodamage sensitizes cells to Pc 4-PDT–induced apoptosis, whether overall cell killing is also sensitized or how up-regulation of Bcl-2 in tumors might make them more or less responsive to Pc 4-PDT. In this study we report on MCF-7c3 cells (human breast cancer cells expressing stably transfected procaspase-3) overexpressing wild-type Bcl-2 or certain deletion mutants in either a transient or a stable mode. By flow cytometric analysis of transiently transfected cells, we found that wild-type Bcl-2, Bcl-2Δ33-54 and Bcl-2Δ37-63 (each of which can be photodamaged) protected cells from apoptosis caused by Pc 4-PDT. In contrast, Bcl-2Δ210-239, which lacks the C-terminal transmembrane domain and cannot be photodamaged, afforded no protection. We then evaluated the PDT sensitivity of transfected cell lines stably overexpressing high levels of wild-type Bcl-2 or one of the Bcl-2 mutants. Overexpression of wild-type Bcl-2, Bcl-2Δ33-54 or Bcl-2Δ37-63 resulted in relative resistance of cells to Pc 4-PDT, as assessed by morphological apoptosis or loss of clonogenicity. Furthermore, overexpression of Bcl-2 also inhibited the activation-associated conformational change of the proapoptotic protein Bax, and higher doses of Pc 4 and light were required to activate Bax in cells expressing high levels of Bcl-2. Many advanced cancer cells have elevated amounts of Bcl-2. Our results show that increasing the dose of Pc 4-PDT can overcome the resistance afforded by either Bcl-2 or the two mutants. PDT regimens that photodamage Bcl-2 lead to activation of Bax, induction of apoptosis and elimination of the otherwise resistant tumor cells.

INTRODUCTION

Photodynamic therapy (PDT), which consists of exposing cancerous lesions to a tumor-localizing photosensitizer and light, is a promising modality for the treatment of a variety of solid tumors (1). Since the first modern clinical trial of PDT by Dougherty (2) was reported in 1978, PDT with Photofrin® has been applied to many solid tumors and is approved by the U.S. Food and Drug Administration for the treatment of advanced esophageal and early and late lung cancers (1,3). To enhance the potential of PDT and to extend its clinical applications, a second generation of photosensitizers, such as the silicon phthalocyanine Pc 4, is now being assessed for cancer therapy, and it is important to elucidate their mechanisms of action in PDT. Some of the second-generation photosensitizers have attained approval for treatment of various cancers in Europe and Japan (4).

PDT generates singlet oxygen and other reactive oxygen species, which cause an oxidative stress and membrane damage in treated cells and eventual cell death (5,6). We have reported that PDT induces a rapid apoptotic response in cancer cells and that apoptosis is a prominent mode of PDT-induced cell death in preclinical tumor models (7–10). Mitochondria are major targets of Pc 4-PDT, and damage to these organelles leads to rapid release of cytochrome *c* and second mitochondria derived activator of caspase into the cytosol and activation of a cascade of apoptosis-mediating caspases (11–13). However, the precise mechanism for the induction of apoptosis by PDT has not yet been elucidated.

Recently, we reported the construction of a series of Bcl-2 mutants by site-directed mutagenesis and the examination of the association between their subcellular localization and their sensitivity to photodestruction by Pc 4-PDT (14). We found that membrane anchorage regions were needed to form the target of Pc 4 photosensitization, *i.e.* in addition to the transmembrane (TM) domain, photodamage required the region between the Bcl-2 homology 1 (BH1) and BH2 domains, which contains two core hydrophobic alpha helices (alpha 5 and 6) that are thought to be inserted into the membrane to form ion channels (15–17). Although we defined the structural features that determine Bcl-2 photosensitivity, how photodamage to Bcl-2 affects its role in apoptosis is still not clear. To elucidate the role of Bcl-2 in PDT-induced apoptosis and to determine whether photodamaged Bcl-2 retains its antiapoptotic function, we studied both transient and stable transfectants that overexpress either the wild-type Bcl-2 or

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*To whom correspondence should be addressed at: Department of Radiation Oncology, School of Medicine (BRB324), Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4942, USA. Fax: 216-368-1142; e-mail: nlo@po.cwru.edu

Abbreviations: BH, Bcl-2 homology; cDNA, complementary DNA; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Pc, phthalocyanine; PDT, photodynamic therapy; PI, propidium iodide; SDS, sodium dodecyl sulfate; STS, staurosporine; TM, transmembrane.

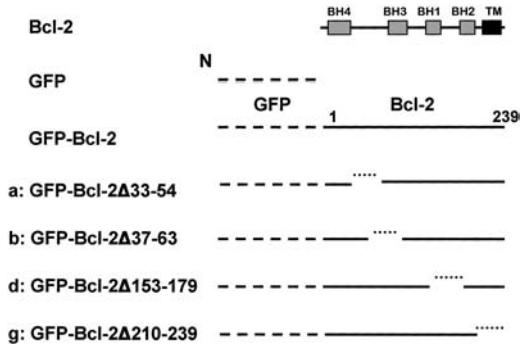

Figure 1. Design of Bcl-2 mutants in pEGFP mammalian expression vector. Human Bcl-2 cDNA (amino acids 1–239; solid line) is tagged at its N-terminus with GFP (dashed line). The positions of the four BH domains and the TM domain of Bcl-2 are shown at the top of the figure. The mutants (a, b, d and g) are indicated with reference to the amino acids deleted from each and their approximate position in Bcl-2 by a dotted line.

a Bcl-2 mutant. We examined the association between the ability of the Bcl-2 protein to be photodamaged and the sensitivity of the cells to apoptosis and to overall cell death in response to Pc 4-PDT.

MATERIALS AND METHODS

Cell culture. Human breast cancer MCF-7 cells transfected with human procaspase-3 complementary DNA (cDNA) (MCF-7c3) were cultured in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum. Human prostate cancer DU-145 cells were grown in Dulbecco modified Eagle medium containing 5% fetal bovine serum. All cultures were maintained in a humidified atmosphere at 37°C with 5% CO_2. The responses of these cells to Pc 4-PDT have been reported previously (8,11,14). Apoptosis is efficiently induced in MCF-7c3 cells by Pc 4-PDT (8,11).

Photodynamic therapy. The phthalocyanine photosensitizer Pc 4, $HOSiPcOSi(CH_3)_2(CH_2)_3N(CH_3)_2$, was provided by Dr. M. E. Kenney (Department of Chemistry, Case Western Reserve University) (18). Cells were loaded with Pc 4 by addition of an aliquot of the stock solution (0.5 m*M* in dimethylformamide) to the culture medium 16 h before irradiation. For all experiments the light source was an EFOS LED array (EFOS, Mississauga, Ontario, Canada) delivering red light (λ_{max}, 670–675 nm; bandwidth at half maximum, 24 nm; fluence rate at the level of the cell monolayer, 6–7 mW/cm^2).

DNA transfection. Generation of the Bcl-2 mutants was described previously (14). Wild-type or mutated Bcl-2 cDNA was digested with *Eco*RI and cloned into the C-terminus of green fluorescent protein (GFP) fusion vector, pEGFP-C (Clontech, Palo Alto, CA) (14). Cells were transfected with 2 μg of plasmid DNA using the transfection reagent Lipofectamine (Invitrogen, Carlsbad, CA). The efficiency of transfection was estimated by fluorescence microscopy and by flow cytometry and was generally about 20–40%.

Isolation of clones expressing Bcl-2 or Bcl-2 mutants. MCF-7c3 cells were transfected with GFP, GFP–Bcl-2, GFP–Bcl-2Δ33-54 (Mutant a), GFP–Bcl-2Δ37-63 (Mutant b) and GFP–Bcl-2Δ210-239 (Mutant g) plasmids (14). Two weeks after the transfections, GFP-positive cells were sorted under sterile conditions by flow cytometry and further cultured. After 14–21 days, the colonies were harvested, and a single clone was obtained using a limiting dilution method.

Nuclear-staining assay for apoptosis. Cells were treated with staurosporine (STS; 0.4 μ*M*) for 0, 5, 9 or 24 h and then collected and fixed in 1% formaldehyde. For PDT, cells were treated with 200 n*M* Pc 4 for 16 h and then irradiated with red light (200 mJ/cm^2). Five or 24 h after PDT, cells were collected and fixed. After the fixation, cells were stained with Hoechst 33342 (Molecular Probes, Eugene, OR). At least 200 cells were counted from each sample, and the yield of apoptotic cells was expressed as a percentage of the total population.

Clonogenic cell survival. Cells were collected from the monolayer with trypsin immediately after PDT. Aliquots of the cells were seeded into 25 cm^2 flasks in amounts sufficient to yield 50–150 colonies. After incubation for 10–14 days, the cells were stained with 0.1% crystal violet in 20% ethanol, and colonies containing at least 50 cells were counted (8,19). The plating efficiency of untreated cells was 30–40%.

Confocal microscopy. All fluorescence images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal fluorescence microscope at the Case Western Reserve University Ireland Comprehensive Cancer Center Confocal Microscopy Facility. For live cell fluorescence imaging of stable transfectants of GFP–Bcl-2 and GFP–Bcl-2 mutants, cells were plated on 35 mm glass-bottom dishes (MatTek Corp., Ashland, MA) and incubated with 100 n*M* MitoTracker Red (Molecular Probes) for 45 min at 37°C. Images of GFP fluorescence were collected using a 488 nm excitation light from an argon laser and a 500–550 nm band-pass barrier filter. Images of MitoTracker Red fluorescence were collected using a 543 nm excitation light from a He–Ne laser and a 560 nm long-pass filter (11,14).

Flow cytometry. After PDT, 1×10^6 cells were fixed in 1% formaldehyde for 30 min at 4°C. After washing twice with phosphate-buffered saline (PBS), the cells were rinsed with PBS containing 0.1% Triton X-100. The cells were stained with a solution containing 25 μg/mL propidium iodide (PI) and 10 μg/mL ribonuclease for 15 min at room temperature. Analysis was carried out at the Flow Cytometry Facility of the Ireland Comprehensive Cancer Center, Case Western Reserve University. Fluorescence measurements were made on an EPICS ESP flow cytometer (Coulter Corp., Miami, FL), activating at 488 nm and monitoring fluorescence emission at 508 nm (GFP) and 623 nm (PI) (11,14).

Western blot analysis. Cells were harvested by centrifugation and washed twice with ice-cold PBS. The cell pellets were incubated in a lysis buffer (50 m*M* Tris–HCl, pH 7.5, 120 m*M* NaCl, 1% Triton X-100, 0.2% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 m*M* phenylmethylsulfonyl fluoride and 100 m*M* NaF) on ice for 30 min and then sonicated. The protein content of the whole-cell lysates was measured using the BCA protein assay reagent (Pierce, Rockford, IL). An aliquot (20 μg) of the whole-cell lysate was separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were incubated with one of the following antibodies at appropriate concentrations for 1 h: mouse monoclonal anti-Xpress (Invitrogen), rabbit polyclonal anti-human Bax, rabbit polyclonal anti-human Bak, mouse monoclonal anti-GFP, mouse monoclonal anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and hamster monoclonal anti-human Bcl-2 (Pharmingen, San Diego, CA). After rinsing with PBS containing 0.1% (vol/vol) Triton X-100, the membranes were incubated with anti-mouse, anti-rabbit or anti-hamster immunoglobulin G conjugated to horseradish peroxidase for 1 h at room temperature. The membranes were washed and developed with Western blotting–enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Independent experiments were repeated at least three times.

Immunoprecipitation. Cells were lysed with precipitation buffer (10 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 8.0, 150 m*M* NaCl, 1% [(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, 10 μg/mL aprotinin and 10 μg/mL leupeptin). Each lysate was sonicated, and the protein content was assayed. Predetermined amounts of protein were preincubated with 30 μL of protein G–sepharose (Sigma Chemical Co., St. Louis, MO) at 4°C for 1 h. After centrifugation, the supernatants were transferred to new tubes and incubated with mouse monoclonal anti-Bax antibody (against amino acids 12–24, clone 6A7) (Pharmingen) for 1 h at 4°C (20). After the addition of protein G–sepharose, the mixture was incubated. Each mixture was centrifuged at 3000 ***g*** for 10 min and washed three times with ice-cold lysis buffer. The immunoprecipitates were separated by SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membranes, and target proteins were detected by Western blotting using rabbit polyclonal anti-human Bax antibody, as described above.

RESULTS

MCF-7c3 cells transiently transfected with Bcl-2 mutants capable of photodamage were resistant to apoptosis induced by Pc 4-PDT

This study uses a subset of Bcl-2 mutants that we constructed previously by site-directed mutagenesis (14) (Fig. 1). The two mutants that we found to be resistant to photodamage are those lacking one of the membrane anchorage regions, *i.e*. Mutant d (Bcl-2Δ153-179) and Mutant g (Bcl-2Δ210-239).

We first studied the ability of each of the GFP-tagged proteins listed in Fig. 1 to alter the induction of apoptosis by Pc 4-PDT, using transient transfection and analysis of MCF-7c3 cells by flow cytometry (Table 1). The MCF-7c3 cell line was chosen for the present study because of its efficient induction of apoptosis in response to Pc 4-PDT (8,11). For the control cells transfected with GFP alone, there was no significant difference in the extent of apoptosis between the GFP-expressing and nonexpressing cells; both subpopulations contained about 6.6% apoptotic cells before PDT, a value that may reflect some toxicity from the transfection agent alone, and 17–18% of the cells were in apoptosis 5 h after PDT. MCF-7c3 cells successfully transfected with GFP–Bcl-2 had a reduced level of apoptosis in the absence of PDT (3.4%), which indicates that Bcl-2 was protecting cells against apoptosis. There was also a reduction in the level of PDT-induced apoptosis, but this difference was not statistically significant ($P = 0.20$). A statistically significant inhibition of baseline and PDT-induced apoptosis was achieved in cells expressing either of the mutants (a or b) with deletions near the N-terminus; both mutant proteins could be photodamaged by Pc 4-PDT (14). This indicates that the deleted regions, which include a caspase-3 cleavage site at Asp-34, are not necessary for the inhibition of PDT-induced apoptosis by Bcl-2. Thus, overexpression of Bcl-2 mutants, Bcl-2Δ33-54 or Bcl-2Δ37-63, or wild-type Bcl-2 can protect against Pc 4-PDT–induced apoptosis. It should be noted that the levels of expression and efficiency of the transfection of each of these mutants were higher, in terms of GFP per expressing cell, than the levels of GFP–Bcl-2 (data not shown), which may explain the greater antiapoptotic effect of the mutants than of the wild-type protein.

The Bcl-2 mutant deleted in the C-terminal TM domain (Mutant g; GFP-Bcl-2Δ210-239) is known from our work (14) and that of others (21,22) not to become membrane bound, and it did not inhibit apoptosis (Table 1). Mutant d (Bcl-2Δ153-179), which is deleted in the region between the BH1 and BH2 domains and therefore lacks the alpha helices 5 and 6, was previously shown to bind to mitochondrial membranes exclusively (14,20) but to be resistant to photodamage, as observed by Western blot analysis (14). Expression of this mutant in MCF-7c3 cells appeared to promote apoptosis both in the absence of PDT and after Pc 4-PDT.

The flow cytometric analyses of the transiently transfected cells reveal that Bcl-2 and Bcl-2 mutants (a and b) that can be damaged by Pc 4-PDT have an antiapoptotic function against PDT. However, it is not clear whether protection against apoptosis translates into protection against overall photocytotoxicity. A valid comparison of the level of Bcl-2 photodamage and sensitivity of the cells to loss of clonogenicity requires the study of cells that are stably overexpressing the transgene. Therefore, we transfected GFP–Bcl-2 or a mutant into MCF-7c3 cells, and after 2 weeks of growth, the transfected cells were sorted by flow cytometry according to their GFP expression. With this procedure, we isolated clones of MCF-7c3 cells stably overexpressing high levels of GFP–Bcl-2 and three of the mutants (a, b and g). We were not able to isolate a clone overexpressing mutant d, probably because it promoted apoptosis in any cell in which it was expressed.

The stable transfectants have been characterized with respect to several properties that could determine the sensitivity of the cells to Pc 4-PDT. By Western blot analysis with an anti–Bcl-2 antibody, it was estimated that the clone expressing GFP–Bcl-2 had on the order of 50 times the level of Bcl-2 as the parental MCF-7c3 cells or cells stably expressing GFP alone (Fig. 2A). Interestingly, it appears that the clone overexpressing GFP–Bcl-2 had also upregulated its endogenous Bcl-2 (Fig. 2A). A similar phenomenon has been observed for Bcl-2 by Wang *et al.* (23). Because each of the mutations eliminated part of the epitope recognized by the anti–Bcl-2 antibody, an anti-GFP antibody was used to estimate the relative levels of mutant protein in cells expressing those molecules. As shown in Fig. 2B, roughly similar amounts of wild-type GFP–Bcl-2 and the C-terminal mutant were expressed in their respective cells, but, as found for the transient transfections, Mutants a and b were expressed at higher levels.

Table 1. Protection by Bcl-2 mutants against PDT-induced apoptosis: transient transfectants*

Cells	%Apoptosis (sub-G1 DNA)			
	GFP(+)		GFP(−)	
	No PDT	PDT + 5 h	No PDT	PDT + 5 h
GFP	6.6 ± 1.8	17.3 ± 3.1	6.6 ± 2.1	17.9 ± 4.3
GFP–Bcl-2	3.4 ± 0.5	13.1 ± 2.3	7.9 ± 1.3	19.1 ± 3.8
GFP–Bcl-2Δ33-54	2.3 ± 0.9	9.7† ± 1.2	8.4 ± 2.4	19.2 ± 3.2
GFP–Bcl-2Δ37-63	3.2 ± 0.6	8.5† ± 0.9	9.3 ± 1.4	17.4 ± 4.6
GFP–Bcl-2Δ210-239	5.3 ± 1.1	16.2 ± 2.9	3.8 ± 1.2	16.8 ± 3.7
GFP–Bcl-2Δ153-179	14.7 ± 3.2	25.2† ± 4.1	7.3 ± 2.6	13.8 ± 4.4

*Eight hours after the transfection, the cells were loaded with 200 n*M* Pc 4 for 16 h, then irradiated with red light (150 mJ/cm^2) and further incubated for 5 h. The dose of PDT used in these experiments (200 n*M* Pc 4 + 150 mJ/cm^2 red light) was demonstrated to produce 93 ± 4% killing of MCF-7c3 cells, as determined by clonogenic assay (11). The cells were fixed and stained with PI to detect the cellular DNA content. Each cell population was gated to separate the GFP-expressing and nonexpressing cells, and within these two populations the percentage of the cells with less than the G1 content of DNA was used as a measure of the content of apoptosis. The data from three experiments are presented.

†Significantly different from GFP(–) cells 5 h after PDT ($P < 0.05$).

GFP–Bcl-2 localizes at the mitochondria, endoplasmic reticulum membranes and nuclear envelopes in MCF-7c3 cells

We next examined the localizations of GFP–Bcl-2 fusion proteins in live cells by confocal microscopy (Fig. 3). Each of the proteins localized in their respective stable transfectants of MCF-7c3 cells in a manner similar to the localization in the transient transfectants of DU-145 cells reported previously (12). Specifically, wild-type GFP–Bcl-2 and Mutants a and b were bound to mitochondrial and endoplasmic reticulum membranes and nuclear envelopes, whereas Mutant g was found dispersed throughout the cells.

The levels of Bax and Bak were unaffected by overexpression of wild-type or mutant Bcl-2

Overexpression of Bcl-2 can lead to upregulation of the proapoptotic protein Bax (24). Accordingly, we evaluated the level of expression of Bax and its homolog Bak in each of the stable transfectants and found no difference in the levels of either proapoptotic protein as a function of Bcl-2 overexpression (Fig. 4).

Bcl-2–overexpressing MCF-7c3 cells were resistant to apoptosis caused by Pc 4-PDT and STS

To examine whether stable expression of Bcl-2 or Bcl-2 mutant protein can protect against apoptosis induced by Pc 4-PDT, we estimated apoptosis by monitoring nuclear morphology after

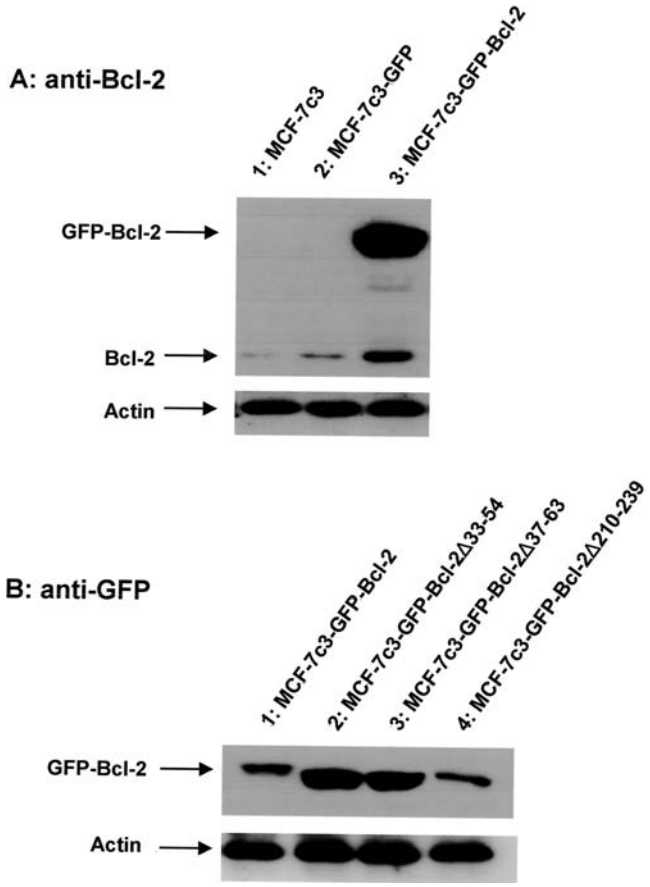

Figure 2. Bcl-2 expression levels in the parental MCF-7c3 cells and the stable transfectants. A: The Bcl-2 level was examined in parental MCF-7c3 cells (Lane 1), MCF-7c3 GFP cells (Lane 2) and MCF-7c3 GFP–Bcl-2 cells (Lane 3) using an anti-human Bcl-2 antibody by Western blot analysis. The membrane was reprobed with anti-actin to control for loading. B: An anti-GFP antibody was used to examine the expression of GFP–Bcl-2 proteins in MCF-7c3 cells stably overexpressing wild-type GFP–Bcl-2 (Lane 1), GFP–Bcl-2Δ33-54 (Mutant a, Lane 2), GFP–Bcl-2Δ37-63 (Mutant b, Lane 3) and GFP–Bcl-2Δ210-239 (Mutant g, Lane 4). The membrane was reprobed with anti-actin to control for loading.

staining with Hoechst 33342 (Table 2). STS, a strong inducer of apoptosis in MCF-7c3 cells, was used as a positive control. STS-induced apoptosis was largely blocked by overexpressed Bcl-2, Bcl-2Δ33-54 or Bcl-2Δ37-63. Pc 4-PDT (200 n*M* Pc 4, 150 mJ/cm^2 red light) was previously shown to induce morphologically typical apoptosis in MCF-7c3 cells within 5 h of photoirradiation (8,11). Of the MCF-7c3 cells overexpressing GFP or GFP–Bcl-2Δ210-239, 20–30% were in apoptosis within 5 h and considerably more within 24 h of Pc 4-PDT. However, the presence of overexpressed Bcl-2, Bcl-2Δ33-54 or Bcl-2Δ37-63 effectively blocked PDT-induced apoptosis in a manner similar to blockage of the response to STS. Thus, as with the transient transfections (Table 1), protection against apoptosis was afforded by overexpression of proteins that were capable of being photodamaged, but not by Bcl-2Δ210-239 protein, which could not be photodamaged (Table 2).

Bcl-2–overexpressing cells were resistant to loss of clonogenicity

To determine whether cells protected from apoptosis remained alive or died by a nonapoptotic process, we evaluated the sensitivity of the stably transfected cells to Pc 4-PDT by clonogenic assay. As shown by the survival curves of Fig. 5, MCF-7c3 cells overexpressing wild-type Bcl-2 or Mutant a or b were considerably more resistant to the lethal effects of Pc 4-PDT than were cells expressing either GFP alone or Mutant g. At the 10% survival level, the presence of Bcl-2 provided a dose-modifying factor of approximately 1.4. Thus, apoptosis appears to be the major mode of cell death in response to Pc 4-PDT for these MCF-7c3 cells, and Bcl-2 exerts a dominant regulatory effect on cell survival.

Bcl-2–overexpressing cells required high doses of Pc 4-PDT to produce observable photodamage

Although the above data provide a link between Bcl-2, apoptosis and cell death in Pc 4-PDT–treated MCF-7c3 cells, it is not clear how photodamage to Bcl-2 affects the cellular sensitivity to apoptosis. To begin to address this issue, we examined the extent of Bcl-2 photodamage in MCF-7c3 GFP–Bcl-2 cells by Western blot. We previously reported that the endogenous Bcl-2 of MCF-7c3 or DU-145 cells was completely lost on treatment with 200 n*M* Pc 4 and 150 mJ/cm^2 red light (9,14). When we applied similar doses of PDT to MCF-7c3 GFP–Bcl-2 cells, which express large amounts of both endogenous and transfected Bcl-2 protein, we found that most of the Bcl-2 protein was unaffected; higher doses of Pc 4 (400 n*M*), red light (600 mJ/cm^2) or both produced severe Bcl-2 photodamage (Fig. 6A). These results suggest that the high levels of Bcl-2 in the stably transfected cells exceed the capacity for photodamage by moderate doses of Pc 4-PDT and that an abundance of residual nonphotodamaged Bcl-2 after PDT provided protection against cell killing.

Bcl-2 overexpression inhibits PDT-induced Bax conformational change

It has been reported that Bax undergoes a conformational change in response to apoptotic signals based on analysis of the three-dimensional structure of Bax (25–27). To elucidate the mechanism underlying Bcl-2 protection against Pc 4-PDT–induced apoptosis, we examined the ability of Bax to undergo the conformational change associated with its activation. MCF-7c3 GFP cells and MCF-7c3 GFP–Bcl-2 cells were treated with PDT or STS, and 5 h later conformationally changed Bax protein was immunoprecipitated from cell lysates with mouse monoclonal anti-Bax (6A7) antibody, which recognizes the N-teminal Bax epitope (amino acids 12–24) (28,29), and was detected on Western blots with rabbit polyclonal anti-human Bax, which recognizes all forms of the protein. As a negative control, we used DU-145 cells, which do not express Bax (11,30). Both PDT (200 n*M* Pc 4 + 200 mJ/cm^2 red light) and STS (0.4 μ*M*, 5 h of incubation) induced Bax conformational change in MCF-7c3 GFP cells but not in MCF-7c3 GFP–Bcl-2 cells. A higher light fluence (600 mJ/cm^2), which produced Bcl-2 photodamage (Fig. 6A), was required to induce the Bax conformational change in MCF-7c3 GFP–Bcl-2 cells. These results suggest that overexpression of Bcl-2 inhibits the activation of Bax in response to Pc 4-PDT or STS.

DISCUSSION

In this study, we have shown that cells overexpressing Bcl-2 or certain Bcl-2 mutants that are capable of photodamage by Pc 4-PDT are resistant to the induction of apoptosis and to the overall lethal effects of Pc 4-PDT. These data are similar to our previous

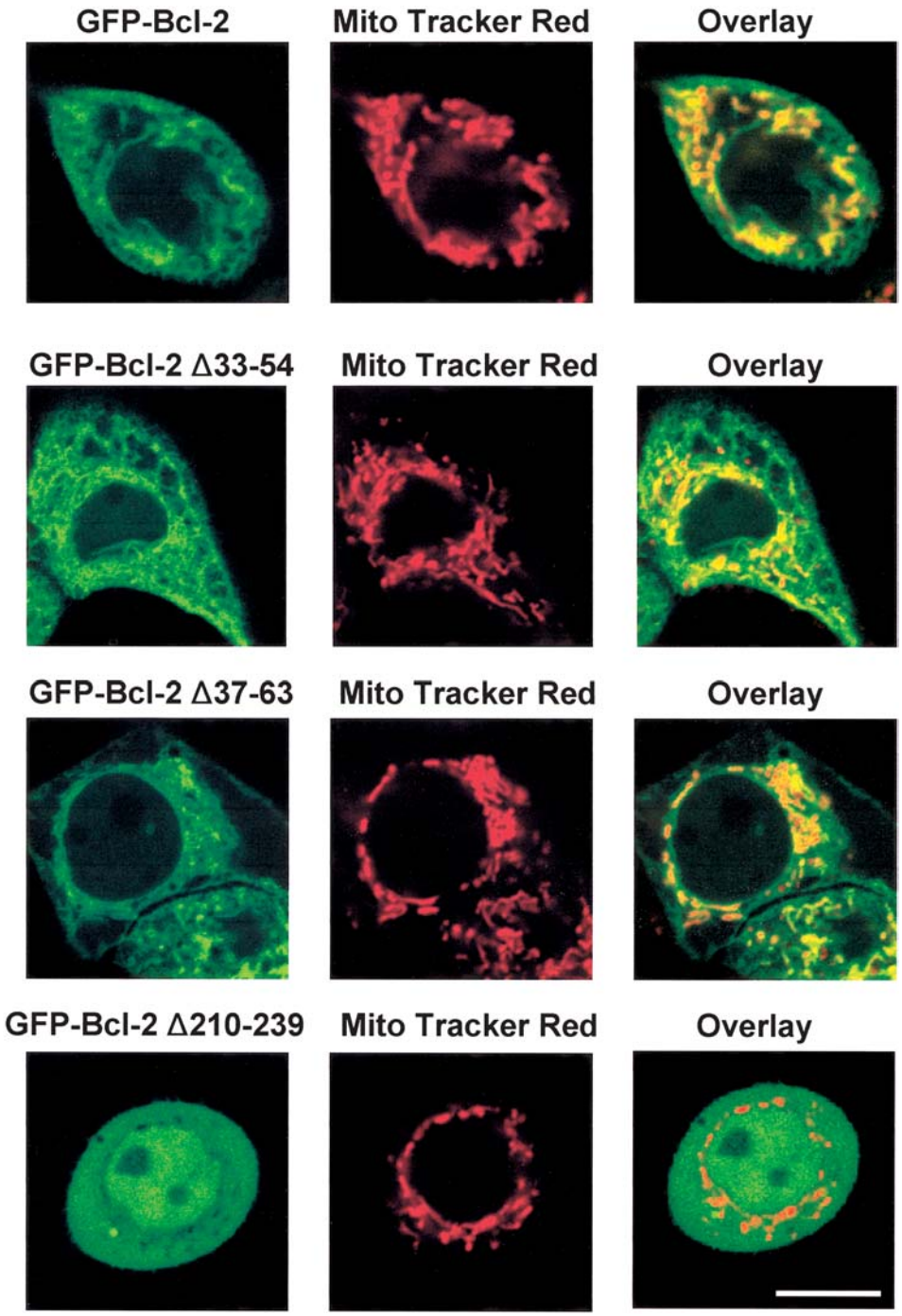

Figure 3. The subcellular localization of stably transfected GFP–Bcl-2 and GFP–Bcl-2 mutants (a, b and d). MCF-7c3 cells stably overexpressing Bcl-2, Bcl-2Δ33-54, Bcl-2Δ37-63 or Bcl-2Δ210-239 as a fusion protein with GFP were imaged by confocal microscopy. In living cells the subcellular localization of each of these proteins was determined by observing GFP fluorescence, and mitochondria were imaged by the fluorescence of MitoTracker Red. GFP and MitoTracker Red images were overlaid to show colocalization in yellow. Scale bar, 5 μm.

observations that overexpressing Bcl-2 made Chinese hamster ovary cells more resistant to apoptosis and to loss of clonogenicity on exposure to Pc 4-PDT (19).

However, cells overexpressing Bcl-2 mutants (d and g) that are not capable of photodamage (14) are not protected from cell death caused by Pc 4-PDT. Mutant d, which lacks alpha helices 5 and 6 of Bcl-2, promoted apoptosis (Fig. 2), in agreement with the observed requirement for that region for the antiapoptotic activity of Bcl-2 (15,16). Mutant g, which is deleted in the C-terminal TM domain, did not inhibit apoptosis, in agreement with previous reports (21,22).

An important question is why cells overexpressing Bcl-2 are resistant to the induction of PDT-induced apoptosis. One possibility is that Bcl-2 or other components of complexes formed by Bcl-2 may scavenge singlet oxygen and other reactive oxygen species produced by Pc 4-PDT. PDT produces reactive oxygen

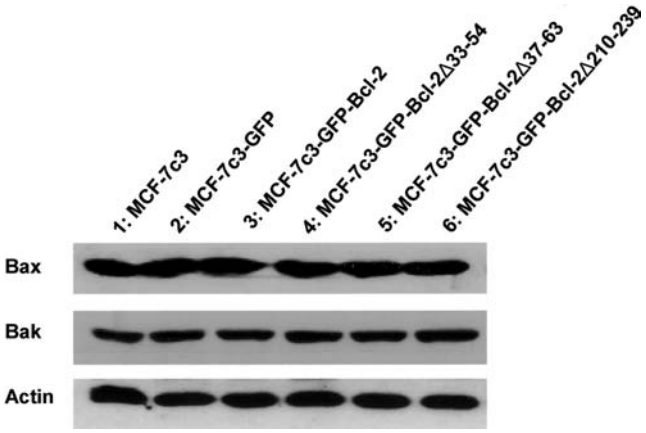

Figure 4. Expression of Bax and Bak in parental MCF-7c3 cells and the derived stable transfectants. Whole-cell lysates were subjected to electrophoresis and blotted onto polyvinylidene difluoride membranes. The levels of Bax and Bak were examined by Western blot analysis. The membrane was reprobed with anti-actin to control for loading. Lane 1, MCF-7c3 cells; Lane 2, MCF-7c3 GFP cells; Lane 3, MCF-7c3 GFP–Bcl-2 cells; Lane 4, MCF-7c3 GFP–Bcl-2Δ33-54 cells; Lane 5, MCF-7c3 GFP–Bcl-2Δ37-63 cells; and Lane 6, MCF-7c3 GFP–Bcl-2Δ210-239 cells.

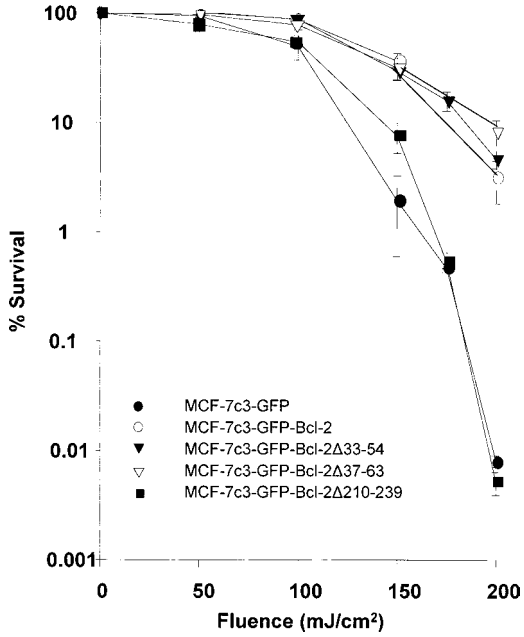

Figure 5. Loss of clonogenicity of MCF-7c3 stable transfectants as a result of Pc 4-PDT. Exponentially growing cultures of each cell line were treated with 200 n*M* Pc 4 for 16 h and then irradiated with red light (0–200 mJ/cm^2), as indicated on the abscissa. Immediately after PDT, cells were trypsinized, collected, diluted and plated at appropriate concentrations. Data for PDT-treated cells were normalized to the plating efficiency of untreated cells of the same cell line. Each datum is the mean ± standard deviation for results from three independent experiments. MCF-7c3 GFP cells (closed circle), MCF-7c3 GFP–Bcl-2 cells (open circle), MCF-7c3 GFP–Bcl-2Δ33-54 cells (closed triangle), MCF-7c3 GFP–Bcl-2Δ37-63 cells (open triangle) and MCF-7c3 GFP–Bcl-2Δ210-239 cells (closed square).

species in membranes and causes photooxidative damage to proteins and lipids that reside within a few nanometers of the photosensitizer-binding sites (5,6). It has been proposed that Bcl-2 protects cells from H_2O_2-induced oxidative death and regulates an antioxidant pathway at sites of free radical generation (31). However, others have suggested that the presumed antioxidant mechanism was in reality due to the prevention of the release of cytochrome *c* from the mitochondria (32). The available data are consistent with Bcl-2 acting either in an antioxidant pathway or as a block to cytochrome *c* release to interfere with apoptosis and cell death caused by Pc 4-PDT.

Another possible reason for resistance to apoptosis after Pc 4-PDT in Bcl-2–overexpressing cells is that the Bax–Bcl-2 ratio may determine the sensitivity to PDT. A high Bax–Bcl-2 ratio is known to promote the initiation of apoptosis (24,33). Srivastava *et al.* (33) reported that antisense Bcl-2 sensitized A431 cells to Pc 4-PDT and attributed the increased apoptotic response to the increased Bax–Bcl-2 ratio. Kim *et al.* (24) found that the enhanced apoptotic response to PDT in Bcl-2–overexpressing MCF-10A cells could be attributed to an elevated Bax–Bcl-2 ratio after PDT, resulting from the upregulation of Bax along with photodamage to Bcl-2. Because we did not observe upregulation of Bax or Bak levels in any of the MCF-7c3 cells in our study (Fig. 4), the difference in these responses might be accounted for by the differences in the response of Bax (24). In our study, MCF-7c3 GFP–Bcl-2 cells express high levels of Bcl-2 protein, and abnormally high doses of Pc 4 or light were required to reduce the Bcl-2 level and thereby elevate the Bax–Bcl-2 ratio. We suggest that the presence of residual nonphotodamaged Bcl-2 may control the sensitivity to apoptosis after Pc 4-PDT.

Recently, Vantieghem *et al.* (34) reported that PDT with hypericin, a nonporphyrin photosensitizer that targets microtubules, does not cause Bcl-2 destruction but instead produces a G2–M delay, during which Bcl-2 becomes transiently phosphorylated.

Table 2. Protection by Bcl-2 mutants against PDT-induced apoptosis: stable transfectants*

Cells	Control	STS			PDT	
		5 h	9 h	24 h	5 h	24 h
GFP	0.5 ± 0.3	65.1 ± 7.6	85.5 ± 5.0	98.5 ± 1.0	28.8 ± 2.6	85.3 ± 1.3
GFP–Bcl-2	0.3 ± 0.3	1.2 ± 0.3	2.8 ± 0.6	10.8 ± 0.8	2.0 ± 0.5	5.3 ± 1.2
GFP–Bcl-2Δ33-54	0.5 ± 0.3	1.4 ± 0.2	2.0 ± 0.5	11.2 ± 1.3	1.7 ± 0.3	9.5 ± 1.5
GFP–Bcl-2Δ37-63	0.3 ± 0.3	1.2 ± 0.3	3.8 ± 0.3	13.5 ± 0.5	2.0 ± 0.5	4.8 ± 0.8
GFP–Bcl-2Δ210-239	0.5 ± 0.3	43.8 ± 1.3	83.6 ± 2.8	86.6 ± 0.8	22.1 ± 1.6	56.0 ± 1.7

*Cells were treated with STS (0.4 μ*M*) for 0, 5, 9 or 24 h, collected and fixed. For PDT, cells were treated with 200 n*M* Pc 4 for 16 h and then irradiated with red light (200 mJ/cm^2). Five or 24 h after PDT, cells were collected and fixed. After fixation, cells were stained with Hoechst 33342. At least 200 cells were counted from each sample, and the yield of apoptotic cells was expressed as the percentage of the total population. Independent experiments were repeated at least three times.

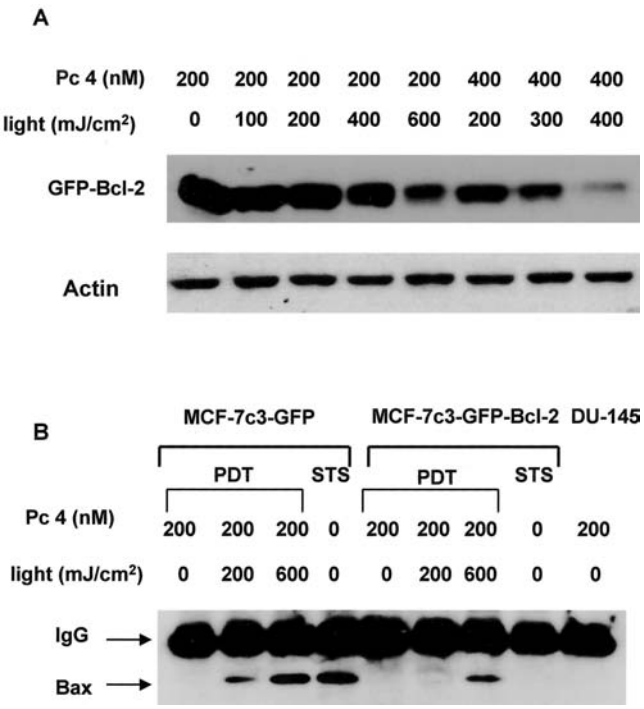

Figure 6. A: The photosensitivity of overexpressed wild-type Bcl-2. MCF-7c3 GFP–Bcl-2 cells were treated with Pc 4 (200 or 400 n*M*) for 16 h and then irradiated with red light (0–600 mJ/cm^2). Immediately after PDT, cells were collected, washed and lysed. An aliquot (20 μg) of the whole-cell lysate was separated by SDS-PAGE. The Bcl-2 level was examined on Western blots using a mouse monoclonal anti-GFP antibody. B: The influence of Bcl-2 overexpression on Bax conformational change after PDT or STS treatment. MCF-7c3 GFP, MCF-7c3 GFP–Bcl-2, and DU-145 cells were treated with 200 n*M* Pc 4 for 16 h and then irradiated with red light (0, 200 or 600 mJ/cm^2). Alternatively, some cells were treated with 0.4 μ*M* STS for 5 h. Five hours after PDT or STS treatment, cells were collected and lysed in [(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate lysis buffer. The conformationally changed Bax was immunoprecipitated with mouse monoclonal anti-Bax antibody (against amino acids 12–24, clone 6A7). The immune complexes were analyzed by SDS-PAGE with a rabbit polyclonal anti-human Bax antibody.

Thus, hypericin-PDT may act like other microtubule-targeting agents such as paclitaxel (35–38). In contrast, for Pc 4-PDT it appears that phosphorylation of Bcl-2 is not important for inhibition of apoptosis because MCF-7c3 cells overexpressing Bcl-2Δ37-63 (Mutant b) were resistant to apoptosis and loss of clonogenicity despite the deletion of a key phosphorylation site on Thr56 (35–38) (Figs. 2 and 5).

Photosensitizers that localize in lysosomes also do not cause photodamage to Bcl-2, but induce apoptosis through release of lysosomal cathepsins (39). However, for mitochondrion-targeting photosensitizers like Pc 4, Bcl-2 and its homolog Bcl-xL are major phototargets. We have shown previously that Pc 4-PDT photodamages Bcl-2 and forms large complexes of Bcl-2, as detected by Western blot analysis (9,14). However, it is unclear whether photodamaged Bcl-2 retains its antiapoptotic function. Our data suggest that photodamage destroys the ability of Bcl-2 to prevent apoptosis, but when high levels of overexpressed Bcl-2 or functional mutants (a or b) are present, high doses are required to produce sufficient photodamage to inactivate the large amount of Bcl-2 protein.

It has been reported that during apoptosis the Bcl-2 protein can be cleaved at Asp34 by caspase-3 and the cleaved Bcl-2 is converted to a Bax-like proapoptotic effector (40,41). Such a cleavage does not appear to be important for Pc 4-PDT–induced apoptosis. First, photodamage was observed in Mutant a, which lacks Asp34 and thus cannot be cleaved at that site (14). Second, if Bcl-2 were converted to a proapoptotic protein, one would expect the sensitivity of cells to increase with Bcl-2 content; however, in the case where Bax expression did not change (Fig. 4), the cells became more resistant when the Bcl-2 content was increased (Fig. 5).

In summary, our data indicate that the extent of Bcl-2 photodamage may determine the sensitivity of cancer cells to apoptosis and to overall cell killing caused by PDT. Pc 4-PDT damages Bcl-2 photochemically, but the photodamaged Bcl-2 is neither phosphorylated nor converted to a proapoptotic molecule. Although overexpression of Bcl-2 protein inhibited Bax activation caused by Pc 4-PDT, further studies are required to elucidate whether the photodamaged Bcl-2 can associate with Bax or Bak.

The application of these findings to clinical PDT may depend on the levels of the Bcl-2 family proteins, especially Bcl-2, Bcl-xL and Bax, in the tumor being treated; the intracellular target of the photosensitizer (mitochondria *vs* lysosomes); the contribution of vascular effects to the overall tumor response and the feasibility of increasing the PDT dose to overcome any resistance afforded by elevated amounts of Bcl-2. Although the present study shows that highly overexpressed Bcl-2 can make tumor cells relatively resistant to PDT, it is possible that a standard PDT dose is sufficient to photodamage all the Bcl-2 found in most tumor cells.

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