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Enhanced Apoptotic Response to Photodynamic Therapy after bcl-2 Transfection[1]

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ABSTRACT

Apoptosis is a cellular death process involving the sequential activation of a series of caspases, endonucleases, and other enzymes. The initiation of apoptosis can be inhibited by overexpression of bcl-2 and certain other members of a related family of proteins. We examined the effects of bcl-2 overexpression on the apoptotic response to photodynamic therapy (PDT), using aluminum phthalocyanine as the photosensitizing agent. In this study, we compared the immortalized human breast epithelial cell line MCF10A with a subline (MCF10A/bcl-2) transfected with the human *bcl-2* gene. The latter was ~2-fold more sensitive to the phototoxic effects of PDT. At a 50 mJ/cm^2 light dose, photodamage to MCF-10A/bcl-2 resulted in a greater loss of the mitochondrial membrane potential ($\Delta\Psi_m$), enhanced release of mitochondrial cytochrome *c*, a more rapid and greater activation of caspase-3, and a greater apoptotic response. Western blot analysis revealed that the transfected cell line showed overexpression of both bcl-2 and bax, and that PDT caused selective destruction of bcl-2, leaving bax unaffected. The greater apoptotic response by the transfected line is, therefore, attributed to the higher bax:bcl-2 ratio after photodamage.

INTRODUCTION

PDT[3] involves the irradiation of photosensitized cells, leading to photodamage at subcellular sites where the photosensitizing agent has accumulated (1). The first report of apoptotic cell death after PDT appeared in 1991 (2). At the time, little was known regarding mechanisms involved in programmed cell death, but we now know that initiation of the apoptotic program can be triggered by the translocation of cytochrome *c* from mitochondria to the cytosol (3). PDT using a photosensitizing agent that mediates selective mitochondrial photodamage can initiate a rapid apoptotic response if other proteins required for expression of apoptosis are undamaged (4, 5); otherwise, a necrotic outcome can result (6). Overexpression of bcl-2 inhibits apoptosis by antagonizing release of mitochondrial cytochrome *c* (7, 8). In a finding consistent with these observations, He et al. (9) reported that *bcl-2* transfection of a Chinese hamster ovary cell line led to partial resistance to apoptotic cell death after PDT. In this report, we describe the effect of *bcl-2* transfection on the immortalized human breast epithelial cell line MCF10A (10), a near-diploid cell line that appeared during long-term culture of breast tissue in low-calcium medium. We had anticipated that this procedure would also antagonize PDT-induced apoptosis in this system, but a contrary result provided the rationale for this investigation.

MATERIALS AND METHODS

Cell Culture Conditions. The development and characterization of the MCF10A cell line have been described elsewhere (10). We reported previously a procedure for preparation of bcl-2-overexpressing MCF10A clones (11). Briefly, the *bcl-2* gene, under the control of cytomegalovirus promoter (provided by Dr. S. Korsmeyer), was introduced into MCF10A cells using Lipofectin (Sigma). Stable transfectants were selected in the presence of 400 μg/ml G418, and individual clones were isolated and characterized.

The human bax cDNA in a pSFFV-neo expression vector (provided by Dr. S. Korsmeyer, Washington University School of Medicine, St. Louis, MO) was introduced into MCF10A cells using FuGENE 6 reagent (Boehringer Mannheim). Fifty % confluent cells in a 60-mm dish were transfected in 3 ml of culture medium containing 100 μl of FuGENE 6 reaction mixture. The FuGENE 6 mixture was prepared as follows; 2 μg of expression vector or control vector was mixed with 5 μl of FuGENE6 in 100 μl of serum free-medium. The mixture was incubated at room temperature for 30 min before transfection. Cells were harvested 36–48 h later.

Cells were cultured in 5% CO_2 in DMEM/F12 supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 0.1 μg/ml cholera enterotoxin, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 0.5 μg/ml fungizone.

Photosensitizer. AlPc was obtained from Kodak and recrystallized from α-chloronaphthalene. Stock solutions of AlPc in ethanol were diluted 1:1000 into cell suspensions for these studies to yield a final concentration of 0.3 μM. Accumulation of AlPc by the two cell lines was measured after incubation for 15 min at 37°C. The ratio of intracellular *versus* extracellular AlPc was determined by comparing the fluorescence in media *versus* extracts of washed cells.

Fluorescent Probes. Fluorescent probes for the mitochondrial membrane potential (MTO) and for nuclear morphology (HO342) were purchased from Molecular Probes (Eugene, OR). Fluorescence studies were carried out with a Nikon Labophot microscope fitted with a digital video camera (Photometrics, Tucson, AZ). A 600-nm low-pass filter was inserted into both the excitation path and the camera entry port to reduce transmission of infra-red light from the mercury source, which will otherwise fog the CCD detector. Images were processed with MetaMorph software (Universal Imaging Corp., West Chester, PA).

Photodynamic Therapy and Its Consequences. Photosensitized cell cultures were irradiated at 10°C, using specified light doses. Light was provided by a 600 W quartz-halogen lamp with IR radiation removed by 10 cm of water and an 850-nm cutoff filter. The bandwidth was further confined to 660 ± 5 nm by an interference filter (Oriel). The effect of photodamage on the mitochondrial membrane potential ($\Delta\Psi_m$) was assessed directly after irradiation or 4 h later, using the fluorescent probe MTO. PDT-induced changes in nuclear morphology were examined 4 and 24 h after irradiation by labeling cells with HO342. For the latter determination, three fields of 100 cells were surveyed, and the percentage of apoptotic nuclei was reported. These procedures have been described previously (4, 12). Viability studies were carried out using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 96 h after PDT (13).

Immunoblot Analysis. Extracts were prepared from 10^6 cells in 125 mM Tris-HCl (pH 6.8) buffer containing 2% SDS and 10% glycerol. The protein concentration was measured using BCA protein assay reagents (Pierce, Inc., Rockford, IL). Protein samples were heated to 100°C for 10 min in the presence of 5% β-mercaptoethanol, chilled on ice, and subjected to SDS-PAGE analysis, followed by electrophoretic transfer to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.02% NaN_3, and 0.2% Tween-20 (T-TBS) for 1 h at room temperature and incubated with anti-bcl-2, anti-bax, or anti-β-actin antibodies in T-TBS. After several washes with T-TBS, the blot was incubated with a horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody. The antigen was detected using the ECL detection system (Pierce) according to the manufacturer's instruction. Release of cytochrome *c* from mitochondria into the cytosol was assessed by Western blot

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[3] The abbreviations used are: PDT, photodynamic therapy; AlPc, aluminum phthalocyanine; MTO, MitoTracker Orange (Molecular Probes); DEVD-afc, asp-glu-val-asp-aminofluoro coumarin; HO342, Höchst dye 33342 (bis-benzamide).

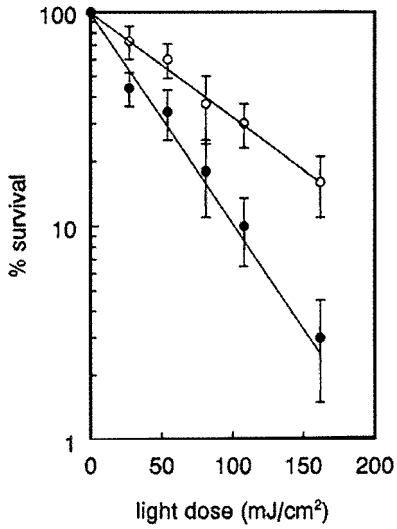

Fig. 1. Loss of viability of MCF10A (○) and MCF10A/bcl-2 (●) after photodynamic therapy. Cells were incubated with 0.3 μM AlPc and then irradiated with the specified light dose. Survival is expressed as a percentage of control (cells exposed to drug in the dark) using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 96 h after irradiation. Data are reported as means of three experiments; *bars*, SD.

analysis (5) using control cells and in cells photosensitized and treated with a light dose of 50 mJ/cm^2.

Caspase-3 Activity. Cells were collected after incubation at 37°C after irradiation (50 or 100 mJ/cm^2) and then lysed in 50 mM Tris buffer (pH 7.5) containing 0.03% Nonidet and 1 mM DTT. Nuclei were removed by low-speed centrifugation (800 × *g*, 5 min), and the cytosol fraction was incubated with 40 μM DEVD-afc, 10 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT in a total volume of 200 μl for 120 min at 37°C. Fluoromethylcoumarin fluorescence, released by caspase activity, was measured using 360-nm excitation. A CCD device (Instaspec IV; Oriel, Stratford, CT) fitted with a monochromator was used to measure the fluorescence emission spectrum. The intensity at the optimum (~450 nm) was measured. Units of enzyme activity are measured as pmols of substrate hydrolyzed/mg cells/mg protein/60 min. In all cases, control determinations were made on extracts of untreated cells. The system was calibrated with known levels of trifluoromethylcoumarin.

RESULTS

MCF10A is an adhering human breast epithelial cell line derived from human fibrocystic tissue and immortalized by long-term passage in calcium-deficient medium (10). We used this line, and a stable subline transfected with *bcl-2*, to examine the effect of bcl-2 overexpression on the apoptotic response to PDT. The photosensitizing agent was AlPc, an agent that initiated apoptosis in either of two murine leukemia cell lines, with mitochondria being the predominant target (6). After a 15-min incubation, AlPc was concentrated ~110-fold by MCF10A or MCF10A/bcl-2, *i.e.*, there was no difference in concentrative ability after transfection. There was, however, a marked difference in PDT responsiveness; *bcl-2* transfection resulted in a decrease in the light dose required for 90% loss of viability (Fig. 1).

Four h after irradiation, using a light dose of 50 mJ/cm^2, we observed <5% apoptotic cells in MCF10A and small but detectable loss in $\Delta\Psi_m$, as distinguished by the MTO labeling pattern (Fig. 2). In contrast, a substantial number of apoptotic cells were detected in the transfected subline, along with a markedly diminished intensity of MTO fluorescence (Fig. 2). When the incubation time was prolonged to 24 h after irradiation, greater numbers of apoptotic nuclei were detected in both cell lines (Table 1). Increasing the light dose promoted the apoptotic response in both cell lines, but MCF10A/bcl always showed the greater response.

Western blot analysis for bcl-2 and bax was carried out before and immediately after irradiation. Compared with the parent MCF10A, the transfected line exhibited enhanced levels of both bcl-2 and bax (Fig. 3). After irradiation at 10°C, there was a complete loss of the bcl-2 signal in MCF10A, and the appearance of a band of lower molecular weight (Fig. 3, *Lane 2*), representing a photodegradation product. In the transfected line, the bcl-2 signal was markedly diminished, and the degradation product was also detected. At a 150 mJ/cm^2 light dose, signals from both bcl-2 and the degradation product were abolished in both cell lines (not shown). In no case was there any effect on bax expression. Western blot analysis for cytosolic cytochrome *c* indicated that the level of translocation from the mitochondria was promoted by PDT and further promoted when MCF10A/bcl-2 cells were used (Fig. 3).

Using the fluorogenic substrate DEVD-afc, we could not detect caspase-3-like activity in either line before irradiation (Table 1). Four h after a 50 mJ/cm^2 light dose to AlPc-sensitized cells, DEVDase activity was detected only in the transfected cell line. After 24 h, both lines exhibited enhanced levels of DEVDase activity; this was ~8-fold higher in the transfected cell line. Use of a 100 mJ/cm^2 light dose increased the level of DEVDase activity in both cell lines.

Transfection of MCF10A with *bax* led to production of an unstable cell line, with DEVDase activation and apoptotic nuclear morphology detectable within 48 h (Table 1). The transfection efficiency was ~20%. These cells showed an enhanced apoptotic response to mitochondrial photodamage. In contrast, levels of DEVDase activity and numbers of apoptotic cells in control cultures and in MCF10A/bcl-2 cells were not elevated significantly.

DISCUSSION

The first report on PDT-initiated apoptosis (2) contained a proposal for a possible mechanism; mitochondrial photodamage could result in degradation of bcl-2 and related proteins that

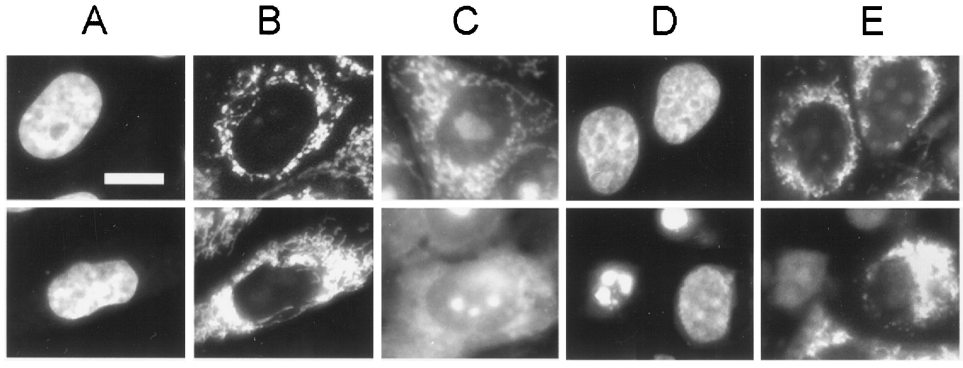

Fig. 2. Effects of photodynamic therapy with AlPc (light dose, 50 mJ/cm^2) on MCF10A (*top*) and MCF10A/bcl2 (*bottom*) labeled with HO342 or MTO. *A*, HO342 control; *B*, MTO control; *C*, MTO label directly after irradiation; *D*, HO342 label 4 h after irradiation; *E*, MTO label 4 h after irradiation. *Bar*, 10 μm.

Table 1 *DEVDase activity and apoptotic morphology in control and photodamaged cells*

Cells were treated with 0.3 μM AlPc for 15 min and then irradiated with 50 or 100 mJ/cm^2 of red light. DEVDase activity (units = pmols/mg protein/60 min) was measured after an additional incubation for 4 or 24 h in growth medium at 37°C. Results are mean ± SD of three determinations. Dark controls were assayed 24 h after transfection. The percentage of apoptotic cells was estimated from a survey of three fields of 100 cells labeled with HO342.

	MCF10A[a]					
	0 mJ/cm^{2b}		50 mJ/cm^{2b}		100 mJ/cm^{2b}	
	DEVDase	% apoptotic	DEVDase	% apoptotic	DEVDase	% apoptotic
Dark controls	0.5 ± 0.1	<2				
PDT + 4 h			1.4 ± 0.3	<5	7 ± 3	31 ± 6
PDT + 24 h			26.3 ± 2.7	25	65 ± 6	60 ± 5
	MCF10A/bcl-2[a]					
Dark controls	0.6 ± 0.3	<2				
PDT + 4 h			13 ± 3.2	41 ± 4	24 ± 5	75 ± 4
PDT + 24 h			102 ± 16	65 ± 7	110 ± 9	83 ± 3
	MCF10A/bax[a]					
Dark controls	25 ± 4.1	21 ± 4				
PDT + 4 h			36 ± 5.9	52 ± 5		

[a] Cell line.
[b] Light dose.

normally function as apoptotic suppressors (14). A later study demonstrated that transfection of a Chinese hamster ovary cell line with *bcl-2* yielded a cell line 1.5-fold more resistant to PDT phototoxicity than the parent cell line (9).

In contrast to the latter report, we observed a different pattern. A subline of MCF10A transfected with *bcl-2* was more sensitive to the lethal effects of PDT (Fig. 1), showed a greater apoptotic response (Fig. 2), and a more rapid and extensive activation of caspase-3-like activity (Table 1), compared with the parent cell line. We had reported previously that PDT, resulting in mitochondrial photodamage, caused the release of cytochrome *c* into the cytosol (5), a translocation that can initiate the apoptotic program (3).

Because bcl-2 inhibits the loss of cytochrome *c* from mitochondria (7, 8), it might be anticipated that bcl-2 overexpression would protect cells from PDT-induced apoptosis. An explanation for the enhanced PDT responsiveness of MCF10A/bcl-2 was suggested by the finding that the transfection led to overexpression of both bcl-2 and bax (Fig. 3). The stabilization of bax by bcl-2 overexpression has been reported before (15). Stabilization of bax in the MCF10A/bcl-2 cell line did not affect the inhibition of apoptosis initiated by a variety of stimuli including ionizing radiation, hydrogen peroxide, and calcium ionophores (11, 16). Because mitochondrial photodamage catalyzed by AlPc resulted in the selective degradation of bcl-2 without affecting bax, the enhanced apoptotic response to PDT in the MCF10A/bcl-2 cell line can therefore be attributed to the elevated bax:bcl-2 ratio after PDT. A high ratio is known to promote the initiation of apoptosis (17).

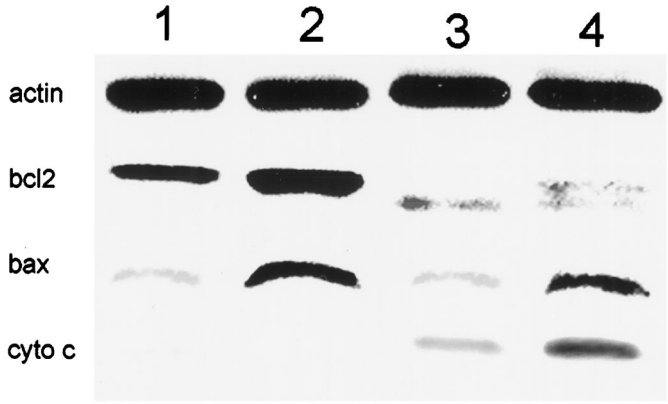

Fig. 3. Western blot analysis of actin, bcl-2 (*bcl2*), bax, and cytochrome *c* (*cyto c*) in control cells and in cells directly after PDT was carried out at 10°C. Cells were photosensitized with AlPc and then irradiated with a light dose of 100 mJ/cm^2.

The critical function of *bcl-2* gene product is the prevention of cytochrome *c* release from mitochondria, inhibiting activation of caspases at the outer mitochondrial membrane (8). However, increasing evidence suggests a pleiotropic role for bcl-2 in apoptosis regulation (16, 18, 19). In addition to the mitochondrial membrane, bcl-2 is also localized to the outer nuclear and endoplasmic reticulum. We showed previously that bcl-2 increased calcium uptake through the up-regulation of the endoplasmic reticulum calcium pump expression and enhanced lumenal Ca^{2+} concentration of endoplasmic reticulum in MCF10A cells (16). A recent report showed that bcl-2 activates the transcription factor nuclear factor-κB through degradation of its inhibitor IκBa (19). bcl-2 was also shown to inhibit activation of c-Jun NH_2-terminal kinases, which are known to play a role in apoptosis and gene expression (18). These results suggest that inhibition of apoptosis by bcl-2 may involve regulation of gene expression through modulation of central signaling molecules, including Ca^{2+} flux, transcription factors, and kinase activity. Consistent with this, we showed previously that bcl-2 down-regulates $p21^{WAF1/CIP1}$ expression, an inhibitor of cyclin-dependent kinases, in MCF10A cells. Although bcl-2 down-regulated $p21^{WAF1/CIP1}$ expression at confluent cells or after irradiation, serum-induced $p21^{WAF1/CIP1}$ was not inhibited by bcl-2 (11). Modulation of $p21^{WAF1/CIP1}$ and calcium pump expression by bcl-2 suggests that the role of bcl-2 in the inhibition of apoptosis may be related to its ability to regulate cell cycle progression. However, it is unlikely that PDT-induced apoptosis depends on cell cycle distribution, because apoptosis is rapidly induced after mitochondrial photodamage.

In the present study, we propose that the enhanced PDT responsiveness of MCF10A/bcl-2 results from bcl-2-mediated increase in the stability of bax, a proapoptotic gene product. A transient bax transfection was found to lead to both spontaneous apoptosis and an enhanced apoptotic response to PDT (Table 1). Bax may promote apoptosis by triggering pore-forming activity in the mitochondrial membrane (20) and can promote release of cytochrome *c* from isolated mitochondria (21). Moreover, bcl-2 can antagonize bax-induced initiation of apoptosis after release of cytochrome *c* (22). It was reported previously that the caspase-mediated conversion of bcl-2 to a bax-like molecule can also increase the apoptotic response (23). Caspase-induced bcl-2 degradation after PDT is not likely to be significant under conditions reported here, because the degradation was observed immediately after irradiation at 10°C (Fig. 3) and is considered to be the direct result of photodamage.

The suggestion made by Agarwal *et al.* (2), that photodamage to bcl-2 could promote an apoptotic response to PDT, can now be put into the proper context. PDT-induced mitochondrial photodamage is now known to cause release of cytochrome *c* into the cytosol and the triggering of apoptosis (4–6). Moreover, bcl-2 overexpression can lead to impaired apoptosis after PDT (9). If, however, bcl-2 overexpression also leads to stabilization of bax, selective bcl-2 photodamage can result in a high bax:bcl-2 ratio and an enhanced apoptotic response to mitochondrial photodamage.

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