Bcl-2 overexpression blocks caspase activation and downstream apoptotic events instigated by photodynamic therapy

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Summary Treatment with the photosensitizer benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin) followed by irradiation with visible light induces apoptosis in human acute myelogenous leukaemia HL-60 cells. Photocactivation of BPD-MA induces procaspase 3 (CPP32/Yama/apopain) and procaspase 6 (Mch2) cleavage into their proteolytically active subunits in these cells. The Bcl-2 proto-oncogene product has been shown to protect cells from a number of proapoptotic stimuli. In the present study, the influence of Bcl-2 overexpression on cellular resistance to photocactivation of BPD-MA was studied. Overexpression of Bcl-2 in HL-60 cells prevented apoptosis-related events including caspase 3 and 6 activation, poly(ADP-ribose) polymerase cleavage and the formation of hypodiploid DNA produced by BPD-MA (0–200 ng ml⁻¹) and light. However, Bcl-2 overexpression was less effective at preventing cell death that occurred after photocactivation at high levels (50–100 ng ml⁻¹) compared with lower doses (10–25 ng ml⁻¹) of BPD-MA. These results indicate that caspase 3 and 6 activation and their regulation by Bcl-2 may play important roles in photodynamic therapy (PDT)-induced cell killing.

Keywords: apoptosis; photodynamic therapy; Bcl-2; caspase; resistance; leukaemic cells.

Photodynamic therapy (PDT) is an approved clinical technique for the treatment of malignancies. PDT involves the topical or systemic application of a photosensitizing agent followed by illumination at a specific ‘activating’ wavelength of light (Gomer et al, 1988; Jamieson et al, 1993; Kick et al, 1995). Light activation of photosensitizers is believed to kill cells by catalysing the production of reactive oxygen intermediates (Gomer et al, 1988). Our laboratory is interested in the biological activity of the chlorin-type photosensitizer, benzoporphyrin derivative monoacid ring A (BPD-MA) (Richter et al, 1987). BPD-MA accumulates to higher levels in leukaemic cells compared with normal blood mononuclear cells (Jamieson et al, 1990, 1993). Further, using light-activated BPD-MA, in vitro experiments have demonstrated a selective killing of leukaemic cells over normal haematopoietic progenitor cells (Jamieson et al, 1990; Gluck et al, 1996). Electron microscopy, histological and biochemical studies have shown that PDT with a variety of photosensitizers induces apoptosis in different cell types (Gomer et al, 1988; Zaidi et al, 1993; Tajiri et al, 1996; Granville et al, 1997). Treatment of human promyelocytic leukaemia HL-60 cells with BPD-MA and light rapidly induces DNA fragmentation, caspase activation and apoptotic cell death (Granville et al, 1997, 1998a). However, it cannot be ruled out that PDT might also inflict damage to cells which promote passive necrotic cell death (Noodt et al, 1996).

It is now established that proteolytic cleavage of key cellular substrates is a fundamental biochemical event underlying the apoptotic process (Casciola-Rosen et al, 1996). One of the most intensively studied of these proteases, caspase 3 (previously termed CPP32/Yama/apopain), normally resides in the cytosolic fraction of cells as an inactive precursor and is proteolytically activated in cells undergoing apoptosis (Schlegel et al, 1996). Increasing numbers of caspase 3 substrates have been identified and include poly(ADP-ribose) polymerase (PARP) (Nicholson et al, 1995), sterol regulatory element binding proteins (Wang et al, 1996), the U1-associated 70-kDa protein (Casciola-Rosen et al, 1996), DNA-dependent protein kinase (Casciola-Rosen et al, 1996) and DNA fragmentation factor (DFF) (Liu et al, 1997). Recent studies have demonstrated that caspase 3 activation and DNA fragmentation are directly linked through the caspase-3-mediated cleavage of DNA fragmentation factor (DFF) (also known as inhibitor of caspase-activated deoxyribonuclease, ICAD), a cytosolic factor which binds to and inhibits the activity of an endonuclease (caspase-activated nuclease, CPAN or caspase-activated deoxyribonuclease, CAD) directly responsible for DNA fragmentation during apoptosis (Liu et al, 1997; Enari et al, 1998; Halenbeck et al, 1998, Sakahira et al, 1998). This endonuclease, CAD/CPAN, is only activated during apoptosis and is, therefore, believed to be responsible for DNA fragmentation (Halenbeck et al, 1998; Sakahira et al, 1998). We have shown that caspase-3-dependent cleavage of DFF occurs in PDT-treated HL-60 (Granville et al, 1998b) and HeLa cells (Carthy et al, 1998).

Caspase 6 (Mch2) is also mobilized in response to certain apoptotic stimuli (Fernandes-Alnemri et al, 1995; Orth et al, 1996; Srinivasula et al, 1996). Caspase 6 is believed to be responsible...
for nuclear lamin cleavage which may contribute to the nuclear degradation observed during apoptosis (Orth et al., 1996; Srinivasula et al., 1996; Takehishi et al., 1996).

One of the most studied cell survival genes is Bcl-2 (Gajewski and Thompson, 1996; Reed, 1997). Bcl-2 was discovered as an overexpressed protein in human B-cell lymphomas arising as a result of a t(14;18) chromosomal translocation (Pegoraro et al., 1984; Tsujimoto et al., 1985). The overexpression of Bcl-2 has been shown to protect different cell types against apoptosis induced by such diverse stimuli as viral infection, hypoxia, ionizing radiation or chemotherapeutic agents (Shimizu et al., 1995; Gajewski and Thompson, 1996; Ibrado et al., 1996, 1997; Reed, 1996). Bcl-2 overexpression in Chinese hamster ovary cells provided partial protection against the loss of clonogenicity produced by PDT using phthalocyanine Pc4 (He et al., 1996), supporting a role for Bcl-2 in the regulation of PDT-induced apoptosis. Several members of the Bcl-2 family have been identified in mammals: Bcl-2, Bcl-XL, A1/Bfl-1, Bcl-w, Nr13 and McI-1 serve to inhibit apoptosis, whereas Bax, Bik, Bak, Bad and Bcl-Xs promote apoptosis (Reed, 1996; Nagata, 1997). Members of the Bcl-2 family have been shown to homo- or heterodimerize with one another to either antagonize or enhance the function of the other (Yang et al., 1995; Reed, 1996). In vivo and in vitro studies showed that Bcl-2 provides protection against apoptosis in the absence of protein translation, suggesting that it does not exert its protective effect through gene regulation (Rowan and Fisher, 1997). Bcl-2 is an integral membrane protein that is situated primarily on the outer membranes of mitochondria, endoplasmic reticulum and nuclei (Nagata, 1997; Yang et al., 1997). In some systems, Bcl-2 regulates intracellular Ca2+ levels and prevents the loss of mitochondrial membrane potential produced by proapoptotic stimuli (Rowan and Fisher, 1997). Recent evidence suggests that Bcl-2 may act as an ion channel and regulate the release of cytochrome c from the mitochondria, an event that may be necessary for caspase 3 activation (Kluck et al., 1997; Yang et al., 1997).

We have previously demonstrated that caspase 3, but not caspase 1, is activated leading to PARP and DNA-PK cleavage in HL-60 cells treated with cytotoxic levels of BPD-MA and light (Granville et al., 1997). The present report shows that although Bcl-2 overexpression effectively blocked caspase 3 and caspase 6 activation as well as hypodiploid DNA formation produced by PDT, Bcl-2 had little or no effect on cell death induced by the photoactivation of BPD-MA at high drug doses but did offer a degree of protection against killing at lower levels of the photosensitizer as determined by 3-day culture studies.

**MATERIALS AND METHODS**

**Reagents and cell culture**

Liposomally formulated BPD-MA was from QLT Photo-Therapeutics (San Francisco, CA, USA). All antibiotics were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The transfected human promyelocytic leukaemia HL-60/neo and HL-60/Bcl-2 cell lines were generously provided by Dr Kapil Bhalla (Emory University School of Medicine, Atlanta, GA, USA). Detailed methods used to generate these clones have been previously described (Ibrado et al., 1996). The HL-60/Bcl-2 cells used in this study contain a fivefold higher level of Bcl-2 than the HL-60/neo control cells, whereas Bcl-XL and Bax levels are comparable for both transfectants (Ibrado et al., 1996). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM l-glutamine, 1 mM sodium pyruvate, 1 mM Hepes, penicillin (100 U ml–1) and streptomycin (100 µg ml–1) (Gibco, Burlington, Ontario, Canada) and G418 (1 mg ml–1) (Geneticin; Life Technologies, Grand Island, NY, USA).

**PDT and inhibitor treatment of cells**

For photodynamic studies, cells were incubated for a total of 60 min at 37°C with BPD-MA (0–200 ng ml–1) in RPMI containing 10% FBS. After incubation with BPD-MA, cells were exposed to fluorescent red light (620–700 nm) delivered at 5.6 mW cm–2 to give a total dose of 2 J cm–2. For caspase 3 inhibition studies, Z-DEVD-fmk (Enzyme Systems Products, Dublin, CA, USA) was added to the cells to give final concentrations of 10, 20 or 25 µM for the final 30 min before photoactivation.

**Analysis of DNA status**

The propidium iodide (PI) fluorescence analysis procedure was used to detect changes in the status of cellular DNA (Darzynkiewicz et al., 1992; Telford et al., 1994). At 3 h, after PDT, 1 x 0.5 cell were washed twice with ice-cold PBS then permeabilized and fixed in 80% ethanol at 4°C for 1 h. Cells were washed twice in ice-cold PBS and treated with RNAase (5 U ml–1, DNase-free) and stained with PI (50 µg ml–1) in PBS. Samples were analysed by flow cytometry. The percentage of cells containing hypodiploid levels of DNA was calculated from single parameter flow cytometry for PI fluorescence (Telford et al., 1994) using an Epics XL flow cytometer (Coulter Electronics, Hialeah, FL, USA).

**Preparation of cellular protein extracts**

To prepare cell lysates, cells were initially washed twice with ice-cold PBS. Cell pellets were treated with 1 ml of lysis buffer [1% Nonidet P-40 detergent (NP-40), 20 mM Tris, pH 8, 137 mM sodium chloride, 10% glycerol] supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF), aprotinin (0.15 µg ml–1) and 1 mM sodium orthovanadate for 20 min on ice. Lysates were centrifuged for 10 min at 15 000 g at 4°C. Protein concentrations of cell extracts were determined with the Pierce BCA protein assay (Pierce, Rockford, IL, USA).

**Protease assay**

To evaluate caspase 3 activity, cell lysates were prepared 1 h after their respective treatment. Assays were performed in 96-well microtitre plates by incubating 25 µl (10 µg protein) of cell lysate in 125 µl of reaction buffer (1% NP-40, 20 mM tris-HCl, pH 7.5, 137 mM sodium chloride, 10% glycerol) containing the caspase-3 substrate [Acetyl-Asp-Glu-Val-Asp-aminomycin (Ac-DEVD-AMC)] (Calbiochem, Cambridge, MA, USA) at 100 µM. Lysates were incubated at 37°C for 16 h and fluorescence levels were determined using a CytoFluor 2350 (PerSeptive Biosystems, Burlington, ON, USA) set at excitation and emission wavelengths of 380 nm and 460 nm respectively.
Immunoblot analysis

Detergent-soluble proteins (30 μg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels, under reducing conditions (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes at 100 V for 1 h. Membranes were blocked for 30 min at room temperature with 5% skimmed milk powder in PBS-0.05% Tween 20 (PBS-T). Membranes were incubated for 45 min using the following polyclonal antibodies: goat-anti-PARP, goat-anti-CPP32, goat-anti-Mch2 or rabbit-anti-Bcl-2 at 1 μg ml–1. Membranes were then probed with anti-goat IgG-HRP or anti-rabbit IgG-HRP (1:5000) in PBS-T with 1% skimmed milk powder for 30 min at room temperature. Membranes were rinsed twice in PBS-T, followed by three 15-min washes with PBS-T. Proteins were detected using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA) and bands visualized by autoradiography.

Assessment of cell viability using MTT

To assess cell viability, 5 × 10^4 cells were loaded into eight replicate wells (0.2 ml per well) of 96-well microtitre plates. After 3, 24, 48 or 72 h, 10 μl of a MTT solution (5 mg ml–1) was added to each well (Mosmann, 1983). Cells were incubated for a further 1 h at 37°C. The reaction was stopped by the addition of 150 μl of acidified isopropanol. The degree of colour development was analysed with an automated densitometer microtitre plate reader (Dynatech, Hamilton, VA, USA) using a 590-nm filter.

RESULTS

Caspase 6 activation occurs downstream of caspase 3

To assess the involvement of caspases in PDT-induced apoptosis, wild-type HL-60 cells were treated with different amounts of the caspase 3 inhibitor Z-DEVD-fmk before BPD-MA photoactivation. The presence of Z-DEVD-fmk did not affect PDT-induced caspase-3 cleavage and the appearance of the 12-kDa cleavage product at the concentrations tested (Figure 1). However, when the membrane was probed with either anti-caspase 6 or anti-PARP antibodies, it was evident that PDT-induced cleavage of these proteins was greatly reduced at a 25 μM concentration of Z-DEVD-fmk. The PDT-induced increase in hypodiploid DNA was also blocked at this concentration of the tetrapeptide inhibitor. A lower concentration of Z-DEVD-fmk (10 μM) did not block the cleavage of procaspase 6 or PARP, nor affect the appearance of hypodiploid levels of DNA (Figure 1). These results demonstrate that caspase 6 is activated by PDT and may occur downstream of caspase-3 processing.

Overexpression of Bcl-2 blocks caspase 3 and caspase 6 cleavage

To assess the influence of Bcl-2 overexpression and susceptibility to photodynamic killing, HL-60/neo or HL-60/Bcl-2 cells were incubated with or without BPD-MA and then exposed to light. Cells were lysed 1 h after light activation of BPD-MA. The status of Bcl-2, caspase 3 and caspase 6 in cytosolic extracts was determined by SDS-PAGE and analysed by Western immunoblotting. Cells were treated with light alone (2 J cm–2) (control), BPD-MA alone (100 ng ml–1), BPD-MA (100 ng ml–1) and light (PDT-100), or PDT-100 and Z-DEVD-AMC at the indicated concentrations.
Overexpression of Bcl-2 blocks caspase 3-like protease activity

Cell lysates were assayed for their capacity to cleave a fluorescently labelled caspase 3 substrate (Ac-DEVD-AMC) (Figure 3). Lysates prepared from HL-60/neo cells treated with BPD-MA and light exhibited high Ac-DEVD-AMC cleavage activity, whereas lysates from HL-60/Bcl-2 cells treated with the same levels of BPD-MA and light exhibited baseline levels of substrate cleavage. These results further support the observation that overexpression of Bcl-2 blocks activation of caspase 3 instigated by BPD-MA photoactivation.

Overexpression of Bcl-2 blocks PDT-induced increases in hypodiploid DNA

HL-60/neo and HL-60/Bcl-2 cells were analysed for their DNA status 3 or 24 h after BPD-MA photoactivation (Figure 4). HL-60/neo cells treated with BPD-MA and light exhibited high levels of hypodiploid DNA, suggestive of DNA fragmentation, compared with untreated cells and cells treated with light or BPD-MA alone. However, there was no evidence of PDT-induced DNA fragmentation (cells < 2n DNA) for HL-60/Bcl-2 cells treated with BPD-MA and light at 3 h after photoactivation and only a slight increase in hypodiploid DNA by 24 h. These results indicate that overexpression of Bcl-2 in HL-60 cells prevents the appearance of hypodiploid DNA induced by BPD-MA and light.

Overexpression of Bcl-2 provides partial protection against cell death at low levels of PDT

Our final step was to examine the effect of Bcl-2 overexpression on the viability of cells treated with BPD-MA and light. HL-60/neo and HL-60/Bcl-2 cells were incubated with titrated amounts of BPD-MA and exposed to light 1 h later. Bioreduction of MTT was used to compare the viability of each cell type at 3, 24, 48 and 72 h after the indicated treatments. Cell viability was assayed 3, 24, 48 or 72 h later by measuring the bioreduction of MTT. Data are expressed as a percentage of the result obtained with untreated control cells. Error bars represent standard deviations (s.d.) of the mean values from two experiments using eight replicates for each treatment sample.

Overexpression of Bcl-2 provides protection against PDT-induced cell death only for cells treated with concentrations of BPD-MA of 25 ng ml⁻¹ or less. HL-60/neo (□) and HL-60/Bcl-2 (●) cells were incubated with BPD-MA (0–100 ng ml⁻¹) and exposed to light 1 h later. Cell viability was assayed 3, 24, 48 or 72 h later by measuring the bioreduction of MTT. Data are expressed as a percentage of the result obtained with untreated control cells. Error bars represent the s.d. of mean values from two experiments using eight replicates for each treatment sample.

Overexpression of Bcl-2 blocks the induction of DEVD-ase activity associated with PDT. AT 1 h after treatment, HL-60/neo (■) or HL-60/Bcl-2 (□) cell lysates were prepared and assayed for their capacity to cleave a fluorescently labelled caspase 3 substrate (Ac-DEVD-AMC). Cytosolic extracts were taken from untreated cells (control) or cells treated with BPD-MA (100 ng ml⁻¹) alone, BPD-MA (100 ng ml⁻¹) and light (2 J cm⁻²) (PDT-100), or BPD-MA (200 ng ml⁻¹) and light (2 J cm⁻²) (PDT-200). Mean values with standard deviations (s.d.) of the mean values obtained in three experiments using replicates of five for each treatment sample. Error bars represent the s.d. of the mean values obtained in three experiments using replicates of five for each treatment sample.
after PDT. Overexpression of Bcl-2 provided no observable protection at concentrations of BPD-MA at 50 or 100 ng ml$^{-1}$, as measured by MTT bioreduction over a 72-h period (Figure 5). However, at lower concentrations of BPD-MA (10 or 25 ng ml$^{-1}$), Bcl-2 overexpression did provide a degree of protection against photodynamic inactivation of a small proportion of cells which exhibited recovery over the 72-h test period.

**DISCUSSION**

Overexpression of Bcl-2 has been shown to protect many different cancer cell lines from apoptosis induced by a wide variety of chemotherapeutic agents including dexamethasone, etoposide, methotrexate, cisplatin, cyclophosphamide, vincristine and 1-beta-D-arabinofuranosylcytosine (Ara-C) (reviewed in Reed, 1996). It has been observed that Bcl-2 overexpression blocks caspase 3 activation and subsequent PARP cleavage in these systems (Ibrado et al, 1996; Estoppey et al, 1997; Perry et al, 1997). In this study, we evaluated caspase activity, DNA fragmentation (as evidenced by an increase in hypodiploid DNA) and the viability of HL-60/neo or HL-60/Bcl-2 cells to determine whether treatment with cytotoxic levels of BPD-MA and light was regulated in a similar fashion in the setting of Bcl-2 overexpression.

Overexpression of Bcl-2 prevented procaspase 3 and PARP cleavage produced by PDT. Such findings correspond to previous results obtained with these cells in which Ara-C was used as the apoptotic stimulus (Ibrado et al, 1996). Procaspase 6 was also not processed in HL-60/Bcl-2 cells, indicating that Bcl-2 also acts upstream of caspase 6 activation in cells treated with BPD-MA and light. It has been demonstrated that caspase 6 may be responsible for degradation of nuclear lamins during apoptosis (Lażebnik et al, 1995; Neamati et al, 1995; Takahashi et al, 1996). Lamin cleavage may be critical for the degradation of the nuclear envelope during apoptotic cell death (Lażebnik et al, 1995). Studies by others have shown that caspase 3 can directly cleave procaspase 6 (Srinivasula et al, 1996), suggesting that procaspase 6 cleavage does not occur in PDT-treated HL-60/Bcl-2 cells because of the absence of caspase 3 activity. Conversely, a recent report by Thornberry et al (1997) suggests that both the optimal amino acid substrate sequence for caspase 6 resembles the activation site in effector caspase proenzymes such as caspase 3, suggesting that caspase 6 may act upstream of caspase 3 (Thornberry et al, 1997). To determine whether caspase 6 activation occurred downstream of caspase 3, wild-type HL-60 cells were incubated with a caspase 3 inhibitor, Z-DEVD-fmk, before photoactivation of BPD-MA. The caspase 3 inhibitor did not block the appearance of the 12-kDa subunit of caspase 3, as produced by PDT; but did prevent the cleavage of procaspase 6 and PARP indicating that procaspase 6 cleavage is probably downstream of caspase 3 activation.

Cytosolic extracts prepared from HL-60/Bcl-2 cells exhibited minimal cleavage of the Ac-DEVD-AMC substrate in contrast to the HL-60/neo cells treated with the same amounts of BPD-MA and light. Interestingly, untreated HL-60/Bcl-2 cells, or those exposed to the photosensitizer in the absence of light, had a lower constitutive capacity to cleave the Ac-DEVD-AMC substrate than the HL-60/neo cells. This observation could be attributable to the presence of a minor proportion of apoptotic cells within the HL-60/neo cell population exhibiting caspase activity. Background levels of protease activity would be less for HL-60/Bcl-2 cells because overexpression of Bcl-2 inhibits caspase 3 activation (Ibrado et al, 1996).

Several studies have now demonstrated that cytochrome c may be necessary for the activation of caspase 3 and that Bcl-2 blocks the release of cytochrome c from mitochondria (Kluck et al, 1997; Yang et al, 1997). Furthermore, it has recently been demonstrated that cytochrome c is involved in the activation of caspase 9, which is believed to then process caspase 3 (Li et al, 1997). Whether this scenario transpires in PDT-treated cells will be examined in future studies.

For HL-60/Bcl-2 cells treated with BPD-MA and light, cell survival was not detected at photosensitizer concentrations of 50 ng ml$^{-1}$ or greater at 72 h post PDT, suggesting that PDT may circumvent the inhibitory function of Bcl-2. The photodynamic treatment did not override the inhibitory effects of Bcl-2 with respect to caspase 3 and caspase 6 activation because procaspase 3 and 6 cleavage or DNA fragmentation did not occur in the HL-60/Bcl-2 cells that were treated with BPD-MA (100 ng ml$^{-1}$) and light. This would suggest that a parallel biochemical pathway unrelated to caspasas may be triggered in cells treated with higher levels of BPD-MA and light. At BPD-MA concentrations of less than 50 ng ml$^{-1}$, a modest protective effect of Bcl-2 overexpression against PDT-mediated cell death was observed in line with observations for other chemotherapeutic treatments (Reed et al, 1996). Although it has been well established by electron microscopy, histological and biochemical analysis that PDT induces apoptosis, we cannot rule out the possibility that PDT inflicts damage to other cell sites promoting passive necrotic cell death at higher drug concentrations (Gomer et al, 1988; Zaidi et al, 1993; Tajiri et al, 1996; Granville et al, 1997). Further understanding towards the mechanisms involved in PDT-induced cell death and how this treatment may be able to overcome the regulatory control of Bcl-2 should provide valuable insights towards the improvement of current cancer treatments.

**ABBREVIATIONS**

Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; BPD-MA, benzoporphyrin monoacid ring A, verteporfin, ICE, interleukin 1 converting enzyme; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PDT, photodynamic therapy; PI, propidium iodide; Z-DEVD-fmk, Z-Asp-Glu-Val-Asp-fluoromethylketone.

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