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The Death of Human Cancer Cells Following Photodynamic Therapy: Apoptosis Competence is Necessary for Bcl-2 Protection but not for Induction of Autophagy†

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ABSTRACT

Photodynamic therapy (PDT) is an efficient inducer of apoptosis in many types of cells, except in cells deficient in one or more of the factors that mediate apoptosis. Recent reports have identified autophagy as a potential alternative cell death process following PDT. Here we investigated the occurrence of autophagy after PDT with the photosensitizer Pc 4 in human cancer cells that are deficient in the pro-apoptotic factor Bax (human prostate cancer DU145 cells) or the apoptosis mediator caspase-3 (human breast cancer MCF-7v cells) and in apoptosis-competent cells (MCF-7c3 cells that stably overexpress human pro-caspase-3 and Chinese hamster ovary CHO 5A100 cells). Further, each of the cell lines was also studied with and without stably overexpressed Bcl-2. Autophagy was identified by electron microscopic observation of the presence of double-membrane-delineated autophagosomal vesicles in the cytosol and by immunoblot observation of the Pc 4-PDT dose- and time-dependent increase in the level of LC3-II, a component of the autophagosomal membrane. Autophagy was observed in all of the cell lines studied, whether or not they were capable of typical apoptosis and whether or not they overexpressed Bcl-2. The presence of stably overexpressed Bcl-2 in the cells protected against PDT-induced apoptosis and loss of clonogenicity in apoptosis-competent cells (MCF-7c3 and CHO 5A100 cells). In contrast, Bcl-2 overexpression did not protect against the development of autophagy in any of the cell lines or against loss of clonogenicity in apoptosis-deficient cells (MCF-7v and DU145 cells). Furthermore, 3-methyladenine and wortmannin, inhibitors of autophagy, provided greater protection against loss of viability to apoptosis-deficient than to apoptosis-competent cells. The results show that autophagy occurs during cell death following PDT in human cancer cells competent or not for normal apoptosis. Only the apoptosis-competent cells are protected by Bcl-2 against cell death.

INTRODUCTION

Autophagy is an evolutionarily conserved, dynamic and lysosome-mediated process that can function as a cell survival or a cell death mechanism. During autophagy, cytoplasmic contents, including mitochondria, endoplasmic reticulum (ER) and ribosomes, are sequestered within double or multimembranous vacuoles, called autophagosomes, which subsequently fuse with lysosomes to become autolysosomes, where the contents are degraded by lysosomal enzymes (1–5). Autophagosomes and autolysosomes are formed during a process called macroautophagy (hereafter referred to as "autophagy").

During nutrient deprivation, autophagic degradation of membrane lipids and proteins generates free fatty acids and amino acids which can be used to maintain mitochondrial ATP production, protein synthesis and cell survival (1–5). Autophagy is also involved in removing damaged mitochondria and organelles (self-destruction), resulting in the promotion of cellular survival during aging, infection and neurodegenerative processes (4,5). In contrast, when cells were treated with various stimuli, such as radiation (6), ceramide (7), rapamycin (8) or photodynamic therapy (PDT) (9,10), autophagy was observed and shown to be cytotoxic. The form of cell death induced by autophagy (called Type II cell death) is distinct from apoptotic Type I cell death (7,8).

Photodynamic therapy is a potent inducer of apoptosis in many types of cells (11). We have reported that PDT with the phthalocyanine photosensitizer, Pc 4, induces apoptosis in mouse lymphoma L5178Y-R (12), Chinese hamster ovary (13,14), human prostate cancer LNCaP (15) and human breast cancer MCF-7c3 (16) cells. However, PDT did not induce typical apoptosis in human prostate cancer DU145 cells which lack Bax expression. Following PDT, the hallmarks of apoptosis, such as the release of cytochrome *c* from mitochondria, loss of mitochondrial membrane potential, caspase activation and chromatin condensation and fragmentation were completely blocked in DU145 cells (17). Caspases are crucial components of most apoptosis pathways (18). We found that PDT induces much more rapid and extensive apoptosis in MCF-7c3 cells, which express a stably transfected CASP-3 gene, than in MCF-7v cells lacking caspase-3. However, the two cell lines were equally sensitive to photodynamic killing when evaluated by a clonogenic assay (16). We hypothesized that a nonapoptotic pathway for cell death must be activated in the caspase-3- or Bax-deficient cells.

The Bcl-2 family of proteins are well-characterized regulators of apoptosis (19). Overexpression of Bcl-2 can enhance cell survival by suppressing apoptosis in cells treated with various stimuli (20,21). Protection by Bcl-2 against Pc

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4-PDT-induced apoptosis and cell killing has been observed in MCF-7c3 cells (22) and CHO 5A100 cells (14). In this study, we investigated the cell death pathways activated in Pc 4-PDT-treated DU145 and MCF-7v cells and the effect of Bcl-2 overexpression. To elucidate the relationship between apoptosis and autophagy, MCF-7c3 cells were also studied with and without Bcl-2 overexpression.

MATERIALS AND METHODS

Cell culture. Human breast cancer MCF-7 cells transfected with human procaspase-3 cDNA (MCF-7c3) or empty vector (MCF-7v) were obtained from Dr. C. Froehlich, Northwestern University, and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Human prostate cancer DU145 cells and these cells transfected with FLAG-Bcl-2 cDNA (DU145-Bcl-2) were cultured in DMEM containing 10% FBS. Monolayer cultures of CHO cells (line 5A100) were maintained in McCoy's 5A medium containing 10% FBS. All cultures were maintained in a humidified atmosphere at 37°C with 5% CO_2.

Transfection. MCF-7c3 cells were transfected with GFP-Bcl2 (MCF-7c3-Bcl2) or GFP alone (MCF-7c3-GFP), as described previously (22). MCF-7v cells were transfected with pSFFVneo expression vector or human Bcl-2 cDNA subcloned into pSFFVneo (provided by Dr. C. Distelhorst, Case Western Reserve University) using the transfection reagent LipofectAMINE PLUS (Invitrogen, Carlsbad, CA). Transfected cells were subjected to G418 (1 mg mL^{-1}) selection.

Photodynamic treatment. The phthalocyanine photosensitizer Pc 4, $HOSiPcOSi(CH_3)_2(CH_2)_3N(CH_3)_2$, was provided by Dr. Malcolm E. Kenney, Case Western Reserve University Department of Chemistry (23). It was dissolved in dimethyl formamide to 0.5 mM. Cells were loaded with Pc 4 by addition of an aliquot of the stock solution to the culture medium ~18 h before irradiation. The light source was an EFOS LED array (EFOS, Mississauga, ON, Canada) delivering red light ($\lambda_{max} \approx 675$ nM). All irradiations were performed at room temperature.

Western blot analysis. Cells were lysed and sonicated as described previously (24). An equal volume of 2 × SDS sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% mercaptoethanol and 20% glycerol) was added to the cell lysate. For LC3 detection, 1 × SDS buffer was added directly to cells rather than preparing lysates first. Equivalent amounts of protein were loaded onto polyacrylamide gels, subjected to electrophoresis, transferred to a PVDF membrane and incubated with anti-LC3 antibody (a kind gift from Dr. T. Yoshimori, National Institute of Genetics, Japan), anti-Bcl-2 antibody (Pharmingen, San Diego, CA), anti-caspase-3 antibody (BD Transduction Laboratories, Lexington, KY), anti-PARP antibody (Pharmingen, San Diego, CA), anti-Bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-actin antibody (NeoMarkers, Fremont, CA). The immune complexes were detected by ECL system (Amersham, Arlington Heights, IL).

Assay of cell viability. Cells were seeded in 96-well plates at 1.5×10^4 cells/well and allowed to attach overnight. The medium was removed and replaced with fresh medium with or without various doses of Pc 4. The cells were incubated for ~18 h, then re-fed with fresh medium with or without 3-methyladenine (3-MA) or wortmannin (Sigma-Aldrich, St. Louis, MO). After 30 min incubation, cells were photoirradiated, then incubated for an additional 24 h. Cell viability was measured using a tetrazolium salt, either MTT (Sigma-Aldrich) or WST-1 (Roche Applied Science), according to the manufacturer's instructions.

Clonogenic assay. Cells were collected from the culture monolayer with trypsin immediately after PDT. Aliquots of the cells were plated in triplicate into 6 cm Petri dishes in amounts sufficient to yield 50–100 colonies per dish. After incubation for ~12 days, the cells were stained with 0.1% crystal violet in 20% ethanol, and colonies containing at least 50 cells were counted. The plating efficiencies of untreated DU145, MCF-7 and CHO 5A100 cells were 35–50%, 34–41% and 95%, respectively.

Electron microscopy. Trypsinized cells were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate, postfixed with 1% osmium tetroxide and dehydrated in ethanol and propylene oxide. The cell pellets were embedded in Poly/Bed-812 resin, and ultrathin sections were cut (100 nM) and stained with 1% uranyl acetate. Images were examined with a JEM 1200EX Transmission Electron Microscope (JEOL) at 80 KV.

RESULTS

We first examined by western blot analysis the level of stable expression of pro-caspase-3, Bcl-2 and Bax in the various cell lines derived from DU-145 and MCF-7 cells. It can be seen from Fig. 1 that MCF-7c3 cells expressed a high level of procaspase-3; in contrast, no pro-caspase-3 was detected in MCF-7v cells and DU145 cells expressed only limited pro-caspase-3. The level of pro-caspase-3 was unaffected by overexpression of Bcl-2. As expected, DU145-Bcl-2, MCF-7v-Bcl-2 and MCF-7c3-Bcl-2 cells expressed much higher levels of Bcl-2 than did those cells transfected with empty vectors. No Bax expression was observed in DU145 or DU145-Bcl-2 cells due to a frameshift insertion mutation in the gene (25,26). In contrast, MCF-7 cells expressed a high constitutive level of Bax which was not affected by overexpression of Bcl-2.

We previously reported an absence of typical apoptosis in PDT-treated DU145 cells even though the cells were killed by PDT with similar sensitivity to apoptosis-competent cells (17). In order to understand how Bax-deficient DU145 cells die and how the process might be affected by Bcl-2, we looked for evidence of autophagy. During autophagy, an isolation membrane forms and sequesters cytoplasmic materials within a double-membrane vacuole (autophagosome) which can be visualized by electron microscopy; this provides the best evidence for autophagy. As shown in Fig. 2a, double-membrane encased vacuoles were abundant in PDT-treated DU145 cells, but not in the untreated control cells. These results indicate that PDT with the photosensitizer Pc 4 induces autophagy in Bax-deficient DU145 cells.

LC3 is a mammalian homolog of the yeast Apg8p, microtubule-associated protein-1 light chain-3, which is localized in the autophagosome membranes after processing and is a well-characterized marker for autophagosome formation (27,28). There are two forms of LC3. LC3-I is cytosolic, whereas LC3-II is membrane bound. Upon the induction of autophagy, the cytosolic LC3-I protein is covalently linked to phosphatidylethanolamine to yield LC3-II, which subsequently associates with the autophagosome (27). Therefore, the amount of LC3-II is correlated with the extent of

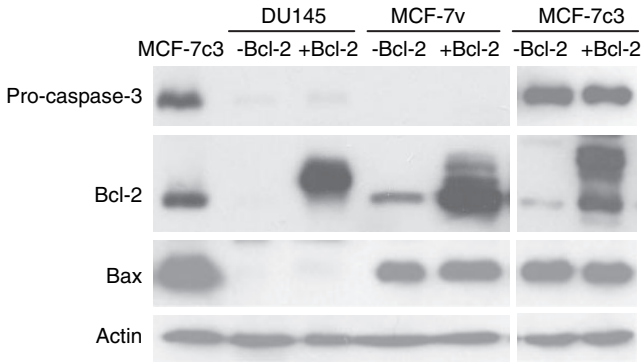


Figure 1. Expression of pro-caspase-3, Bcl-2 and Bax in MCF-7c3, DU145, DU145-Bcl-2, MCF-7v, MCF-7v-Bcl-2, MCF-7c3 and MCF-7c3-Bcl-2 cells. Equal amounts of protein from whole cell lysates were loaded onto a minigel, separated, transferred to a PVDF membrane, and then probed with various antibodies. Actin was used as a loading control.

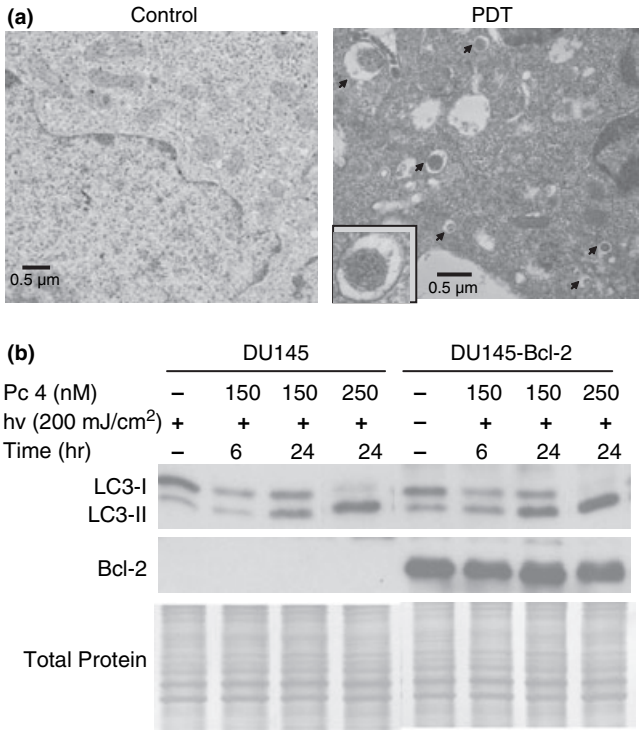


Figure 2. Autophagy in PDT-treated DU145 cells. (a) Double-membrane vacuoles in PDT-treated DU145 cells observed by electron microscopy. DU145 cells were untreated or PDT-treated with 300 nM Pc 4 and 200 mJ cm^{-2} and then further incubated for 24 h. Cells were collected and samples were prepared for EM as described in Materials and Methods. (b) LC3 levels in control and PDT-treated DU145 and DU145-Bcl-2 cells. Cells were loaded with 150 or 250 nM Pc 4 overnight, then irradiated with 200 mJ cm^{-2} of red light and incubated for an additional 6 or 24 h. The cells were collected, and western blot analysis was performed with anti-LC3 antibody. The blot was also probed with anti-Bcl-2, and loading was monitored by staining the blot.

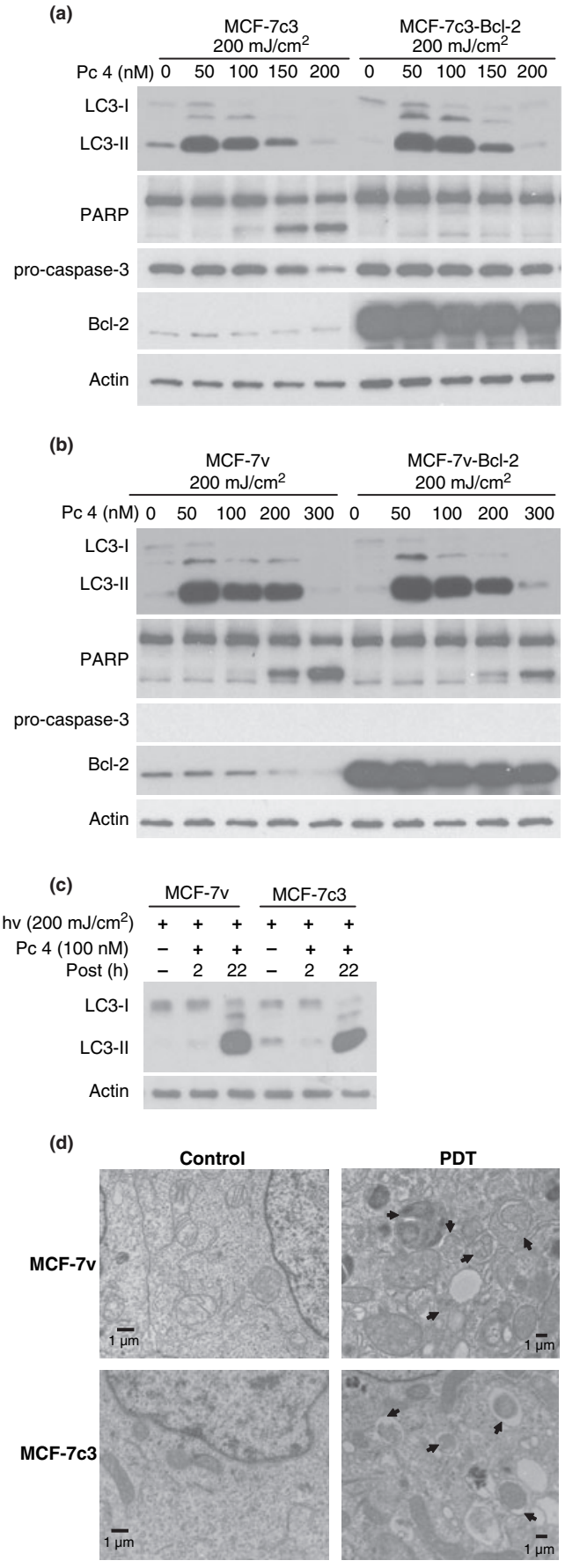
autophagosome formation and thus with the progress of autophagy (27,28). In order to better quantify PDT-induced autophagy in DU-145 cells, we examined LC3 levels by immunoblot analysis. As shown in Fig. 2b, lysates from untreated cells exhibited predominantly an 18-kDa protein band representing LC3-I, whereas the LC3-II (16 kDa) band had a weaker intensity. However, lysates from PDT-treated cells revealed the presence of processed LC3-II, and the density of the LC3-II band increased with PDT dose and with post-PDT time. Furthermore, the results were similar for DU145 and DU145-Bcl-2 cells, suggesting that Bcl-2 did not protect against PDT-induced autophagy.

Photodynamic therapy induces faster and more extensive apoptosis in MCF-7c3 cells than in MCF-7v cells (16). To better understand the mechanism of cell death in caspase-3-deficient MCF-7v cells and how Bcl-2 regulates cell killing, four cell lines (MCF-7v and MCF-7c3 cells each stably transfected with empty vector or with Bcl-2) were compared with respect to the generation of markers of PDT-induced apoptosis and autophagy (Fig. 3a,b). The cells were treated with increasing doses of Pc 4, then irradiated with 200 mJ cm^{-2} of red light and incubated for an additional 17 (MCF-7c3) or 24 (MCF-7v) h. Total cellular protein was analyzed for LC3-II increase and PARP cleavage. For cells exposed only to light, there was no or only a faint LC3-II band; however, the LC3-II level increased markedly with PDT dose and reached a maximum at 50–100 nM Pc 4, then decreased. Notably, there was no significant difference in the PDT-induced increase in LC3-II level as a function of Bcl-2 overexpression in either MCF-7c3 or MCF-7v cells, implying that Bcl-2 does not protect against autophagy following PDT.

We next examined PARP, a DNA-repair-associated protein which is cleaved by one or more caspases during many forms of apoptosis. As expected, PARP cleavage was observed when MCF-7c3 cells were exposed to a dose of PDT high enough to induce significant apoptosis in 17 h; however, no PARP cleavage was detected in MCF-7c3-Bcl-2 cells, as Bcl-2 prevents PDT-induced apoptosis (Fig. 3a). Activation of caspase-3 also occurs during PDT-induced apoptosis (16). When the same blot was reprobed with a monoclonal anti-procaspase-3 antibody, there was a dose-dependent loss of procaspase-3 in MCF-7c3 cells, but not in MCF-7c3-Bcl-2 cells, which is consistent with the PARP cleavage results. In contrast, no procaspase-3 was found in either untreated or PDT-treated MCF-7v cells with or without Bcl-2 (Fig. 3b), confirming the deficiency of procaspase-3. Although no caspase-3 was detected in MCF-7v cells, PARP cleavage was still observed at the higher PDT doses (200 and 300 nM Pc 4), probably resulting from caspase-7, which cleaves proteins at the same consensus sequence as does caspase-3 (29). Procaspase 7 is associated with the ER and activated by ER stress. As Pc 4 localizes to the ER as well as mitochondria, Pc 4-PDT also causes ER stress which can activate caspase-7. In comparison with MCF-7c3-Bcl-2 cells, Bcl-2-overexpressing MCF-7v cells were only partially protected against PDT-induced PARP cleavage (Fig. 3b). The blots were also probed with an anti-Bcl-2 antibody to monitor Bcl-2 levels and with an anti-actin antibody as a loading control. It is clear that Bcl-2 levels in Bcl-2-overexpressing cells were much higher than in those expressing only endogenous Bcl-2. The PDT-induced decrease in Bcl-2 level is indicative of photodamage, as we have previously reported (24), which occurs in all four cell lines but is only clearly observed for the overexpressing cells in underexposed blots (data not shown).

Figure 3c shows a time course of PDT-induced autophagy, as monitored by the LC3-II level in MCF-7 cells. PDT induced an increase in the LC3-II level detectable as early as 2 h after treatment, and reaching high levels by 22 h. The results suggest that PDT-induced autophagy begins early and steadily expands. It has been suggested that the marked accumulation of LC3-II, such as observed here, may indicate a partial blockage in the processing and degradation of this protein once the autophagosomes have fused with lysosomes (30). We attempted to evaluate that possibility using the protease inhibitors E64d and pepstatin A to further inhibit proteolytic degradation of LC3-II (31). In MCF-7v and MCF-7c3 cells, the inhibitors caused extensive accumulation of LC3-II by themselves and did not alter the level of LC3-II produced in response to PDT (data not shown).

At present, electron microscopy is the only absolute test of autophagy; accordingly, the ultrastructure of MCF-7 cells was examined before and after PDT (Fig. 3d). Double membrane-enclosed vacuoles, containing what appeared to be mitochondria or other cellular contents, were abundant in MCF-7v and in MCF-7c3 cells after PDT, but not in the untreated cells of



either line, which is consistent with the results shown in Fig. 3a–c.

It has been reported that PDT kills cells by either apoptosis or nonapoptotic pathways, depending on the cell type, the photosensitizer and its localization, and the PDT dose (32,33). Recently, two groups have found that PDT can induce autophagy (9,10). We reported that DU145 cells lacking Bax and MCF-7v cells lacking procaspase-3 did not undergo typical apoptosis and must die mainly by a nonapoptotic mechanism after PDT. The Kessel group demonstrated autophagy in DU145 cells, and we have confirmed that result (Fig. 2). We attempted to estimate the fraction of PDT-induced cell death that may be associated with autophagy, by evaluating the dose-dependent loss of viability of MCF-7 cells using the tetrazolium dye WST-1 and the ability of 3-methyladenine, an inhibitor of phosphatidylinositol-3-kinase (PI3K), to protect the cells. 3-MA was toxic to these cells, producing about 34% cell kill during the 24 h period of exposure. As shown in Fig. 4a,b, when the viability results were corrected for the effect of 3-MA alone, a PDT dose-dependent loss of cell viability was observed for both MCF-7v and MCF-7c3 cells, and 3-MA provided greater protection for MCF-7v cells. 3-MA is a commonly used inhibitor for autophagy; however, it is not specific and may also inhibit apoptosis. Therefore, the small protection of MCF-7c3 cells by 3-MA may result from either a partial inhibition of apoptosis or a small role of autophagy in the death of these cells. The effect of wortmannin, another PI3K inhibitor, was also examined (Fig. 4c,d). Wortmannin itself is also toxic, producing about 40% cell killing in these experiments; however, it acted similarly to 3-MA but was slightly less effective. These results suggest that a PI3K-influenced process(es), possibly including autophagy, may be more relevant to MCF-7v cells.

Short-term viability assays, such as tetrazolium dye reduction assays, are necessary for evaluating cell killing using a drug (*e.g.* 3-MA) that is itself toxic, but the assay may underestimate the extent of cell death, especially missing deaths that occur late after toxic treatment (16,34). DU-145 cells, which lack Bax, die slowly after PDT probably due to their inability to undergo typical apoptosis (35). As we were interested in determining the ability of overexpressed Bcl-2 to protect against PDT-induced cell killing, we next turned to clonogenic assays to compare the overall photocytotoxicity of Pc 4-PDT against matched cell lines either with or without stably transfected Bcl-2 (Fig. 5). In the case of DU145 and

Figure 3. PDT-induced autophagy and apoptosis in MCF-7 cells. (a, b) Comparison of autophagy and apoptosis protein markers in PDT-treated MCF-7c3 and MCF-7c3-Bcl-2 cells (a) and MCF-7v and MCF-7v-Bcl-2 cells (b). Cells were treated with various doses of Pc 4 and irradiated with 200 mJ cm^{-2} red light, then postincubated for 17 h (MCF-7c3) or 24 h (MCF-7v). Protein from whole cell lysates was separated on SDS-PAGE gels, transferred to PVDF membranes, and probed with antibodies to LC3, PARP, pro-caspase-3, Bcl-2 and actin. (c) Time course of PDT-induced LC3-II increase in MCF-7v and MCF-7c3 cells. The cells were incubated in 100 nM Pc 4 overnight, then irradiated with 200 mJ cm^{-2} red light and postincubated for the indicated times. Western blot analysis was performed as described above. (d) Autophagy in PDT-treated MCF-7v and MCF-7c3 cells examined by electron microscopy. The cells were untreated or exposed to 150 nM Pc 4 and 200 mJ cm^{-2} red light and postincubated for 24 h, then prepared for EM. Arrows indicate examples of double-membrane-encased autophagosomal vacuoles.

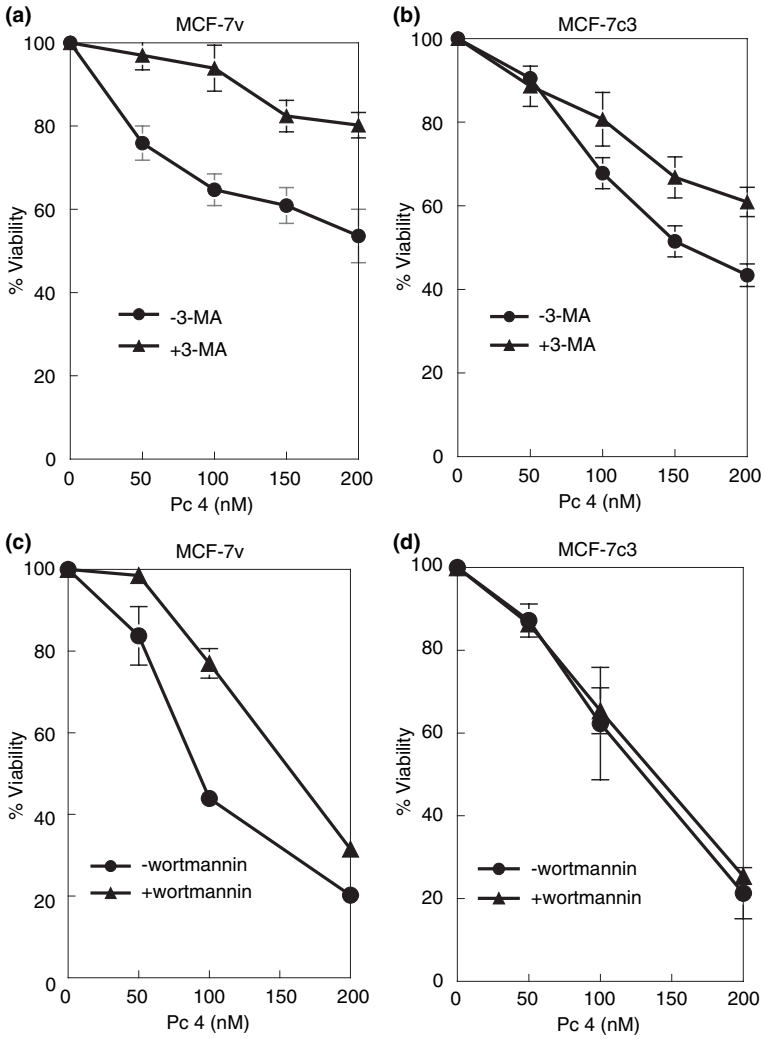


Figure 4. The effect of the autophagy inhibitors 3-MA (a, b) or wortmannin (c, d) on Pc 4-PDT-induced cell death examined by WST-1 (a, b) or MTT (c, d) assay. For 3-MA, MCF-7v (a) and MCF-7c3 (b) cells were loaded with the indicated doses of Pc 4 overnight, then the medium was removed and replaced with fresh medium with or without 5 mM 3-MA for 30 min before exposure of the cells to 200 mJ cm^{-2} of red light. Wortmannin (100 nM) and Pc 4 were added to cells for 3 h before light exposure (c, d). After photoirradiation, the cells were returned to the incubator for 24 h before assay for viability. The viability of cells exposed to 3-MA alone was 63.3% and 68.5% of the untreated control cells for MCF-7v and MCF-7c3, respectively. The viability of cells exposed to wortmannin alone was 58.7% and 62.5% of the untreated control cells for MCF-7v and MCF-7c3, respectively. The viability data for cells exposed to 3-MA or wortmannin plus PDT were normalized to that of the corresponding drug control. Data are the mean ± standard deviation of the results of six to eight wells of a 96-well plate for each condition. Similar results were obtained in one to two repeat experiments.

MCF-7v cells, which are deficient in factors mediating the intrinsic pathway of apoptosis, overexpression of Bcl-2 did not protect against PDT-induced loss of clonogenicity. In contrast, for CHO 5A100 and MCF-7c3 cells, which efficiently undergo apoptosis in response to Pc 4-PDT, overexpression of Bcl-2 provided substantial protection against reproductive cell death, as we have previously demonstrated (14,22).

DISCUSSION

In this study that examined autophagosome formation by electron microscopy and LC3-II generation by western blot analysis, autophagy was found to occur in all Pc 4-PDT-treated cells. For human prostate cancer DU145 cells, which lack Bax and which we previously found to die following Pc 4-PDT by a nonapoptotic pathway (17), autophagy was prominent, in agreement with the results of Kessel *et al.* (9). For human breast cancer MCF-7 cells, autophagy was observed in both procaspase-3-deficient MCF-7v and procaspase-3-expressing MCF-7c3 cells. Furthermore, the extent of induction of autophagy was dose dependent and occurred as early as 2 h post-PDT. The results imply that PDT-induced autophagy is a common phenomenon and that neither Bax nor procaspase-3 is required for this process. The extensive accumulation of LC3-II in PDT-treated cells may indicate that the later steps in autophagy are blocked or at least slowed (31). Because we were unable to affect LC3-II accumulation with the protease inhibitors E64d and pepstatin A, it is conceivable that PDT activates the early steps but inhibits the later steps of the pathway, a possibility that will be tested in further work.

It has been reported that autophagy occurs earlier than apoptosis (36,37); however, autophagy is probably not

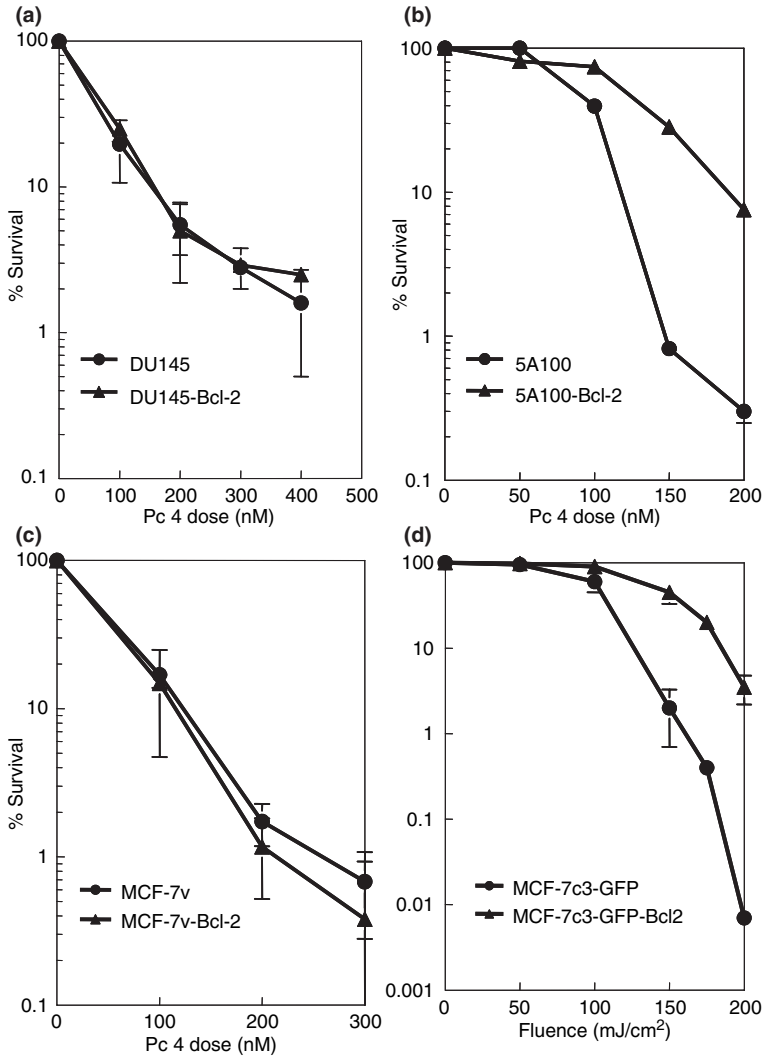


Figure 5. Clonogenic survival of PDT-treated (a) DU145, (b) 5A100, (c) MCF-7v and (d) MCF7c3 cells and the corresponding cells stably overexpressing Bcl-2. Cells were incubated in the indicated concentrations of Pc 4 for 18 h, then irradiated with 200 mJ cm^{-2} red light. Cells were collected, diluted and plated at appropriate concentrations for colony formation. Data for PDT-treated cells were normalized to the plating efficiency of the untreated control (on average, 45% for DU145; 95% for 5A100; 33%–41% for MCF-7). Each datum is the mean ± standard deviation of at least triplicate results from at least two independent experiments. Where error bars are not shown, they were smaller than the size of the symbol. Data for MCF-7c3 cells are replotted from Usuda *et al.* (22).

involved in the death process unless apoptosis is blocked (38). According to the theory of Lockshin and Zakeri, cells preferentially die by apoptosis but in the absence of apoptosis will die by any alternative available route, including autophagy (39). For DU145 and MCF-7v cells in which apoptosis was blocked, PDT-exposed cells appeared to die by a nonapoptotic mechanism. In the case of MCF-7c3 cells, both apoptosis and autophagy were induced by PDT; however, the apoptotic process became the predominant death mechanism, probably because caspase-mediated proteolysis can destroy a cell more rapidly than self-degradation by autophagy. We have reported (16) that MCF-7c3 cells were more sensitive to PDT than MCF-7v cells when assayed for loss of viability by the WST-1 assay, implying that apoptotic cell death proceeds faster than nonapoptotic pathways.

The present study demonstrates that Bcl-2 overexpression has no effect on the progress of PDT-induced autophagy, as assessed by the increase in LC3-II level; this result was found with DU145 cells and with both MCF-7v and MCF-7c3 cells. In addition, Bcl-2 does not protect against overall cell killing by PDT, as assessed by the loss of clonogenicity, in those cells deficient in apoptosis (DU145 and MCF-7v), whereas a marked protection is afforded by overexpressed Bcl-2 in apoptosis-competent cells (5A100 and MCF-7c3). Thus, overexpression of Bcl-2 protects against cell death by apoptosis, but not by nonapoptotic mechanisms, following PDT. The two processes are distinct and regulated differently. Apoptosis is regulated by Bcl-2 family proteins through control of the release of apoptogenic proteins, such as cytochrome *c* (40) and Smac (Second mitochondria-derived activator of caspase) (41), from mitochondria into the cytosol. Anti-apoptotic Bcl-2 family member proteins, Bcl-2 and Bcl-xL, suppress the release (42,43) and protect against apoptotic cell death. For autophagy, cells die through degradation of cytoplasmic contents by lysosomal proteins, a process regulated by phosphoinositol-3-kinase Types I and III (1,4,5), as well as other factors. In

cases when both apoptosis and autophagy occur in response to PDT, *i.e*. in 5A100 and MCF-7c3 cells, apoptotic cell death is dominant, and Bcl-2 overexpression protects against apoptosis as well as overall cell death. For cells undergoing autophagy but not typical apoptosis, *i.e*. DU145 and MCF-7v cells, Bcl-2 was not protective. Similarly, Shimizu *et al*. (38) have reported that when Bax/Bak double-knockout murine embryonic fibroblasts were treated with apoptosis stimuli, although the cells were resistant to apoptosis, they underwent a nonapoptotic cell death (Type II autophagic death). Overexpression of Bcl-2 or Bcl-xL in those cells resulted in enhancement of the nonapoptotic cell death. Our results differ from those of Shimizu *et al*. (38) in that Bcl-2 did not appear to enhance the extent or rate of autophagy in human cancer cells as it did in murine embryonic fibroblasts. Both studies show, however, that there was no inhibition of autophagy by Bcl-2. In contrast, Pattingre and Levine (44) recently demonstrated an opposite role for Bcl-2 in the regulation of autophagy. They reported that Bcl-2 can protect against starvation-induced autophagy by binding to Beclin 1, an autophagy and tumor-suppressor protein, disrupting its autophagy function. The difference between their data and our finding may result from the different stimuli. According to Pattingre and Levine (44), Bcl-2 blocks autophagic cell death by binding to Beclin 1, and only ER-targeted Bcl-2, but not mitochondrion-targeted Bcl-2, inhibits autophagy. We and others (24,45,46) have found that PDT, with a variety of photosensitizers that localize in the mitochondria and ER, directly and immediately damages Bcl-2. It is possible that PDT-induced Bcl-2 photodamage disrupts the binding of Bcl-2 to Beclin1, resulting in elimination of an effect of Bcl-2 on PDT-induced autophagy. It seems that the role of Bcl-2 in PDT-treated cells may represent an exception to what has been observed by others who used other stimuli to induce autophagy.

Autophagic degradation has been proposed to be triggered during the early stages of cell death and to function upstream of the principal apoptotic events (37). According to Jia *et al*. (47), autophagy may promote apoptosis in some systems; for example, the formation of autophagosomes has been shown to be associated with TNF-α-induced apoptosis in human T lymphoblastic leukemia cells. Recently, Yousefi *et al*. (48) reported that cleavage of Atg5 (an autophagy-related gene) by calpain can switch autophagy to apoptosis. In contrast, Herman-Antosiewicz *et al*. (36) found that apoptosis induction by sulforaphane in PC-3 and LNCaP human prostate cancer cells is prevented by induction of autophagy. It is possible that the effect of autophagy on apoptosis is cell line- and stimulus-dependent. For the advance of any of these agents in the clinical treatment of cancer, it is very important to determine whether autophagy promotes or prevents apoptosis. If autophagy prevents apoptosis, the efficiency of killing cancer cells by PDT or other anticancer agents may be enhanced by the simultaneous treatment with an inhibitor of autophagy; if autophagy promotes apoptosis, an inducer of autophagy may be more efficacious.

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