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Differential responses of Mcl-1 in photosensitized epithelial *vs* lymphoid-derived human cancer cells

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The antiapoptotic Bcl-2-family proteins, Bcl-2 and Bcl-xL, are recognized phototargets of photodynamic therapy (PDT) with the mitochondrion-targeting phthalocyanine photosensitizer Pc 4. In the present study, we found that myeloid cell leukemia 1 (Mcl-1), another antiapoptotic member of the Bcl-2 family, was not photodamaged in Pc 4-PDT-treated human carcinoma cells MCF-7c3, MDA-MB468, DU145, and A431, although Mcl-1 turnover was observed after exposure of HeLa or MCF-7c3 cells to a supralethal dose of UVC. In contrast, when human lymphoma U937 and Jurkat cells were treated with Pc 4-PDT, staurosporine (STS) or UVC, Mcl-1 was cleaved to generate a 28-kDa fragment over a 2–4 h period. The cleavage of Mcl-1 was accompanied by the activation of caspases-3, -9, and -8. The broad-specificity caspase inhibitor z-VAD-fmk completely blocked Mcl-1 cleavage induced by PDT, STS or UVC, providing evidence for Mcl-1 as a substrate for caspases. Western blot analysis localized Mcl-1 to mitochondria, ER, and cytosol of both MCF-7c3 and U937 cells, suggesting that Mcl-1 protein, unlike Bcl-2 and Bcl-xL, is not a target for Pc 4-PDT, probably due to its localization to sites removed from those of Pc 4 binding. The 28-kDa cleaved fragment of Mcl-1, which has proapoptotic activity, was produced in PDT-treated lymphoid-derived cells, but not in cells of epithelial origin, suggesting that PDT-induced rapid and extensive apoptosis in lymphoma cells may result in part from the sensitivity of their Mcl-1 to caspase cleavage, removing an important negative control on apoptosis.
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The Bcl-2 family of intracellular proteins is the central regulator of apoptosis (Chao and Korsmeyer, 1998; Gross *et al*., 1999). In mammals, Bcl-2 has at least 20 relatives, all of which share at least one conserved Bcl-2 homology (BH) domain (Cory and Adams, 2002). The family is composed of both pro and antiapoptotic members. The antiapoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-xL, and myeloid cell leukemia 1 (Mcl-1), protect against apoptosis by forming heterodimers with proapoptotic Bcl-2 family members, such as Bax and Bad (Yin *et al*., 1994; Sedlak *et al*., 1995; Zha *et al*., 1997; Bae *et al*., 2000). Recently, Leu *et al*. (2004) and Cuconati *et al*. (2003) reported that Mcl-1 is complexed with Bak in untreated cells. It has been well known that expression of Bcl-2 and Bcl-xL enhances the survival of cells treated with various apoptosis-inducing stimuli (Chao *et al*., 1995; Reed, 1995; White, 1996). Limited information is available regarding the other three antiapoptotic proteins, that is, Mcl-1, Bcl-w, and A1; however, recent work has demonstrated the role of Mcl-1 in promoting cell survival and in controlling cell death in response to cancer therapy (Craig, 2002; Vrana *et al*., 2002; Michels *et al*., 2004).

Photodynamic therapy (PDT) is a relatively new cancer treatment that employs photosensitizers and visible light to kill cells and ablate tumors (Dougherty *et al*., 1998; Oleinick *et al*., 2002). PDT with mitochondrion-localizing photosensitizers, such as the phthalocyanine Pc 4, induce apoptosis in many types of cells (Oleinick and Evans, 1998; Kessel and Luo, 1999; Oleinick *et al*., 2002). PDT with such photosensitizers can directly damage Bcl-2, which is detected upon Western blot analysis as the loss of the 26-kDa protein (Kim *et al*., 1999; Kessel and Castelli, 2001; Xue *et al*., 2001a). The photodamage to Bcl-2 occurs in a dose-dependent manner and can be observed in cells irradiated in the cold and collected immediately upon Pc 4-PDT, indicating that it is a manifestation of immediate photodamage rather than a delayed metabolic effect (Xue *et al*., 2001a). We also found that Bcl-xL, a close homolog of Bcl-2, can be photodamaged by Pc 4-PDT as well (Xue *et al*., 2003a).

Mcl-1has a sequence similar to Bcl-2 family members. It contains two highly conserved regions, designated as the BH1 and BH2 domains, which have been shown to be required for the antiapoptotic function of Bcl-2 and for heterodimerization with Bax (Yin *et al*., 1994). Overexpression of Mcl-1 was found to delay apoptosis induced by various stimuli (Reynolds *et al*., 1994; Zhou *et al*., 1997; Leu *et al*., 2000). As a result of the similarity in sequence and function of Bcl-2 and Mcl-1, we have investigated whether or not PDT affects Mcl-1 expression and whether Pc 4-PDT can photodamage Mcl-1 as

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it does Bcl-2 and Bcl-xL. The response of Mcl-1 to Pc 4-PDT was studied in several human carcinoma cell lines as well as human lymphoid U937 and Jurkat cells.

In order to determine whether Mcl-1 responds to Pc 4-PDT as does Bcl-2 and Bcl-xL, human breast cancer MCF-7c3 cells (MCF-7 cells overexpressing human procaspase-3) were left untreated or were treated with 50–300 nM Pc 4 followed by 200 mJ/cm^2 red light to photoactivate the photosensitizer. Cells were collected 10 min after irradiation, and total cell protein was analysed by Western blot with antibodies to Bcl-xL, Bcl-2 or Mcl-1. As shown in Figure 1a, there was a marked dose-dependent reduction in the amounts of Bcl-xL and Bcl-2, as previously reported (Xue *et al*., 2001a, 2003a). However, the Mcl-1 levels were the same in all cell samples, irrespective of PDT dose. PDT with 200 or 300 nM Pc 4 and 200 mJ/cm^2 red light resulted in 90 or 99% loss of clonogenicity (LD90 or LD99, respectively) and 31 or 38% apoptotic cells, respectively, as examined at 4 h post-PDT by staining with propidium iodide. The absence of response of Mcl-1 to PDT was not limited to MCF-7 cells, but was also observed for human breast epithelial MDA-MB468 cells, human prostate DU145 cells, and human epidermoid carcinoma A431 cells treated with LD90-LD99 doses (Figure 1b). For these cell lines, Bcl-xL was lost in all PDT-treated cells, whereas there was no significant change in the Mcl-1 level. The results indicate that although both Bcl-2 and Bcl-xL were photodamaged, as defined by loss of the native protein, Mcl-1 was not photodamaged.

Pc 4 is found in intracellular membranes, especially mitochondria, endoplasmic reticulum (ER), and the nuclear periphery (Trivedi *et al*., 2000; Lam *et al*., 2001; Usuda *et al*., 2003). Bcl-2 has been demonstrated to localize to the nuclear envelope, ER, and mitochondrial membranes (Hockenbery *et al*., 1990; Krajewski *et al*., 1993), whereas Bcl-xL is found in mitochondria as well as cytosol (Wang *et al*., 2001). Thus, Pc 4 localizes to the same membrane systems housing Bcl-2 and Bcl-xL, resulting in direct photodamage to these proteins. When Bcl-2 is overexpressed with its C-terminal transmembrane domain deleted, the protein is unable to bind to membranes and is completely resistant to photodamage by Pc 4-PDT (Usuda *et al*., 2003). Since membrane binding is one factor that determines the ability of Bcl-2 to undergo photodamage, we examined the subcellular distribution of Mcl-1. Mitochondria, ER, and cytosol were isolated from MCF-7c3 cells, as well as human monocyte U937 cells, and subjected to immunoblot analysis, probing first with the Mcl-1 antibody (Figure 1c), and then reprobing with antibodies to the mitochondrion-specific F1α-ATPase, to ER-specific ERp57, and to the cytosol-specific actin. Separation of mitochondria from cytosol and ER was nearly complete, as there was little cross contamination of the marker proteins. Mcl-1 is localized in all three fractions, although the level is lowest in the ER. The distribution of Mcl-1 in mitochondria, ER, and cytosol is similar for MCF-7c3 and U937 cells. However, the localization of Mcl-1 may be different in other cells, as it has been reported to be predominantly mitochondrial in

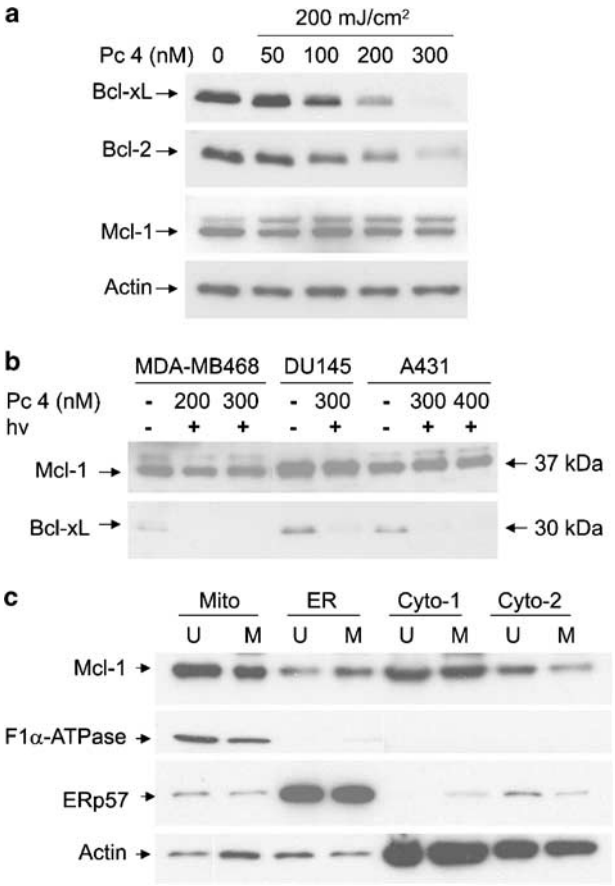

Figure 1 PDT induces photodamage to Bcl-2 and Bcl-xL but not to Mcl-1. (**a**, **b**) MCF-7c3 and MDA-MB-468 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). A431 and DU145 cells were grown in DMEM containing 10% (A431) or 5% (DU145) FBS. The cells were untreated or exposed to various doses of the phthalocyanine photosensitizer Pc 4 ($HOSiPcOSi(CH_3)_2(CH_2)_3N(CH_3)_2$, provided by Dr Malcolm E Kenney, Case Western Reserve University Department of Chemistry (Oleinick *et al*., 1993)), and irradiated with 200 mJ/cm^2 of red light using a diode array (EFOS, Mississauga, Ont., Canada) as described (Xue *et al*., 2001a, 2003a). All irradiations were performed at room temperature, and cells were collected 10 min later. Whole-cell lysates (Xue *et al*., 2001a) were separated by SDS–PAGE, transferred to PVDF membranes and probed with monoclonal hamster antihuman Bcl-2 (6C8, BD PharMingen, San Diego, CA, USA), anti-Bcl-xL (Cell Signaling, Beverly, MA, USA), anti-Mcl-1 (S-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and antiactin (NeoMarkers, Fremont, CA, USA). The immune complexes were detected by ECL system (Amersham, Arlington Heights, IL, USA). (**a**) MCF-7c3 cells. (**b**) MDA-MB468, DU145, and A431 cells. (**c**) Subcellular distribution of Mcl-1 in MCF-7c3 and U937 cells. Mitochondria, ER, and cytosol fractions were separated as described (Varnes *et al*., 1999) with some modifications. Briefly, cells were permeabilized with digitonin (100 μg/ml in 0.25 M sucrose buffer) on ice for 5 min and then centrifuged. The supernate from digitonin treatment was collected and called cyto-1. The permeabilized cells were homogenized and subjected to differential centrifugation, and mitochondria were collected as the pellet following centrifugation at 13 000 *g* for 15 min. The supernate was subjected to ultracentrifugation at 100 000 *g* for 40 min. The resulting pellet contains the ER fraction, and the supernate is cyto-2. All fractions were analysed by Western blotting using antibodies against Mcl-1, F1α-ATPase (Molecular Probes, Eugene, OR, USA), ERp57 (StressGen, Victoria, Canada), and actin

unstressed Saos2 cells (Leu *et al*., 2004), but mainly near the internal face of the plasma membrane and colocalized with α-tubulin in HeLa cells (Herrant *et al*., 2004). Although nonmembrane-associated Mcl-1 would be expected to be resistant to photodamage by Pc 4-PDT, there must be another explanation for the resistance of the Mcl-1 residing in mitochondria.

Recent reports have demonstrated that mitochondrial Mcl-1 is bound to Bak in healthy cells (Cuconati *et al*., 2003; Leu *et al*., 2004); treatment with doxorubicin can activate p53 resulting in disruption of the Bak-Mcl-1 complex. In the case of Pc 4-PDT, p53 was not activated (data not shown), so that Mcl-1 may remain bound to Bak. It is possible that the conformation or suborganellar distribution of Mcl-1 in mitochondria is different from that of Bcl-2 and Bcl-xL, and as a result, Mcl-1 resides far enough from the sites of binding of Pc 4 that it might be out of range of the photodynamically generated singlet oxygen that is the mediator of the photodamage. Alternatively, the resistance of Mcl-1 to photodamage may be due to structural differences between Mcl-1 and Bcl-2. Usuda *et al*. (2003) reported that both the transmembrane domain and α-helices 5 and 6 of Bcl-2 were critical for the induction of photodamage by Pc 4-PDT. Mcl-1 and Bcl-2 share only 25.6% amino-acid identity and 63% similarity in the α5/α6 region (Kozopas *et al*., 1993), which may be insufficient to form a photosensitive structure in Mcl-1. The transmembrane domain of Mcl-1 is more hydrophobic than that of Bcl-2, possibly directing the proteins to different membrane sites. Any of the suggested differences with Bcl-2 or other mechanisms may be responsible for the photodamage resistance of Mcl-1. Indeed, some other mitochondrion-located proteins, such as the voltage-dependent anion channel or Bax after translocation from cytosol to mitochondria during apoptosis, are not targets of photodamage (Usuda *et al*., 2003).

Although Mcl-1 has sequence similarity to other Bcl-2 family members, it contains a PEST sequence (Kozopas *et al*., 1993), which confers lability on the protein, with an estimated half-life of 1–3 h (Yang *et al*., 1995). It has been reported that Mcl-1 levels decrease in response to various toxic agents (Kitada *et al*., 2000; Iglesias-Serret *et al*., 2003; Nijhawan *et al*., 2003). We therefore investigated the turnover of Mcl-1. Following the procedure of Nijhawan *et al*. (2003), HeLa cells were irradiated with 200 mJ/cm^2 UVC light, followed by incubation for up to 4.5 h. The level of Mcl-1 was markedly reduced in a time-dependent manner after this highly supralethal UVC dose delivered to HeLa cells, as previously found, or to MCF-7c3 cells (Figure 2a). However, when HeLa or MCF-7c3 cells were treated with a dose of PDT that results in 90% loss of clonogenicity (LD90, 200 nM Pc 4, and 200 mJ/cm^2 red light), no change in Mcl-1 level was observed over the ensuing 4.5 h (Figure 2b). In contrast, the Bcl-2 level was dramatically reduced at the earliest time (10 min) postirradiation and remained suppressed. Because the UVC dose used in Figure 2a was supralethal, we tested doses that produced levels of toxicity similar to that of the PDT dose of Figure 2b (LD90). The LD90 for UVC-

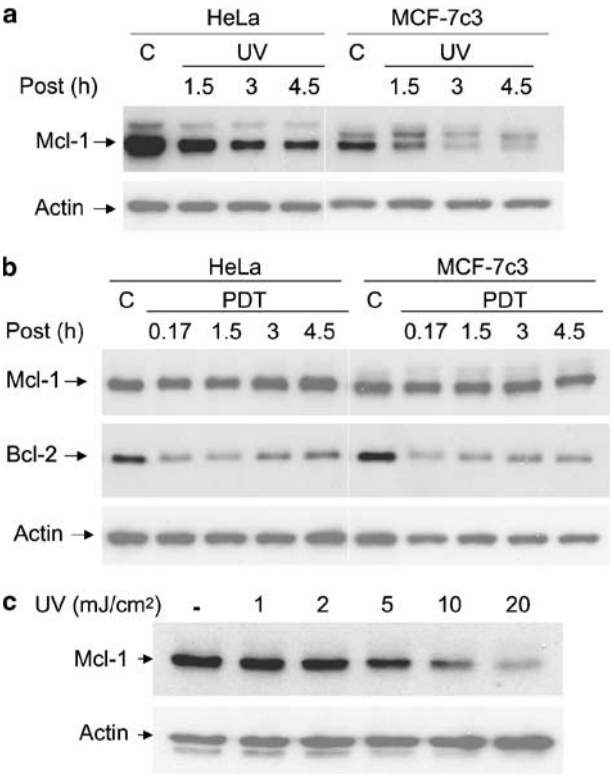

Figure 2 Comparison of Mcl-1 levels in control and UVC- or PDT-treated HeLa and MCF-7c3 cells. Cells, cultured as described for Figure 1, were untreated or exposed to 200 mJ/cm^2 UVC light (**a**) or PDT with 200 nM Pc 4 and 200 mJ/cm^2 of red light (**b**). For UVC radiation, cells were cultured in six-well plates. The medium was replaced with fresh medium 2 h before irradiation. The cells were irradiated with a Stratalinker (Stratagene, La Jolla, CA, USA) with the lid off, postincubated for various times, then collected for Western blot analysis of Mcl-1, Bcl-2, and actin. (**a**) Mcl-1 levels decreased in UVC-treated HeLa and MCF-7c3 cells. (**b**) PDT did not change Mcl-1 levels, but caused photodamage to Bcl-2 in both HeLa and MCF-7c3 cells. (**c**) MCF-7c3 cells were exposed to lower UVC doses (0–20 mJ/cm^2), then postincubated for 4 h, and Mcl-1 levels were examined by Western blotting

treated human GM38 fibroblasts is reported to be ~1.6 mJ/cm^2 (Bohr *et al*., 1986). Exposure of MCF-7c3 cells to UVC doses in this range of cytotoxicity resulted in no significant change of Mcl-1 level within 4 h, although loss of the protein was apparent after doses higher than 5 mJ/cm^2. Thus, when UVC and PDT are compared at equitoxic but not supralethal doses, no change in the Mcl-1 level is observed. A time-dependent Mcl-1 degradation was also observed in MCF-7c3 cells treated with staurosporine (STS), a protein kinase inhibitor (data not shown). These results indicate that the mechanism of high-dose UVC-induced Mcl-1 loss differs substantially from PDT-induced Bcl-2 and Bcl-xL photodamage. In fact, the decrease in Mcl-1 level in response to UVC has been attributed to inhibition of protein synthesis (Nijhawan *et al*., 2003). In contrast, in response to agents that do not inhibit protein synthesis, such as temperature shift or the DNA-damaging agent doxorubicin, Mcl-1 levels did not change (Leu *et al*., 2004). Thus, Pc 4-PDT has no effect on the steady-state

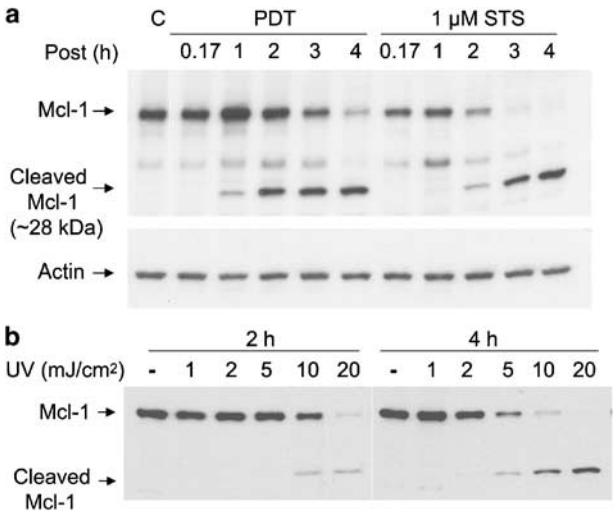

Figure 3 Mcl-1 cleavage in PDT-, STS-, or UVC-treated U937 cells. U937 cells, cultured in RPMI-1640 medium containing 10% FBS, were untreated or treated with PDT (100 nM Pc 4 and 200 mJ/cm^2) or 1 μM STS (**a**) or various UVC doses (**b**) and harvested at various times up to 4 h later. Total cellular protein was analysed by Western blot with Mcl-1 and actin antibodies. The ~28-kDa fragment of Mcl-1 is marked

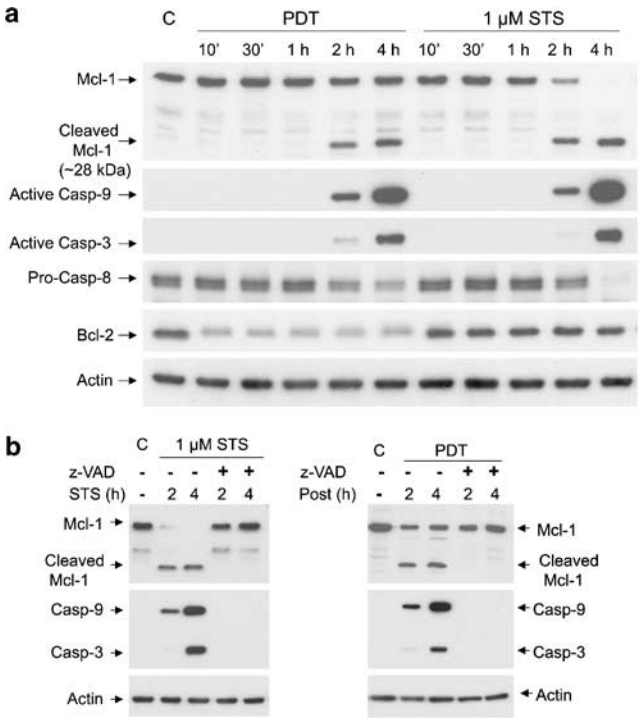

Figure 4 Mcl-1 cleavage and caspase activation in PDT- or STS-treated Jurkat cells. (**a**) Jurkat cells were cultured and treated as described for Figure 3. Samples were collected at different time points, and Western blot analysis was performed with Mcl-1, active caspase-9 (New England BioLabs, Beverly, MA, USA), active caspase-3, and caspase-8 from BD PharMingen (San Diego, CA, USA) antibodies. The blots were also probed with anti-Bcl-2 and actin. (**b**) Caspase inhibitor z-VAD completely blocks PDT- or STS-induced Mcl-1 cleavage in Jurkat cells. Jurkat cells were cultured and treated as above, except that some of the cultures were pre-incubated with 50 μM z-VAD-fmk (Biomol, Plymouth Meeting, PA, USA) for 1 h before exposure of the cells to PDT or STS. z-VAD, when present, remained in the culture throughout the posttreatment period. Western blot analysis of total cellular protein employed antibodies to Mcl-1, active caspase-9 and -3, and actin

levels of Mcl-1, because protein synthesis is not inhibited and Mcl-1 is not susceptible to direct photodamage.

It has been reported that Bcl-2 proteins can be cleaved to ~23-kDa fragment in interferon (IFN)-γ- or VP-16-treated human monocytic U937 cells (Fujita and Tsuruo, 1998; Varela *et al*., 2001). In our previous study, U937 cells were found to be very sensitive to PDT (Separovic *et al*., 1998). In order to determine whether Mcl-1 could be downregulated or cleaved to small fragments in these PDT-sensitive cells, U937 cells were treated with a PDT dose (100 nM Pc 4 and 200 mJ/cm^2 red light), which resulted in ~99% cell killing (Separovic *et al*., 1998; Chiu *et al*., 2001) and 46% apoptotic cells 4 h post-PDT. For comparison, cells were also treated with STS, a strong inducer of apoptosis. After various post-treatment incubation periods, total cellular protein was subjected to Western blot analysis with anti-Mcl-1 and antiactin. As shown in Figure 3a, a ~28-kDa Mcl-1 cleavage fragment was seen as early as 1 h post-PDT and increased in amount at later times, as the full-size Mcl-1 level decreased. In STS-treated U937 cells, Mcl-1 cleavage began by 2 h and increased with time, such that by 3–4 h, essentially all Mcl-1 was cleaved. U937 cells were also examined after the same range of UVC doses used for MCF7c3 cells (Figure 2c) and analysed for Mcl-1 level 2 or 4 h later (Figure 3b). Similar to responses of U937 cells to PDT or STS, UVC also induced a time- and dose-dependent cleavage of Mcl-1.

With regard to PDT, clearly the responses of Mcl-1 differ in lymphoid-derived cells (Figure 3a) from those in epithelium-derived cells (Figures 1a, b, 2b). This difference is unlikely to be due to different Pc 4 localization or uptake, because we have found similar Pc 4 uptake in A431 and Jurkat cells and similar distribution of the photosensitizer in mitochondria and ER/Golgi in both lymphoma cells (L5178Y-R) and carcinoid cells (A431, MCF-7, DU-145) (Trivedi *et al*., 2000; Lam *et al*., 2001; Usuda *et al*., 2003).

Like U937 cells, Jurkat human lymphoblasts are sensitive to PDT-induced cell killing (data not shown) and to the cleavage of Mcl-1, which is observed 2–4 h after either PDT or STS exposure (Figure 4a). To investigate the relationship between Mcl-1 cleavage and caspase activation, the blots were also probed with antibodies to activated caspase-9, activated caspase-3, and procaspase-8. Figure 4a shows that all three caspases were activated in the same time period as for Mcl-1 cleavage in response to either treatment. As expected, immediate Bcl-2 loss (photodamage) was observed in PDT-treated but not in STS-treated Jurkat cells. Interestingly, no Bcl-2 cleavage was found in response to either treatment, implying that Mcl-1 is a more sensitive target for caspase cleavage than Bcl-2.

To better link Mcl-1 cleavage with caspase activation, Jurkat cells were preincubated with or without the broad spectrum caspase inhibitor z-VAD-fmk for 1 h, then exposed to PDT or STS and incubated for 2 and 4 h. Total cell lysates were Western blotted with anti-Mcl-1, activated caspase-9 or -3, and actin antibodies. Figure 4b shows that z-VAD completely blocked activation of caspases-3 and -9 and prevented Mcl-1 cleavage induced by PDT or STS. Mcl-1 cleavage induced by UVC was also blocked by z-VAD (data not shown). Our results indicate that Mcl-1 cleavage was mediated by caspases. Caspase cleavage of Mcl-1 has been observed in other cell systems (Herrant *et al*., 2002; Snowden *et al*., 2003). Human Mcl-1 is cleaved at two sites *in vitro*, Asp^{127} and Asp^{157} (Clohessy *et al*., 2004; Herrant *et al*., 2004; Michels *et al*., 2004). The resulting protein that lacks the first 127 or 157 amino acids contains only the BH1–BH3 domains and may have a reduced antiapoptotic potential (Herrant *et al*., 2004). Another Mcl-1 mutant, Mcl-1 Δ127, exhibited an increased association with the BH3-only protein Bim and lesser ability to inhibit apoptosis (Herrant *et al*., 2004).

Although the levels of both Bcl-2 and Mcl-1 are reduced in PDT-treated Jurkat cells (Figure 4a), the mechanisms operating on these two proteins must be different. As we have reported (Xue *et al*., 2001a), Bcl-2 photodamage occurs immediately upon photoirradiation, even when cells were kept on ice or in the presence of protease inhibitors, including those blocking caspases or the proteasome, implying that Bcl-2 is a primary target of the photochemical generation of singlet oxygen. In this study, the observation of Bcl-2 photodamage in PDT-treated, but not in STS-treated, U937 and Jurkat cells further confirms that this unique phenomenon occurs in many cell types. In contrast, Mcl-1 cleavage is a delayed response, requires caspase activation, occurs in response to PDT, STS, and UVC, but is limited to cells of lymphoid origin. The Mcl-1 cleavage fragment is reported to be pro-apoptotic (Zhou *et al*., 1997; Michels *et al*., 2004). The initial PDT-induced photodamage to Bcl-2 may promote caspase activation, which is amplified as Mcl-1 is converted to a proapoptotic cleaved fragment. The contribution of apoptosis to the overall killing of lymphoid cells by PDT and other agents may be more important than that contribution is to the photocytotoxicity of carcinoid cells (Xue *et al*., 2001b, 2003b), and the presence of cleaved Mcl-1 may then be associated with the rapid and extensive apoptosis of these cells induced by PDT and other agents. The lack of appearance of the 28-kDa Mcl-1 cleavage product in carcinoma cells, either in response to low (Figure 2c) or high (Figure 2a) doses of UVC (Nijhawan *et al*., 2003) or to an LD90 dose of PDT (Figures 1 and 2) may contribute to the greater resistance of carcinomas to these and other toxic treatments. It should also be noted that after the highly supralethal UVC dose, cells may die through necrosis, for which caspase activity would not be required; however, even with the more modest PDT dose we have employed, which is known to induce caspase activation and apoptosis, Mcl-1 cleavage was not found in carcinoma cells. Thus, the factors controlling the susceptibility of Mcl-1 to caspase cleavage during apoptosis may be dependent upon cell type and stimulus; these factors remain to be defined. Understanding those controls may suggest means to increase the sensitivity of carcinomas to PDT to more closely match the greater sensitivity of lymphoid tumors. Alternatively, it may allow PDT to be used more effectively to treat lymphomas with minimum damage to surrounding epithelial tissues.

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