Rapid cytochrome *c* release, activation of caspases 3, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy

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Abstract Photodynamic therapy (PDT) is a clinical approach that utilizes light-activated drugs for the treatment of a variety of pathologic conditions. The initiating events of PDT-induced apoptosis are poorly defined. It has been shown for other pro-apoptotic stimuli that the integral endoplasmic reticulum protein Bap31 is cleaved by caspases 1 and 8, but not by caspase-3. Further, a 20 kDa Bap31 cleavage fragment is generated which can induce apoptosis. In the current report, we sought to determine whether Bap31 cleavage and generation of p20 is an early event in PDT-induced apoptosis. The mitochondrial release of cytochrome *c*, involvement of caspases 1, 2, 3, 4, 6, 7, 8, and 10 and the status of several known caspase substrates, including Bap31, were evaluated in PDT-treated HeLa cells. Cytochrome *c* appeared in the cytosol immediately following light activation of the photosensitizer benzoporphyrin derivative monoacid ring A. Activation of caspases 3, 6, 7, and 8 was evident within 1–2 h post PDT. Processing of caspases 1, 2, 4, and 10 was not observed. Cleavage of Bap31 was observed at 2–3 h post PDT. The caspase-3 inhibitor DEVD-fmk blocked caspase-8 and Bap31 cleavage suggesting that caspase-8 and Bap31 processing occur downstream of caspase-3 activation in PDT-induced apoptosis. These results demonstrate that release of mitochondrial cytochrome *c* into the cytoplasm is a primary event following PDT, preceding caspase activation and cleavage of Bap31. To our knowledge, this is the first example of a chemotherapeutic agent inducing caspase-8 activation and demonstrates that caspase-8 activation can occur after cytochrome *c* release.

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Key words: Apoptosis; Photodynamic therapy;
Benzoporphyrin derivative monoacid ring A; Caspase;
Cytochrome *c*; Mitochondrion

1. Introduction

It is becoming increasingly evident that the same genetic defects that promote the growth and survival of tumor cells may also contribute to their resistance against various anti-cancer agents [1,2]. Thus, intense research has been devoted to understanding the molecular processes involved in apoptotic cell death and devising therapeutic strategies that can selectively restore the apoptotic potential to tumor cells.

Disruption of mitochondrial function is a critical event in the apoptotic process leading to the elimination of cells treated with chemotherapeutic agents [2]. Opening of the mitochondrial megachannel (also called permeability transition pore) has been implicated as a key event in the disruption of mitochondrial membrane integrity during apoptosis [2]. Many intrinsic factors can induce opening of the megachannel including members of the Bcl-2/Bax family, cellular redox status, cytosolic Ca^{2+} levels, ceramide, and amphipathic peptides [3]. Disruption of mitochondrial membrane integrity involves the loss of metabolic functions and release of proteins from the mitochondrial intermembrane space into the cytosol. Cytochrome *c* and AIF (apoptosis inducing factor) represent two such proteins, which when released into the cytosol, promote caspase and/or nuclease activation [4–7].

Proteolytic cleavage of certain cellular substrates by caspases plays a central role in the apoptotic process. Caspases (cysteinyl aspartate-specific proteinases), present in the cytoplasm as zymogens, require processing by other proteases, often another caspase, or by autocatalytic cleavage to produce the active form [1]. Caspase precursors are cleaved at internally conserved sequences of amino acids residues with Asp residing in the P1 cleavage site [8]. Phylogenetic analysis has revealed that caspases may be grouped into two major subfamilies: the ICE and CED-3 subfamilies [9]. Members of the ICE subfamily (caspases 1, 4, 5, 11 and 13) may have subsidiary roles in pro-inflammatory events such as cytokine mobilization, whereas members of the CED-3 subfamily (caspases 2, 3, 6, 7, 8, 9, and 10) are primarily involved in apoptosis [9]. Caspase-3, considered one of the central executioner molecules, is responsible for cleaving various proteins thereby disabling important cellular structural, functional and repair processes [9–11]. Three mitochondria-related apoptotic protease activating factors (Apafs) may play a key role in the activation of caspase-3 [12]. Apaf-1 contains a conserved amino acid sequence in its prodomain termed the caspase recruitment domain (CARD) that binds caspases containing similar CARDs at their NH_2 terminus [12,13]. In the presence of cytochrome *c* (Apaf-2) and dATP, Apaf-1 binds to and facilitates caspase-9 (Apaf-3) processing which then cleaves caspase-3 [4].

Although the mitochondrial events pertaining to the onset of apoptosis have been well characterized, limited research has been done concerning the possible role of the endoplasmic reticulum (ER) in this process. A role for Bcl-2 in ER function has been proposed recently [14]. Bap31, a 28 kDa integral ER protein, was originally described as a co-precipitant with membrane-bound immunoglobulin D in B cell lysates [15]. Bap31 may act as a shuttle protein between the ER and the intermediate compartment and/or *cis*-Golgi complex [16].

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Bap31 also has been shown to bind to caspases 1 and 8, but not caspase-3 and forms complexes with Bcl-2 or Bcl-X$_L$ [14]. During apoptosis, Bap31 is cleaved, generating a 20 kDa product which itself can induce apoptosis when expressed ectopically in otherwise normal cells [14].

Photodynamic therapy (PDT) involves the topical or systemic application of a photosensitizing agent followed by illumination with a specific wavelength of light which catalyzes the production of reactive oxygen species to produce the cytotoxic effect [17]. PDT, using benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin) as the photosensitizer, induces apoptosis in several cell types [18–20]. It has also been demonstrated that overexpression of Bcl-2 or Bcl-X$_L$ prevents caspase-3 activation, cleavage of specific cellular proteins, and DNA fragmentation implicating caspase-3 as a key player in PDT-induced apoptosis [18,21,22].

In the present report, we show that cytochrome c release occurs immediately following light activation of BPD-MA while caspase activation and Bap31 cleavage occur at a significantly later time. In particular, caspase-8 activation occurred after mitochondrial cytochrome c release and activation was inhibited by the general caspase inhibitor ZVAD-fmk, as well as by the caspase-3/7 inhibitor DEVD-fmk, indicating that caspase-8 may be activated at a later stage during PDT-induced apoptosis.

2. Materials and methods

2.1. Reagents

Liposomally formulated BPD-MA was provided by QLT Photo-Therapeutics Inc. (Vancouver, BC). Antibodies were obtained from the following sources: rabbit anti-caspases 1, 3 and 6 and mouse anti-caspase-8 (Upstate Biotechnology Inc., Lake Placid, NY), mouse anti-caspases 2 and 7 and Ras GTPase-activating protein (Ras-GAP) (Transduction Laboratories, Mississauga, Ont.), rabbit anti-caspase-4 (Oncogene Research Products, Cambridge, MA), mouse anti-cytochrome c and caspase-9 (Pharmingen, Mississauga, Ont.), goat anti-caspase-10 (Research Diagnostics Inc., Flanders, NJ), rabbit anti-DNA fragmentation factor (DFF) (Dr. Xiaodong Wang, University of Texas Southwestern Medical School, Dallas, TX), mouse anti-poly(ADP-ribose) polymerase (PARP) (Biomol Research Laboratories, Plymouth Meeting, PA), rabbit anti-Bap31. All other drugs and chemicals, unless specified, were from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

HeLa cells (ATCC, Rockford, MD) were maintained in DMEM 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) and streptomycin (100 μg/ml) (Gibco BRL, Burlington, Ont.).

2.3. Photoactivation of BPD-MA

For photodynamic studies, HeLa cells were incubated for a total of 60 min at 37°C with or without BPD-MA (0 or 200 ng/ml) in DMEM supplemented with 2% FBS. For caspase inhibition studies, Z-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-fmk) (50 μM) (Enzyme Systems Products, Dublin, CA) or Z-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk) (50, 100 or 200 μM) (Bachem, Torrance, CA) was added to cells for the final 30 min of the BPD-MA incubation period prior to light activation. Following drug incubation, cells were exposed to fluorescent red light (620–700 nm) delivered at a rate of 5.6 mW/cm^2 to give a total dose of 2 J/cm^2. Cells were maintained in petri dishes at 37°C until further analysis.

2.4. Preparation of whole protein extracts

To obtain whole cell lysates, cells were washed twice in PBS and lysed in 1 ml of lysis buffer (1% Nonidet P-40 (NP-40), 20 mM Tris, pH 8, 137 mM NaCl, 10% glycerol) supplemented with 1 mM phenyl-methylsulfonyl fluoride, aprotinin (0.15 U/ml), and 1 mM sodium orthovanadate for 20 min on ice as described [18].

2.5. Preparation of cytosolic protein extracts

To evaluate mitochondrial cytochrome c release, cytosolic protein extracts (S-100) were obtained as previously described [23]. Briefly, cells were washed twice with PBS and cell pellets were resuspended in 700 μl ice-cold buffer containing 20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF. Cells were disrupted using a Kontes dounce homogenizer. Lysate was centrifuged at 10 000×g for 10 min and the resultant supernatant was further centrifuged at 100 000×g for 1 h in a Beckman Optima TLX ultracentrifuge (Beckman, Palo Alto, CA) using a TL-100 rotor.

2.6. Protease assay

A cell-free protease assay was performed by incubating 50 μg of cell lysate protein in 150 μl of reaction buffer (1% NP-40, 20 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol) containing 100 μM of caspase-3 (acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC)) (Calbiochem, Cambridge, MA) or caspase-8 (Z-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin (Z-IETD-AFC)) substrate in 96-well microtiter plates [18]. Lysates were incubated at 37°C for 4 h and cleaved substrate fluorescence levels were determined using a CytoFluor 2350 (PerSeptive Biosystems, Ont.) set at excitation and emission wavelengths of 360 nm and 460 nm for caspase-3 and 360 nm and 490 nm for caspase-8.
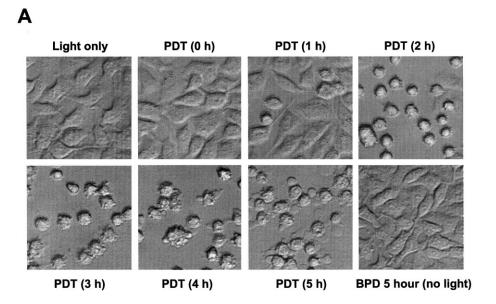
2.7. Immunoblot analysis

Detergent soluble proteins (30 μg) were separated by SDS-PAGE in 10% acrylamide gels, under reducing conditions followed by Western blotting [18]. Membranes were incubated with primary antibody for 45 min at room temperature. Membranes were probed with horse-radish peroxidase-labelled anti-goat IgG, anti-mouse IgG or anti-rabbit IgG antibodies (1:5000) in PBS, 0.05% Tween 20, 5% (w/v) skim milk powder for 30 min at room temperature. Proteins were detected using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) and bands visualized by autoradiography.

3. Results and discussion

3.1. PDT induces rapid apoptosis which is inhibited by ZVAD-fmk

HeLa cells were examined by differential interference contrast (DIC) microscopy to assess their morphology following PDT. Cell shrinkage and membrane blebbing, characteristic of apoptotic cells, were observed for some cells within 1 h following light irradiation of cells treated with BPD-MA (Fig. 1). These changes were clearly evident for the majority of cells by 2 h post PDT. DEVD-ase activity, indicative of caspase-3 and 7-like cleavage activity, paralleled the DIC microscopy observations (Fig. 1B). DEVD-ase activity was detected in cell lysates by 1 h post PDT. PDT-treated cells were also annexin-V-positive after 2 h (data not shown), providing further evidence of apoptosis. Treatment of cells with BPD-MA in the absence of light had no effect on caspase activity or cell viability. We have previously demonstrated that pretreatment with caspase inhibitors or overexpression of Bcl-2 or Bcl-X$_L$ blocked PDT-induced caspase-3 activation and DNA fragmentation in HL-60 cells [18,21,22]. To confirm that caspases were involved in PDT-induced cell killing of HeLa cells, cells were pretreated with increasing concentrations of the general caspase inhibitor ZVAD-fmk prior to PDT. With increasing concentrations of ZVAD-fmk, morphologic apoptotic changes such as membrane blebbing and cell shrinkage became less evident for PDT-treated cells (Fig. 2). These results show that caspases are intimately involved in the formation of the apoptotic phenotype observed for HeLa cells following PDT.

A

Light only   **PDT (0 h)**   **PDT (1 h)**   **PDT (2 h)**

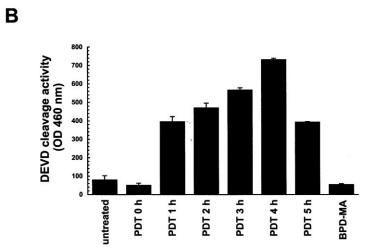**PDT (3 h)**   **PDT (4 h)**   **PDT (5 h)**   **BPD 5 hour (no light)**

B

Fig. 1. PDT induces changes in HeLa cell morphology associated with apoptosis and increased caspase-3-like (DEVD-ase) activity. Cells were incubated without BPD-MA (untreated), with BPD-MA in the absence of light (BPD-MA), or with BPD-MA followed by light irradiation (PDT). A: Cells were examined 0, 1, 2, 3, 4, and 5 h post PDT. B: Cells were lysed 0, 1, 2, 3, 4 and 5 h post PDT and cell extracts were analyzed for DEVD-ase cleavage activity by a cell-free protease assay.

Light treated   **0 μM**   **50 μM**   **100 μM**   **200 μM**   **ZVAD-fmk**
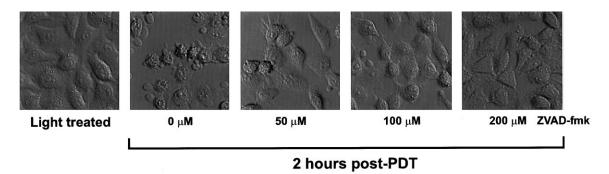
2 hours post-PDT

Fig. 2. Caspases are involved in PDT-induced HeLa cell morphological changes. Prior to light activation of BPD-MA, cells were incubated with different concentrations of the general caspase family inhibitor ZVAD-fmk. Following PDT, cells were maintained for a further 2 h and their phenotype was assessed by DIC microscopy.
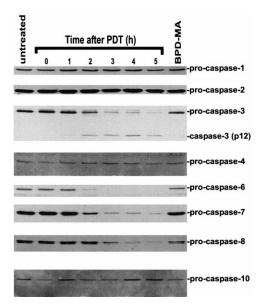
Fig. 3. Processing of caspases 3, 6, 7 and 8 but not caspases 1, 2, 4, and 10 occurs in PDT-treated HeLa cells. Cell lysates were prepared at the indicated times following PDT. These lysates were separated by SDS-PAGE in 10% acrylamide gels followed by Western immunoblotting.

3.2. PDT induces caspases 3, 6, 7 and 8 cleavage in HeLa cells

Our next step was to assess the status of several members of the caspase family during PDT-induced apoptosis of HeLa cells. Caspases 3, 6, 7, and 8, but not caspases 1, 2, 4 and 10, were processed following PDT (Fig. 3). Caspase-9 was not detectable with the antibody utilized. Interestingly, caspase-8 processing was not evident prior to cleavage of caspases 3, 6 and 7. The involvement of caspase-8 as an early upstream effector caspase in receptor-mediated apoptosis instigated by FasL and tumor necrosis factor (TNF) has been well documented [24–26]. However, with the exception of UV irradiation, which may initiate the Fas pathway [27], we were unable to identify a chemotherapeutic agent that invokes caspase-8 activation.

In co-transfected human cells, pro-caspase-8 has been shown to interact with Ced-4, an adaptor molecule found in *Caenorhabditis elegans* that binds to and activates the caspase-3 homologue Ced-3 [28]. Furthermore, the predicted death effector homology domain within the cytosolic region of Bap31 interacts with Ced-4 and contributes to recruitment of pro-caspase-8 [29]. It is conceivable that Apaf-1, the human homologue of Ced-4, may bind to and activate caspase-8 upon recruitment to a Bap31/Apaf-1 complex during apoptosis. This hypothesis will be tested in future studies. To further discern the role for caspases in PDT-induced apoptosis, we examined cleavage of the known caspase substrates PARP, DFF and Ras-GAP [30–32]. As expected, these substrates were all cleaved following PDT (Fig. 4).

3.3. Cytochrome c is released immediately following PDT and prior to caspase-8 processing

Since it has been well documented that caspase-8 is the first caspase activated in Fas-, TRAIL- and TNF-induced apoptosis [26,33,34], we sought to determine whether caspase-8 activation occurs prior to, as in Fas- and TNF-induced apoptosis, or after cytochrome c release. Whole cell extracts and cytosolic (S-100) protein extracts were analyzed for cytochrome c
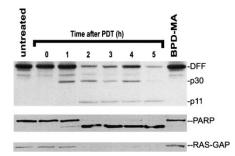

Fig. 4. PDT induces caspase-mediated cleavage of DFF, PARP and Ras-GAP. HeLa cells were lysed at the indicated times following PDT. Cell lysates were separated by SDS-PAGE followed by Western immunoblotting.

content by Western blotting (Fig. 5). Detection of cytochrome c in the cytosolic S-100 fraction corresponds to the release of cytochrome c from the mitochondria [4]. Cytochrome c was readily detectable within the cytosolic fraction prepared immediately following light activation indicating that cytochrome c release is an early event in PDT-induced apoptosis. Cytochrome c was undetectable in the S-100 cytosolic fractions of untreated cells or cells treated with BPD-MA in the absence of light. The rapid appearance of cytochrome c in the cytosol following PDT may be attributed to the direct action and localization of photosensitizers to mitochondria [17].

Caspase-8 activation was assessed by Western blotting and by a cell-free protease assay that measures the cleavage activity of the caspase-8-specific fluorogenically labeled IETD tetrapeptide (Fig. 5) [35]. Bap31 cleavage was evaluated in parallel over the 5 h period following light activation. Caspase-8 cleavage and activation, as well as Bap31 cleavage, occurred
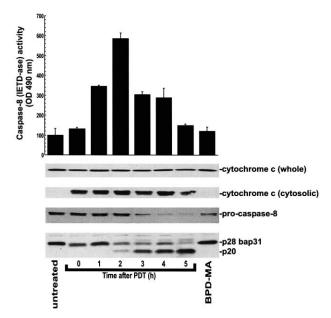

Fig. 5. Cytochrome c is detectable within the cytosol immediately after light irradiation. HeLa cells were lysed at the indicated times following PDT. Cell lysates were analyzed for caspase-8-like (IETD-ase) cleavage activity using a cell-free protease assay. Whole cell lysates were separated by SDS-PAGE followed by Western immunoblotting and probed for cytochrome c, caspase-8 or Bap31. S-100 cytosolic extracts were prepared to evaluate the presence of cytochrome c in the cytosol.
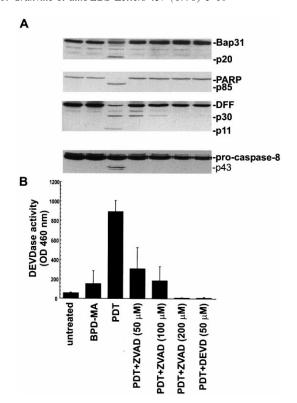
Fig. 6. PDT induces processing of caspase-8 processing that is inhibited by pretreatment with the general caspase (ZVAD-fmk) or specific caspase-3/7 (DEVD-fmk) inhibitors. A: Cells were pretreated with ZVAD-fmk or DEVD (as indicated) for 30 min prior to photosensitization. Cells were then lysed and proteins were separated by SDS-PAGE and immunoblotted for Bap31, PARP, DFF and caspase-8. B: Caspase-3/7-like cleavage activity was measured using a cell-free protease assay.

at least 1–2 h after the appearance of cytochrome *c* in the cytosol indicating that caspase-8 activation is not an initiating event in PDT-induced apoptosis. Since caspase-1 is not activated and caspase-8 is the only other caspase known to bind to and cleave Bap31 [14], Bap31 cleavage during PDT-induced apoptosis provides further support of the existence of caspase-8 activity in these cells.

3.4. ZVAD-fmk and DEVD-fmk inhibit PDT-induced caspase-8 processing

Prolonged exposure of the Western blots during autoradiography allowed the detection of both pro-caspase-8/a (55 kDa) and caspase-8/b (54 kDa) processing and the appearance of 41 and 43 kDa caspase-8 intermediate cleavage products, respectively [24] in PDT-treated cell lysates (Fig. 6). Cells were pretreated with the general caspase family inhibitor ZVAD-fmk or the caspase-3/7-specific inhibitor DEVD-fmk prior to light activation of BPD-MA. ZVAD-fmk and DEVD-fmk inhibited pro-caspase-8/a (55 kDa) and caspase-8/b (54 kDa) processing and the appearance of 43 and 41 kDa caspase-8 intermediate cleavage products, respectively [24]. Both ZVAD-fmk and DEVD-fmk inhibitors blocked the appearance of Bap31 as well as PARP and DFF cleavage fragments in PDT-treated cell lysates (Fig. 6).

In summary, mitochondrial cytochrome *c* release represents an extremely early following PDT. Caspases 3, 6 and 7 were activated in PDT-treated HeLa cells similar to events triggered by other chemotherapeutic agents [36–38]. Caspase-8

activation followed cytochrome *c* release in PDT-treated cells. Although the HeLa cells used in these experiments are Fas-positive (unpublished results) and caspase-8 was activated, due to the rapid release of mitochondrial cytochrome *c* into the cytoplasm we do not believe that the dominant mechanism of apoptotic cell death instigated by PDT occurs via the Fas pathway. It is possible that triggering of the Fas/caspase-8 pathway represents a secondary means of ensuring the elimination of damaged cells. A detailed analysis of the role of the Fas receptor system in PDT-induced apoptosis will be examined in future studies.

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