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Photodynamic therapy induces caspase-3 activation in HL-60 cells

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Abstract

Caspases have been shown to play a crucial role in apoptosis induced by various deleterious and physiologic stimuli. In this study, we show for the first time that photodynamic therapy (PDT), using benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin) as the photosensitizer, induces the complete cleavage and subsequent activation of caspase-3 (CPP32/Yama/Apopain) but not caspase-1 (ICE) in human promyelocytic leukemia HL-60 cells. Poly(ADP-ribose) polymerase (PARP) and the catalytic subunit of DNA dependent protein kinase (DNA PK_{CS}) were cleaved within 60 min of light activation of BPD-MA. The general caspase inhibitor Z-Asp-2,6 dichlorobenzoyloxymethylketone (Z-Asp-DCB) blocked PARP cleavage while the serine protease inhibitors 3,4-dichloroisocoumarin (DCI) and N-tosyl-lysyl chloromethyl ketone (TLCK) blocked the cleavage of caspase-3 suggesting that they act upstream of caspase-3 activation. All three inhibitors were able to block DNA fragmentation that was induced by treatment with BPD-MA followed by light application. These studies demonstrate that protease activity, particularly that of caspase-3, is triggered in HL-60 cells treated with lethal levels of BPD-MA and visible light.

Keywords: PDT; CPP32/Yama/Apopain; PARP; DNA-PK_{CS}; BPD-MA

Abbreviations: BPD-MA, benzoporphyrin derivative monoacid ring A (verteporfin); HRP, horseradish peroxidase; PDT, photodynamic therapy; ICE, interleukin 1β-converting enzyme; DNA-PK_{CS}, DNA dependent protein kinase (catalytic subunit); PARP, Poly (ADP-ribose) polymerase; Z-Asp-DCB, Z-Asp-2,6 dichlorobenzoyloxymethylketone; Z-AAD-CMK, Z-Ala-Ala-chloromethyl ketone; DCI, 3,4-dichloroisocoumarin; TLCK, N-tosyl-lysyl-chloromethyl ketone

Introduction

Photodynamic therapy (PDT) is a cancer treatment which involves the topical or systemic application of a light-absorbing compound (photosensitizer), usually a porphyrin derivative, which accumulates within the target tissue (Jameison *et al*, 1993; Gomer *et al*, 1988). Upon irradiation with visible light of a specific wavelength, reactive oxygen species are produced in cells containing photosensitizer, promoting cell death and tissue necrosis. Our laboratory has been interested in the biological activity of the potent photosensitizer benzoporphyrin derivative monoacid ring A (BPD-MA). BPD-MA is a chlorin-type photosensitizer exhibiting distinct advances over its hematoporphyrin forerunners in that the active component is clearly defined, it is effective at low concentrations and can be activated with longer, more penetrating wavelengths of light (Gluck *et al*, 1996). Evidence has been presented that PDT using other photosensitizers causes cells to die via apoptosis, although the specific cellular events triggered by photoactivation have not been clearly defined (Kessel and Luo, 1996; Oleinik *et al*, 1991).

Apoptosis is a morphologically distinct form of cell death that plays an important role during normal development and homeostasis, and its dysregulation may be involved in the pathogenesis of a number of diseases including cancer, acquired immunodeficiency syndrome (AIDS) and neurodegenerative disorders (Thompson, 1995). When most cell types receive irreversible damage, they activate an intrinsic cell suicide program, characterized by nuclear and cytoplasmic condensation, blebbing off of apoptotic bodies and DNA fragmentation (Kerr *et al*, 1972). These morphological and biochemical events are well conserved throughout evolution (Martin and Green, 1995). The concept that apoptosis is a finely regulated process is now well established (Martin and Green, 1995). Many of the genes involved in apoptotic cell death were initially discovered in the nematode *Caenorhabditis elegans* (Stellar, 1995). In the past few years it has been shown that the proteolytic cleavage of key cellular substrates represent fundamental biochemical events underlying the apoptotic process (Casciola-Rosen *et al*, 1996). One of the best described proapoptotic genes, CED-3, encodes a protein that is highly homologous to the mammalian interleukin-1β converting enzyme (ICE) (Yuan *et al*, 1993). ICE was the first identified member of a class of cysteine proteases with near absolute specificity for aspartic acid residues (Martin and Green, 1995). To date, ten human ICE homologs have been identified leading to the development of the 'caspase' nomenclature to describe this distinct family of proteases (Alnemri *et al*, 1996).

The involvement of caspases in PDT-induced apoptosis has not been described. The following paper describes the

contribution of caspase-3 to the proteolytic events which occur in HL-60 cells treated with BPD-MA and light.

Results

A high proportion of cells treated with PDT (BPD-MA and light) exhibited DNA fragmentation (91.9 $\pm$ 5.3%; *n*=11) within 3 h of photosensitization compared to only 4.3 $\pm$ 2.9% (*n*=11) of untreated cells. DNA fragmentation was not induced in cells exposed only to the light source or in cells incubated with BPD-MA and protected from light (Figure 1).

To determine whether non-specific proteolytic activity might be involved with PDT-induced apoptosis, various broad range biochemical inhibitors were added to the cells after BPD-MA incubation but prior to their exposure to light. Preliminary experiments indicated that DNA fragmentation was not evident for at least 40 min following light treatment. The extent of DNA fragmentation was determined 3 h post-irradiation since PDT-treated HL-60 cells exhibited high levels of DNA fragmentation by this time. The caspase family inhibitor, Z-Asp-DCB as well as the serine protease inhibitors DCI and TLCK completely blocked PDT-induced DNA fragmentation. The tripeptide granzyme B inhibitor (Z-AAD-CMK) was used to determine whether granzyme B (GraB) may be involved in PDT-induced apoptosis. GraB has been shown to be involved in the cell death pathway mediated by cytotoxic T lymphocytes (Shi *et al*, 1996; Quan *et al*, 1996). It has been demonstrated by Quan *et al* (1996) that caspase-3 can be proteolytically activated by GraB in cytotoxic lymphocytes. In our system, the GraB inhibitor did not block DNA fragmentation, thus decreasing the likelihood of GraB involvement in PDT-induced apoptosis. Other inhibitors (PMSF and Aprotinin) and vehicle solutions also had no effect on DNA fragmentation (Figure 1).

To further examine the nature of the proteolytic activity induced by PDT, protease assays were performed to measure caspase-1 (YVAD-ase) and caspase-3 (DEVD-ase) activity. There was no evidence of cleavage of the DABCYL-YVADAPV-EDANS (caspase-1) substrate (Figure 2A) which was supported by the absence of caspase-1 cleavage by Western blot analysis (Figure 3A). However, DEVD-ase activity, indicative of caspase-3 activity, was present in lysates from cells treated with BPD-MA and light but not in control cell lysates (Figure 2B).

To examine whether caspase-3 processing and PARP cleavage were induced by PDT, HL-60 cells were treated with PDT and lysed 0, 15, 30, 60, or 120 min after light activation. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-caspase-1 (ICE), caspase-3 (CPP32) or anti-PARP antibodies. Pro-caspase-1 was not cleaved following PDT (Figure 3A). The pro-caspase-3 protein appears to be fully cleaved within 15 min, followed by the appearance of the 12 kD subunit (Figure 3B), while PARP was fully cleaved by 60 min following light treatment (Figure 3C). There was a small

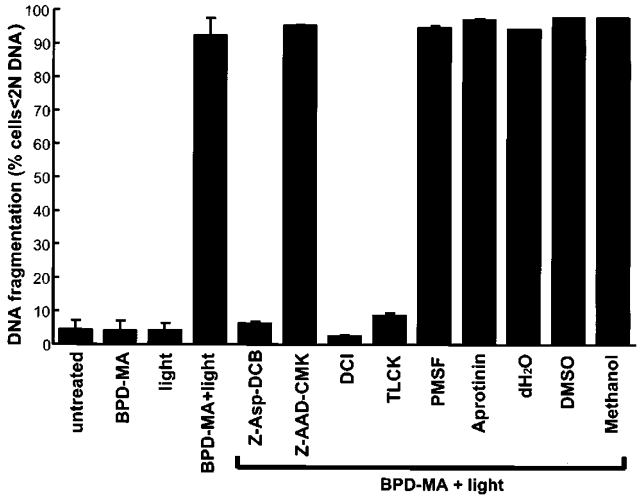

Figure 1 Influence of various protease inhibitors on DNA fragmentation 3 h after PDT. HL-60 cells were stained with PI and the extent of DNA fragmentation determined by flow cytometry. Cells were incubated with BPD-MA (100 ng/ml) for 40 min. At 40 min, inhibitor was added to the cells for a further 20 min. Cells were then exposed to red fluorescent light at 5.6 mW/cm^2 to give a total dose of 2 J/cm^2. Cells were exposed to the following inhibitor concentrations: Z-Asp-DCB (110 μM), Z-AAD-CMK (100 μM), DCI (230 μM), TLCK (135 μM), PMSF (100 μM) aprotinin (100 μM), or appropriately-matched vehicle concentration of dH_2O, methanol or DMSO. Summarized results from a series of experiments are shown. (medium: *n*=11; BPD-MA: *n*=11; light: *n*=3; BPD-MA+light: *n*=11; each protease inhibitor: *n*=6; vehicle: *n*=2)

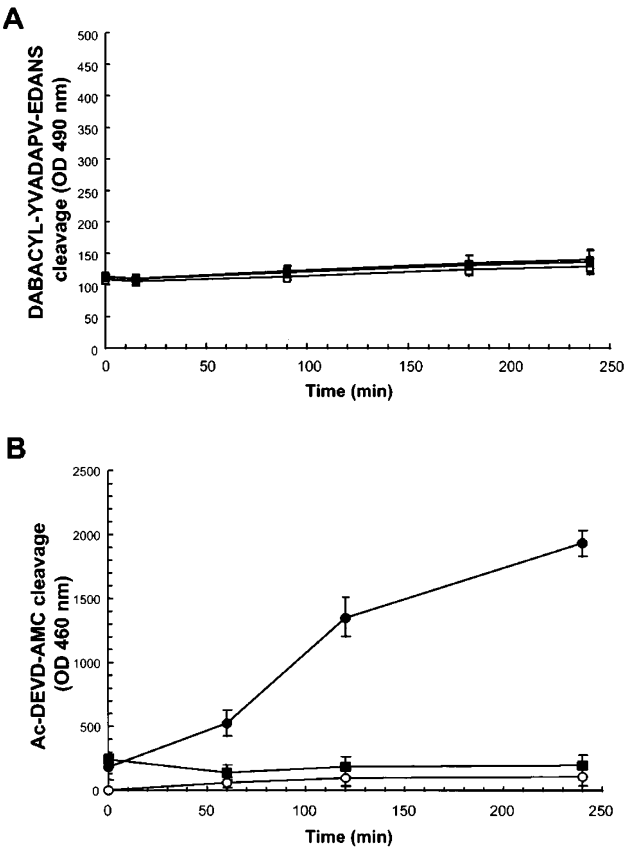

Figure 2 Impact of PDT on caspase-1 and caspase-3 activation. (**A**) Untreated (○), BPD-MA (■) and BPD-MA+light (PDT) (●)-treated cell lysates were assayed for protease activity towards (**A**) YVADAPV (caspase-1) or (**B**) DEVD (caspase-3) cleavage using fluorogenically-labelled peptides

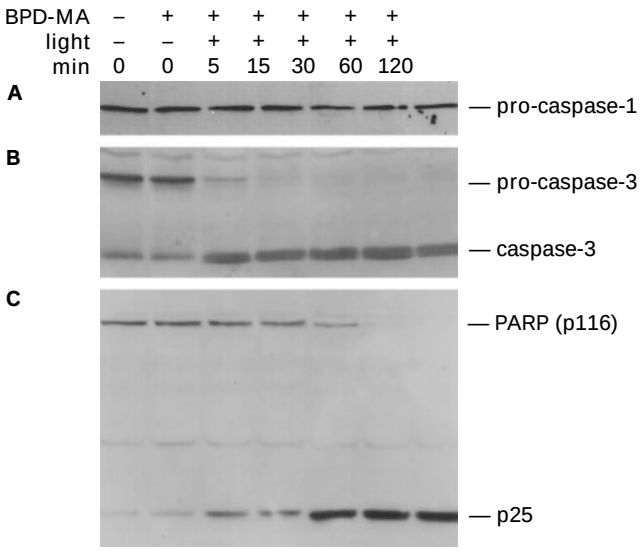

Figure 3 Proteolytic cleavage of caspase-3 and PARP but not caspase-1 precedes endonucleocytic DNA degradation in PDT-treated HL-60 cells. HL-60 cells were lysed at the indicated times following light treatment. Cell lysates were subjected to SDS–PAGE, transferred to nitrocellulose and probed with polyclonal (**A**) anti-caspase-1, (**B**) anti-caspase-3 or (**C**) anti-PARP antibodies

amount of the p12 subunit that was detected in the untreated cells, likely due to some background levels of apoptosis in the culture, however, very little PARP cleavage was detected. The antibody did not detect the 17 kD fragment of caspase-3 since it is specific for the amino terminus of the p12 subunit of the 32 kD pro-caspase-3 protein. The peptide inhibitor Z-Asp-DCB did not block the cleavage of caspase-3 at the inhibitor concentrations employed (Figure 4A). However, the peptide did block the cleavage of PARP (Figure 4B). Cleavage of both caspase-3 and PARP were sensitive to both TLCK and DCI treatment (Figures 5 and 6).

DNA-PK_{CS} cleavage was evident in PDT-treated cells and the breakdown of DNA-PK_{CS} was blocked by Z-Asp-DCB, indicating the action of a caspase-like protease (Figure 7).

Discussion

The involvement of caspases in other systems has been well documented (Martin and Green, 1995; Muzio *et al*, 1996; Alnemri *et al*, 1996). In view of the importance of caspases in these systems, it seemed plausible that these enzymes may also be involved in PDT-induced cell death. Addition of the caspase family inhibitor Z-Asp-DCB to HL-60 cells prior to light activation prevented DNA fragmentation induced by PDT, indicating that caspases are likely involved in the PDT cell death pathway. The serine protease inhibitors DCI and TLCK also blocked DNA fragmentation, while Aprotinin and PMSF had no effect.

The involvement of caspase-1 in PDT-induced apoptosis of HL-60 cells was ruled out since there was no cleavage of the DABACYL-YVADAPV-EDANS substrate in PDT-treated cell lysates. Furthermore, when examined via Western blot

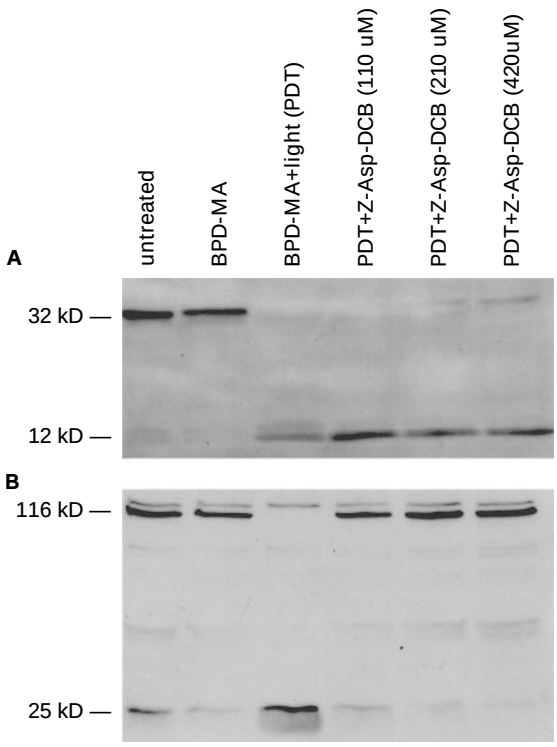

Figure 4 Proteolytic cleavage of PARP but not caspase-3 is inhibited by Z-Asp-DCB. HL-60 cells were lysed 60 min after light exposure. Cell lysates were subjected to SDS–PAGE, transferred to nitrocellulose and probed with either polyclonal (**A**) anti-caspase-3 or (**B**) anti-PARP antibodies

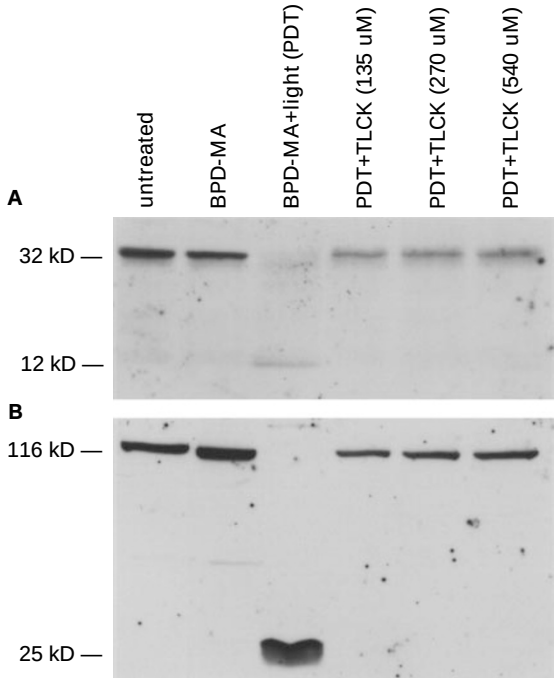

Figure 5 Proteolytic cleavage of caspase-3 and PARP is inhibited by TLCK. PDT-treated HL-60 cells were lysed 60 min after light exposure. Cell lysates were separated by SDS–PAGE, transferred to nitrocellulose and probed with either polyclonal (**A**) anti-caspase-3 or (**B**) anti-PARP antibodies

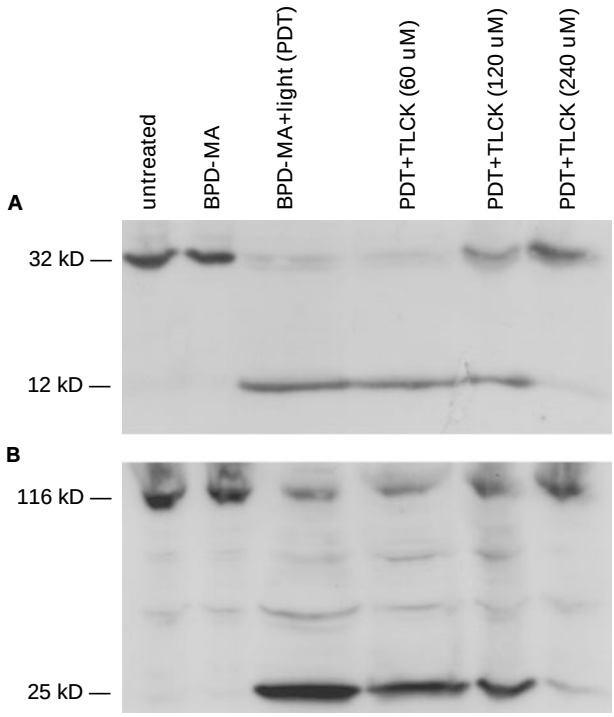

Figure 6 Proteolytic cleavage of caspase-3 and PARP is inhibited by DCI. PDT-treated HL-60 cells were lysed 60 min after light exposure. Cell lysates were separated by SDS–PAGE, transferred to nitrocellulose and probed with either polyclonal (**A**) anti-caspase-3 or (**B**) anti-PARP antibodies

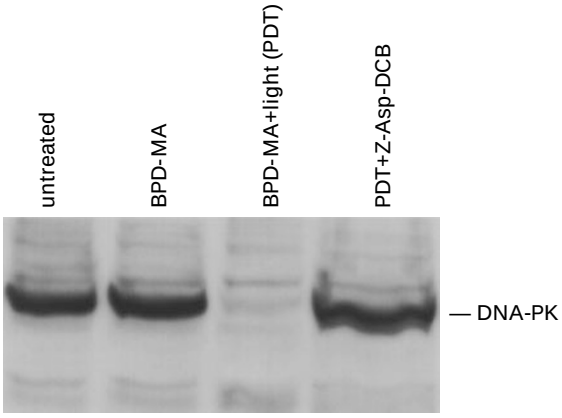

Figure 7 Proteolytic cleavage of DNA-PK_{CS} is inhibited by Z-Asp-DCB, DCI and TLCK. PDT-treated HL-60 cells were lysed 60 min after light exposure. Cell lysates were subjected to SDS–PAGE, transferred to nitrocellulose and probed with polyclonal anti-DNA-PK_{CS} antibody

analysis, the p45 (pro-caspase-1) proenzyme was not cleaved into its active subunits following PDT. However, cleavage of the fluorogenic Ac-DEVD-AMC (caspase-3) substrate was evident in the cells treated with BPD-MA and light suggesting that the treatment may induce capase-3 activation.

During UV and Fas-mediated apoptosis, Caspase-3 is proteolytically cleaved from its precursor 32 kD form to an active enzyme composed of 17 kD and 12 kD subunits (Casciola-Rosen *et al*, 1996; Schlegel *et al*, 1996). Since the protease assay suggested the involvement of caspases, in particular DEVD-ases, it seemed likely that caspase-3 may be activated during PDT-induced cell death. When lysates from PDT-treated cells were analyzed by Western blot analysis, the cleavage of the caspase-3 precursor into its 17 kD and 12 kD active subunits was evident. Moreover, Z-Asp-DCB did not block the processing of caspase-3 but did disable PARP cleavage, suggesting that Z-Asp-DCB may inhibit DNA fragmentation by blocking the proteolytic action of caspase-3. Since Z-Asp-DCB is a general caspase inhibitor, we cannot rule out the possibility that other proteases are involved. It has also been shown that other caspases are capable of cleaving PARP (Fernandes-Alnemri *et al*, 1995). The serine protease inhibitors, DCI and TLCK, also blocked the cleavage of caspase-3, possibly preventing PARP cleavage. These results suggest the existence of a serine protease upstream of caspase-3 activation or an upstream caspase that is sensitive to DCI or TLCK treatment.

PARP serves in the surveillance and enzymatic repair of DNA damage caused by environmental stress (Kaufmann *et al*, 1993; Lazebnik *et al*, 1994). Furthermore, the Ca^{++}/Mg^{++}-dependent endonucleases, involved in the internucleosomal cleavage of DNA within apoptotic cells, are negatively regulated by poly(ADP-ribos)ylation (Nicholson *et al*, 1995). It has been postulated that loss of normal PARP function may unleash endonuclease activity within dying cells (Kaufmann *et al*, 1993; Nicholson *et al*, 1995). It has been demonstrated that caspase-3 may be responsible for the cleavage of PARP, although, caspase-7 (Mch-3) has also been shown to cleave PARP (Nicholson *et al*, 1995; Fernandes-Alnemri *et al*, 1995). PARP was cleaved within 60 min of light treatment in PDT-treated HL-60 cells. PARP cleavage was blocked directly by Z-Asp-DCB, DCI and TLCK. It has recently been shown that precursors of certain caspases can be processed by granzyme B (GraB) in apoptosis mediated by cytotoxic T lymphocytes (Shi *et al*, 1996; Quan *et al*, 1996). However, since GraB is present only in cytotoxic T lymphocytes and natural killer cells, it is unlikely that it is involved in the PDT-induced apoptosis of promyelocytic leukemia HL-60 cells. Furthermore, the GraB inhibitor peptide (Shi *et al*, 1992), Z-AAD-CMK, had no inhibitory effect on the DNA fragmentation caused by PDT. Caspase-6, 8 and 9 may also have a role upstream of caspase-3 activation. In future experiments, the state of caspase-6, 8 and 9 activation will be evaluated to determine their role and position in the PDT-induced apoptotic pathway.

The cleavage of DNA-PK_{CS} was also observed providing further evidence of proteolytic activity in PDT-treated cells. Cleavage of DNA-PK_{CS} was blocked by Z-Asp-DCB suggesting caspase-like activity. It has been suggested that DNA-PK_{CS}, which appears to be involved in regulating the cell cycle in response to DNA damage, may also be another substrate of caspase-3 (Teraoka *et al*, 1996; Han *et al*, 1996).

In conclusion, our results demonstrate that caspase-3 is activated during PDT-induced apoptosis. By inhibiting caspase activity, PDT-induced DNA fragmentation does not occur. We propose that PDT causes apoptotic cell death by evoking a cascade of proteolytic events involving

one or more members of the caspase family. Other photosensitizers may initiate a similar sequence of cellular events although the intracellular localization of these compounds may influence the nature of this response upon light activation. It will be important to identify the cell signalling events that link the formation of reactive oxygen species following PDT with the proteolytic events which lead to apoptosis. The recent demonstration of the activation of stress-activated protein kinase and p38 HOG1 kinase in murine PAM 212 keratinocytes in response to the oxidative stress produced by BPD-MA and light may indicate that these signalling molecules could be intermediataries in the cell death pathway (Tao *et al*, 1996). It is not known whether kinase activation occurs prior to caspase activation or as a consequence of the proteolytic cleavage of various substrates. Future studies will examine the relationship between protein kinase and caspase activation in PDT-treated cells. The rapid induction of proteolysis, followed by DNA fragmentation in cells treated with BPD-MA and light comprises a useful model with which to study the biochemistry of apoptosis.

Materials and Methods

Cell lines, chemicals, and antibodies

The human promyelocytic leukemia HL-60 cell line was obtained from the American Type Culture Collection (Rockville, MA) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine, 1 mM pyruvate, 1 mM HEPES, penicillin (100 U/ml) and streptomycin (100 mg/ml) purchased from Gibco BRL (Burlington, Ontario). Liposomally-formulated benzoporphyrin derivative monoacid ring A (BPD-MA) was provided by QLT PhotoTherapeutics Inc. (Vancouver, BC). All antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). TLCK, 3,4-dichloroisocoumarin and Z-Ala-Ala-Asp-CH_2Cl (Z-AAD-CMK) were obtained from Calbiochem (Cambridge, MA). The ICE inhibitor III (Z-Asp-2,6 dichlorobenzoyloxymethylketone) was provided by Bachem (Torrance, CA). All other drugs and chemicals, unless, specified, were obtained from Sigma Chemical Co. (St. Louis, MO).

Photodynamic (PDT) and inhibitor treatment of cells

HL-60 cells were incubated for a total of 60 min at 37°C with or without BPD-MA (100 ng/ml) in RPMI; 2% FBS. Specific inhibitors were added to the cells after 40 min of BPD-MA incubation for a further 20 min. Unless indicated, cells were exposed to the following inhibitor concentrations: Z-Asp-DCB (110 μM), Z-AAD-CMK (100 μM), dichloroisocoumarin (230 μM), TLCK (135 μM), phenylmethylsulfonyl fluoride (PMSF, 100 μM), aprotinin (100 μM), or appropriately-matched vehicle concentration of dH_2O, methanol or dimethyl sulfoxide (DMSO). Following incubation with BPD-MA, cells were exposed to fluorescent red light (620-700 nm) at 5.6 mW/cm^2 to give a total dose of 2 J/cm^2. Cells incubated with BPD-MA and treated with light are referred to as PDT-treated cells.

Flow cytometric detection of apoptosis

The propidium iodide (PI) fluorescence analysis procedure was used to detect changes in the status of cellular DNA (Telford *et al*, 1994; Darzynkieicz *et al*, 1992). At 3 h, following PDT, 1×10^6 cells 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 stained with PI (50 mg/ml) in PBS with simultaneous RNAse treatment (5 U/ml, DNAse-free). Samples were analyzed by flow cytometry. The percentage of hypodiploid apoptotic cells was calculated from single parameter flow cytometry for PI fluorescence, since the separation between the apoptotic population and cells within the G_0/G_1 region is considerable (Telford *et al*, 1994). Cell fluorescence was analyzed with an Epics XL flow cytometer (Coulter Electronics Inc., Hialeah, FA).

Preparation of cellular protein extracts

Cells were initially washed twice with ice-cold PBS. Cell pellets were treated within 1 ml of lysis buffer (1% Nonidet P-40 detergent, 20 mM Tris, pH 8, 137 mM NaCl, 10% glycerol) supplemented with 1 mM PMSF, aprotinin (0.15 U/ml) and 1 mM sodium orthovanadate for 20 min on ice. Lysates were then centrifuged for 10 min at 15 800 $\times$ *g* at 4°C. Protein concentrations of cell extracts were determined with the Pierce BCA Protein Assay (Pierce, Rockford, Illinois).

Protease assays

For protease assays, cells were lysed 1 h following their respective treatments. Assays were performed in 96-well plates by incubating 25 μl (10 μg) of cell lysate with 125 μl of reaction buffer (1% NP-40, 20 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol) and 100 μM of peptidic substrate. The assays included 4-(4-dimethylaminophenylazo) benzoyl-Tyr-Val-Ala-Asp-Ala-Pro-Val-5-[(2-aminoethyl) amino]-naphthalene-1-sulfonic acid (DABCYL-YVADAPV-EDANS) or Acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC) (Calbiochem, Cambride, MA). Lysates were incubated at 37°C for 4 h and the fluorescence of the cleaved substrates was determined hourly using a CytoFluor^{TM}2350 (PerSeptive Biosystems, ON, Canada) set at excitation and emission wavelengths of 340 nm and 490 nm (DABCYL-YVADAPV-EDANS) or 380 nm and 460 nm (AC-DEVD-AMC).

Western immunoblotting

Detergent soluble proteins (30 mg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS – PAGE) in 10% gels, under reducing conditions as previously described (Laemmli, 1970). Proteins were transferred to nitrocellulose membrane at 100 V for 1 h. Nitrocellulose membranes were blocked for 30 min at room temperature with 5% skim milk in PBS. Membranes were incubated for 45 min using the following antibody concentrations: polyclonal goat-anti-PARP (1 μg/ml), polyclonal goat-anti-DNA-PK_{CS} (1 μg/ml), polyclonal rabbit-anti-ICE (p20) and polyclonal goat-anti-CPP32 (1 μg/ml). Membranes were probed for primary antibodies for 30 min at room temperature with their respective secondary antibody at the following concentrations: anti-goat-IgG-HRP (1 : 2000), anti-rabbit-IgG-HRP (1 : 2000) or anti-mouse IgG-HRP (1 : 2000) for 30 min at room temperature. Biotinylated molecular weight standards were probed with streptavidin-HRP (1 : 5000) conjugate (Amersham, Canada) in PBS-0.1% Tween 20 (PBS-T) for 30 min. The membrane was rinsed twice in PBS-T, followed by three 15 min washes with PBS-T. Proteins were detected using the enhanced chemiluminescence detection system (Amersham, Canada) and bands visualized by autoradiography.

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