Photodynamic therapy: shedding light on the biochemical pathways regulating porphyrin-mediated cell death

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Summary. Photodynamic therapy (PDT) is a clinically approved treatment for the ocular condition age-related macular degeneration, and certain types of cancer. PDT is also under investigation for other ocular, as well as, immune-mediated and cardiovascular indications. PDT is a two step procedure. In the first step, the photosensitizer, usually a porphyrin derivative, is administered and taken up by cells. The second step involves activation of the photosensitizer with a specific wavelength of visible light. Exposure to light of an activating wavelength generates reactive oxygen species within cells containing photosensitizer. PDT with porphyrin photosensitizers induces rapid apoptotic cell death, an event which may be attributed to the close association of these compounds with mitochondria. Thus, PDT is an attractive method to treat ailments such as cancer, viral infections, autoimmune disorders and certain cardiovascular diseases in which the apoptotic program may be compromised. The present review examines the cellular events triggered at lethal and sublethal PDT doses and their relationship to the subsequent effects exerted upon cells.

Key words: Apoptosis, Bcl-2, Caspases, Mitochondria, Photodynamic therapy, Photosensitizer, Verteporfin, Visudyne

Introduction

The original use of photosensitizers for the treatment of disease dates back to ancient times when certain plant extracts were used to treat psoriasis and vitiligo (McCaughan, 1999; Kalka et al., 2000). Photodynamic therapy (PDT) is now an approved treatment for several types of cancer and recently gained approval for the treatment of age-related macular degeneration (AMD), the leading cause of blindness in the elderly. PHOTOFRIN® (Axcan Pharma Inc., Quebec, Canada) was the first approved photosensitizer and is currently used clinically for the treatment of different forms of cancer (lung, esophageal, cervical, bladder, gastric) in North America, Europe and Japan. More recently, a major milestone in the field of ophthalmology was achieved when PDT gained regulatory approval in multiple jurisdictions for the use of Visudyne (benzoporphyrin derivative monoacid ring A, verteporfin, QLT Inc., Vancouver, BC) for the treatment of "wet" AMD, the leading cause of blindness among people over the age of 50. In the treatment of AMD, red laser light is directed into the eye shortly after the systemic administration of Visudyne (Bressler, 1999). The abnormal blood vessels responsible for the loss of visual acuity in this condition rapidly take up Visudyne and are destroyed upon light irradiation, most likely due to a combination of PDT-induced endothelial cell apoptosis, vaso-occlusion and thrombosis. In summary, Visudyne therapy of subfoveal choroidal neovascularization (CNV) from AMD was shown to safely reduce the risk of vision loss in patients with predominantly classic CNV from AMD (Bressler, 1999). In addition, Visudyne is being assessed for its ability to alleviate CNV not related to AMD, including pathologic myopia, ocular histoplasmosis, angiod streaks, and idiopathic causes (Sickenberg et al., 2000).

PDT is a two-step procedure requiring 3 elements: photosensitizer, light and oxygen (Dougherty et al., 1998). In the first step, a photosensitising agent (usually a porphyrin derivative) is administered intravenously (Fig. 1). The photosensitizer then circulates and accumulates in most cells, including the cells of interest. In the next step, the photosensitizer is exposed to non-thermal light of a specific wavelength which 'activates' the drug (Levy, 1995; Moore et al., 1997; Bressler and Bressler, 2000). According to the application, a specific light delivery source may be required. Typical light sources include light emitting diodes (LED), fluorescent tubes, or lasers for greater specificity. The initiating step of photosensitization is the absorption of a light photon by the photosensitizer elevating the molecule from its energy ground state to a highly unstable excited singlet state. The excited singlet photosensitizer either returns
Apoptotic debris is cleared by macrophages or other inflammatory cells associated with the synovial tissue of arthritic rabbits treated with PDT (Ratkay et al., 1998). In PDT-treated human sarcoma xenografts transplanted into nude mice, the mechanism of tumor destruction in this model appears to be vascular damage with initial apoptosis in tumor endothelial cells and delayed tumor cell apoptosis (Engbrecht et al., 1999).

In addition to ocular disorders and cancer, PDT is under investigation for the treatment of atherosclerosis, restenosis, allograft rejection, and immune disorders (LaMuraglia et al., 1994, 1995; Sluiter et al., 1996; Obochi et al., 1997; Hunt and Chan, 1999b; Sessler and Miller, 2000). For the treatment of immune disorders such as psoriasis, the systemic delivery of photosensitizer is followed by broad exposure of a large surface area of the patient to activating light. Low, sublethal levels of PDT can modify immune responses while limiting skin inflammation or erythema (Chowdhary et al., 1994; Leong et al., 1996; Simkin et al., 1997). Large populations of infiltrating T cells are present within the skin plaques of psoriatic patients (Bos and De Rie, 1999). Although the mechanisms by which PDT acts to alleviate psoriasis are not known, PDT may either have a direct impact on psoriatic keratinocytes, or may act indirectly by inducing apoptosis of plaque-associated T cells that release cytokines that alter keratinocyte growth and differentiation in this disease. To this extent, activated T cells are more susceptible to PDT-induced apoptosis than resting T cells (Hunt et al., 1999a). Likely, the effects of PDT on psoriatic skin include both keratinocytes and T cells.

Understanding of the biochemical mechanisms of PDT-induced apoptosis has advanced significantly over the past decade. PDT-induced apoptosis has been demonstrated using both in vitro and in vivo models. Further, PDT has the capacity to induce apoptosis in most normal and tumour cell types. The mitochondrial localization of certain second generation porphyrin derivatives permits rapid activation of the apoptotic program following photosensitization. The present review highlights recent advances in delineating the biochemical mechanisms of porphyrin-based PDT.

Administration of photosensitizer

Irradiation with non-thermal light source

Modification of cell function and/or induction of cell death

**Fig. 1.** PDT is a two-step procedure. In the first step, a photosensitizing agent is administered. This is followed by an interval in which the drug circulates within the blood and accumulates within the target cells. In the second step, the photosensitizer is exposed to non-thermal light at a specific wavelength that "activates" the drug. Photosensitizer-containing cells can undergo rapid apoptotic cell death following exposure to an activating wavelength of light.
induced apoptosis. In addition, we will discuss the cellular signalling events associated with the much less understood sublethal effects of PDT.

**Mitochondrial regulation of PDT-induced apoptosis**

In addition to acting as the main site of cellular ATP production, mitochondria may also act as gatekeepers between life and cell death (Green and Reed, 1998). Since the initial observation that Bel-2 inhibits apoptosis by preventing mitochondrial cytochrome c (cyt c) release (Kluck et al., 1997; Yang et al., 1997), the role of mitochondria in apoptosis has been the subject of intense research. Thus, the search for chemical agents that target mitochondria to induce apoptosis has gained much attention in recent years. Porphyrin-derived photosensitizers may localise to mitochondria (Kessel et al., 1997; McEnery et al., 1999). In addition, a number of groups have now shown that cyt c is released into the cytosol immediately following photosensitisation using different photosensitisers (Granville et al., 1998b, 1999b; Vantieghem et al., 1998; Carthy et al., 1999; Inanami et al., 1999; Kessel and Luo, 1999; Varnes et al., 1999). Further evidence for a direct effect of certain photosensitisers, such as verteporfin, on mitochondria stems from the observation that unmasking of the mitochondrial antigen is not understood. A detailed description of intracellular apoptotic events associated with PDT are illustrated in Fig. 2.

Porphyrin-derived photosensitizers can bind mitochondrial peripheral-type benzodiazepine receptors (PBR) (Snyder et al., 1987; Verma and Snyder, 1988; Verma et al., 1998). PBR are located at the junction of the inner and outer mitochondrial membranes (Anholt et al., 1986; McEnery et al., 1992), a pivotal site for the integration of apoptosis-related signals (Kroemer et al., 1997). Interestingly, PBR expression levels correlated with cell sensitivity to photodynamic killing with porphyrins (Verma et al., 1998). Thus, the localization of porphyrin-derived photosensitizers to mitochondria indicates how PDT with porphyrin photosensitizers may be capable of inducing rapid cyt c release and initiation of the apoptotic cascade. Identification of other mitochondrial structures that bind porphyrin photosensitizers will be a future area of keen interest.

**Role of caspases in PDT-induced apoptosis**

Mitochondrial release of cyt c triggers a cascade of activities resulting in the activation of a family of proteases known as caspases (cysteinyl aspartate specific proteases). These enzymes define a group of cysteine proteases comprising a multi-gene family of which more than a dozen different mammalian members have been identified (Nicholson, 1999). Caspases reside within cells as inactive zymogens. The apoptotic program is amplified through a cascade of caspase activity. Caspases are believed to account for the majority of cellular and morphological events that occur during apoptosis by their cleavage of specific cellular proteins.
with structural, signalling, reductive or enzymatic function (Nicholson, 1999). Caspases recognize a specific tetrapeptide sequence within their target substrates. These amino acid motifs form the basis for inhibitor and synthetic substrate design (Nicholson, 1999). Inhibition of caspase activity prevents many of the biochemical and morphological events that occur during apoptosis (Nicholson, 1999; Zheng et al., 1999). To date, approximately 70 different caspase substrates have been identified (Nicholson, 1999). One of the hallmarks of apoptotic death is genomic disassembly and DNA fragmentation. Caspases disrupt normal DNA repair processes by proteolytically cleaving and inactivating at least two key proteins involved in the maintenance of genomic integrity, poly (ADP-ribose) polymerase (PARP) and DNA dependent protein kinase (DNA-PK). Simultaneously, caspases provoke the onset of DNA fragmentation by indirectly activating an apoptosis-specific endonuclease (caspase activated DNA-PK and DFF) (Nicholson, 1999). In PDT-induced apoptosis, caspase-dependent cleavage of PARP, DNA-PK and DFF have been observed (Granville et al., 1997, 1998c).

Although multiple caspases are active during apoptosis and possibly redundant in different cell types, lessons obtained from caspase knockout mice indicate that certain caspases are essential for the normal functioning and development of specific tissues (Zheng et al., 1999). For instance, caspases -3 and -6 are critical for proper neuronal development, while caspase-8 is necessary in cardiac development and erythropoiesis (Zheng et al., 1999). Caspase-2, on the other hand, has two isoforms, caspase-2L and caspase-2S, which promote or inhibit apoptosis, respectively (Zheng et al., 1999). Thus, deletion of this gene had differential positive and negative effects in a tissue-specific pattern (Zheng et al., 1999). For a more detailed description of the different caspase knockout mice, Zheng et al. (1999) recently published an extensive review outlining the phenotypes of different caspase knockout mice.

Following the release of mitochondrial cyt c into the cytosol, activation of caspases-2, -3, -6, -7, -8, and -9 has been described for several cell types treated with PDT (Granville et al., 1998b, 1999b, 2000; Carthy et al., 1999). During receptor-mediated apoptosis, caspase-8 is mobilized prior to cyt c release. However, in PDT-induced apoptosis caspase-8 is activated after cyt c release in both normal and transformed cell types during PDT-induced apoptosis (Granville et al., 1998b, 1999b), an event likely triggered by caspase-3 (Granville et al., 1998b). The later activation of caspase-8 during PDT-induced apoptosis may amplify cyt c release through caspase-8 mediated cleavage of Bid into a truncated form (tBid) that can cause cyt c release (Granville et al., 1998b, 1999b; Slee et al., 1999, 2000). In addition, recent studies suggested that caspase-3 catalyzes Bid cleavage and triggers an amplification loop by which tBid induces further cyt c release (Slee et al., 2000).

**Role of Bcl-2 related proteins in the regulation of PDT-induced cell death**

The Bcl-2 proto-oncogene was initially discovered as a highly expressed protein in human B-cell lymphomas arising from a t(14;18) chromosomal translocation (Pegoraro et al., 1984; Tsujimoto et al., 1985). Bcl-2 was subsequently found to promote the survival of many cell types exposed to a diverse range of pro-apoptotic stimuli including chemotherapeutics, viruses, hypoxia and ionising radiation (Reed, 1997, 1998a,b). Since then, a number of pro-apoptotic (Bax, Bid, Bak, Bik, Bad, Bcl-XS, Bik, Bok) as well as anti-apoptotic (Bcl-2, Bcl-XL, A1, Bcl-w, Mcl-1) Bcl-2 family members have been identified. Bcl-2 co-localizes to the outer membranes of mitochondria, endoplasmic reticuli (ER) and nuclei. Since the discovery that Bcl-2 inhibits mitochondrial cyt c release (Kruck et al., 1997; Yang et al., 1997), the relationship between Bcl-2 and mitochondria has been an area of great interest.

Conflicting reports have been forwarded concerning the role of Bcl-2 and related proteins in PDT-induced apoptosis. Bcl-2 expression in Chinese hamster ovary cells inhibited apoptosis and partially protected against a loss of clonogenicity in P44-based PDT (He et al., 1996), while in breast epithelial cells Bcl-2 promotes PDT-induced apoptosis using the photosensitizer Pca1 (Kim et al., 1999). The increased susceptibility of Bcl-2 over-expressing cells to PDT was believed to be due to a photodynamic destruction of Bcl-2 that allowed Bax to exert its pro-apoptotic activity (Kim et al., 1999). Conversely, Bcl-2 or Bcl-XL over-expression in HL-60 cells suppressed verteporfin-based PDT-induced caspase activation and DNA fragmentation (Granville et al., 1998c, 1999a). Bcl-XL overexpression inhibited DNA fragmentation indirectly by suppressing caspase-3 activity and subsequent DFF cleavage after PDT (Granville et al., 1999c). However, Bcl-2/XL does not appear to prevent PDT-mediated mitochondrial alterations. In fact, over-expression of Bcl-2 or Bcl-XL in HeLa cells did not prevent the release of mitochondrial cyt c, the unmasking of the mitochondrial 7A6 antigen or cell death induced by PDT with verteporfin (Carthey et al., 1999). Furthermore, studies of hypericin-based PDT indicated that human glioma cell viability was unrelated to expression levels of Bcl-2 or Bax (Weller et al., 1997). In summary, the role of Bcl-2 and related anti-apoptotic proteins in the regulation of PDT-induced apoptosis is unclear but these proteins may act in a cell type- and photosensitizer-specific manner.

The status of pro-apoptotic Bcl-2 family members has been assessed for normal and transformed cell types treated with PDT. Treatment of human umbilical venous endothelial cells (HUVEC) with verteporfin-based PDT induced cleavage of the Bcl-2 homologue Bid into its pro-apoptotic 1Bid form (Granville et al., 1999b). However, in contrast to receptor-mediated forms of
apoptosis, Bid cleavage occurred downstream of mitochondrial cyt c release (Granville et al., 1999b). Since Bid is a caspase-8 and caspase-3 substrate (Li et al., 1998; Luo et al., 1998; Slee et al., 2000) and both are activated downstream of cyt c release following PDT (Granville et al., 1999b), it is possible that caspase-mediated Bid cleavage may amplify cyt c release in certain PDT-treated cell types.

Cellular redistribution of Bax from the cytosol to mitochondria may also enhance cyt c release after PDT in normal, but not transformed cell types. In primary HUVEC, cytosolic Bax levels gradually decreased following PDT (Granville et al., 1999b) but were unchanged in HeLa cells undergoing PDT-induced apoptosis (Carthy et al., 1999). The explanation for this difference is unclear. Further, cytosolic cyt c levels increase over 2 h in PDT-treated HUVEC, but not in PDT-treated HeLa cells (Carthy et al., 1999; Granville et al., 1999b). Many tumour cell types are known to contain abnormal expression or mutations in one or more Bcl-2-related genes (Reed et al., 1996; Reed, 1998a,b). Therefore, it is possible that pro-apoptotic Bax-mediated signalling pathways may have been impaired in the transformed HeLa cell line that was used for these experiments. Thus, it is possible that in PDT-treated non-transformed cells that immediate cyt c release is attributed to direct mitochondrial effects while further cyt c release is a result of Bax and/or Bid-mediated activity.

Effects of PDT on intracellular calcium regulation

The impact of PDT on ER integrity and intracellular calcium regulation are poorly understood. Evidence suggesting a role for ER in PDT-induced apoptosis stems from studies demonstrating caspase-8-mediated cleavage of the integral ER Bap31 protein in HeLa cells treated with verteporfin and light (Granville et al., 1998b). Furthermore, certain porphyrin photosensitizer (haematoporphyrin, HP and protoporphin IX, PPIX) associate with ER to a greater degree than mitochondria in rat liver cells (Ricchelli et al., 1999). With these photosensitizers, altered intracellular Ca\textsuperscript{2+} levels were mediated as a consequence of PDT-mediated ER damage as opposed to mitochondrial damage (Ricchelli et al., 1999). In support of a role of Ca\textsuperscript{2+} in PDT-induced apoptosis, for phophoramide-treated Chinese hamster V79 cells, the intracellular calcium chelator BAPTA-AM inhibited cyt c release, caspase-3 activation and apoptosis following light activation (Inanami et al., 1999). Thus, in this system, the PDT-induced increase in intracellular Ca\textsuperscript{2+} levels may instigate mitochondrial cyt c release. It has not been determined whether BAPTA-AM would prevent cyt c release induced by PDT using other photosensitizers.

PDT, cell signalling and apoptosis

The role of protein kinases in the regulation of cell death and survival is unclear. However, their involvement in the regulation of cell death and survival has been described in recent years. Although protein phosphorylation and kinase activation have been described for PDT-treated cells (Tao et al., 1996; Xue et al., 1997, 1999a,b; Granville et al., 1998d; Klotsz et al., 1998; Assafa et al., 1999), their influence on cell death and/or cell survival signalling pathways is unresolved. The non-receptor tyrosine kinase Etk provided a degree of protection against PDT-induced apoptosis with Pc4 in prostate cancer cells (Xue et al., 1999b). Increased activity of stress-activated protein kinase (SAPK)/c-jun NH2-terminal kinase (JNK) and p38 high osmolality glycerol protein kinase 1 (p38), in mouse (Tao et al., 1996) and human keratinocytes (Klotz et al., 1998) has been demonstrated. The significance of these events in relationship to cell death or cell survival in PDT-treated cells is yet unclear. However, a recent study by Davis and colleagues suggests a requirement for SAPK/JNK in triggering cyt c release in UV-induced apoptosis in murine embryonic fibroblasts (Tournier et al., 2000). A role for SAPK/JNK in Fas-induced apoptosis has also been described (Costa-Pereira et al., 2000). Hypericin-based PDT activated SAPK/JNK while irreversibly inhibiting extracellulary-regulated kinase-1 (ERK1) activity in tumour cells (Assafa et al., 1999). This latter report also indicated that PDT-induced activation of SAPK/JNK was caspase independent (Assafa et al., 1999). Inhibition of p38 kinase activity impaired Pc4 mediated PDT-induced apoptosis in LY-R cells but did not affect PDT-induced apoptosis for Chinese hamster ovary cells (Xue et al., 1999a,b). By contrast, inhibition of p38 promoted apoptosis for HeLa cells treated with hypericin-based PDT (Assafa et al., 1999). PDT-induced apoptosis was enhanced by expression of the dual specificity MKP-1 phosphatase (Assafa et al., 1999). The apparent differential activities of MAP kinases during PDT-induced apoptosis may be attributable to the cell type, the photosensitizer used and/or the intensity of light applied. Clearly, the role of kinases in PDT-induced apoptosis requires further resolution.

Transcriptional regulators

Nuclear factor-kappaB (NF-kB) DNA binding activity occurs in response to various stimuli including cytokines, viral infection, phorbol esters, oxidants or radiation (Karim, 1998). NF-kB is not a single protein, but is comprised of dimers of Rel family DNA binding proteins that bind closely related NF-kB recognition motifs (Karim, 1998). NF-kB resides in the cytoplasm in an inactive state in association with inhibitory kB (I kB) proteins (Karim, 1998). Upon activation, via degradation of I kB, NF-kB translocates to the nucleus where it activates the transcription of a variety of genes involved in immune and inflammatory responses (Karim, 1998). PhotoFRIN® based PDT induced NF-kB nuclear translocation in murine L1210 lymphoma cells (Ryter and Gomer, 1993) as well as increased c-fos and c-jun
gene expression (Kick et al., 1996). The c-fos heterodimerises with c-jun to form the AP-1 transcription factor. In HeLa cells treated with Photofrin®-based PDT, activation of AP-1 but not NF-κB was observed (Kick et al., 1995). With verteporfin, as well as pyropheophorbide-a methyl ester (PPME) based PDT, decreased IkBα levels and NF-κB activation were observed (Matroule et al., 1999; Granville et al., 2000). Furthermore, increased sensitivity to apoptosis was described for PDT (PPME)-treated HCT-116 carcinoma cells in which the NF-κB pathway was impaired due to an overexpression of an IkB super repressor protein suggesting that NF-κB influences cell sensitivity to PDT (Matroule et al., 1999a,b).

In addition to its role in immune responses, NF-κB stimulates the transcription of pro-survival genes such as A1, A20, Bcl-X1, TNFR-associated factors 1 and 2 (TRAF1 and 2) and inhibitor of apoptosis proteins (IAPs) (Wang et al., 1998; Lee et al., 1999; Zong et al., 1999). Thus, it is possible that low-level PDT may activate the NF-κB pathway and subsequent transcription of anti-apoptotic genes. Conversely, NF-κB could activate genes such as FasL, thereby stimulating a ‘counterattack’ mechanism against cells expressing Fas (Kasibhatla et al., 1999). Such an effect on keratinocytes may be beneficial for the treatment of psoriasis with PDT in which high levels of pathogenic, infiltrating, Fas-positive T cells are present in the skin.

The tumour suppressor gene p53 is involved in the regulation of cell division and cell death. Altered p53 function or expression is associated with many forms of cancer and this may impact cell resistance to chemotherapy and γ-irradiation. HL-60 p53 deficient cells were less sensitive to Photofrin®-based PDT than those transfected with wild type p53 (Fisher et al., 1997). This phenomenon may be explained by the observation that p53 gene expression can, in certain cell types, upregulate Bax expression while downregulating Bcl-2 expression (Miyashita et al., 1994; Miyashita and Reed, 1995; McCurrach et al., 1997; Budhram-Mahadeo et al., 1999) which may account for the increased sensitivity of these cells to PDT. In further support of this concept, human colon cancer cell lines with a mutated p53 gene exhibited a greater sensitivity to PDT following transfection with wild type p53 (Zhang et al., 1999). Conversely, when p53 was specifically targeted for ubiquitin degradation in cells transfected with the E6 viral oncoprotein, no change in tumour cell sensitivity to PDT with Photofrin® was evident (Fisher et al., 1999). For a number of human glioma cell lines, susceptibility to PDT with hypericin was unrelated to p53 expression level (Weller et al., 1997). Although in certain cases p53 transfection may heighten cell sensitivity to PDT, the effect is most likely due to the restoration of p53-regulated apoptosis pathways since p53 null cells are still susceptible to PDT-induced apoptosis.

Conclusion

The emergence of PDT, a treatment that primarily affects cells containing drug exposed to activating light, provides several advantages over standard systemic cytotoxic agents since its activity can be controlled by the degree and targeting of light. In addition, porphyrin-derived photosensitizers have the added benefit in that they may directly affect mitochondria to induce apoptosis. This property suggests that this form of treatment may circumvent anti-apoptotic mechanisms that are present in certain types of cancer, viral infection, or diseases in which immune-mediated apoptotic elimination of damaged, injured, or unwanted cells is hindered. Over the past few years, great advances have been made in the understanding of the biochemical pathways that are activated during PDT-induced apoptosis. However, further studies are necessary to delineate and understand the significance of signalling pathways that are activated in different cell types in response to sub-lethal levels of PDT. Additionally, it will be important to determine the characteristics that render certain cell types, such as activated T cells, more susceptible to PDT-induced apoptosis. With these recent advances in our understanding of PDT mechanisms, as well as the increasing approvals for the use of PDT to treat various types of disease, the advantages of this form of treatment are finally being recognised. This bodes well for further advances in the years ahead.

References


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Accepted October 9, 2000