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Death pathways associated with photodynamic therapy

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Abstract

When the mitochondria and/or the endoplasmic reticulum were targeted by photodynamic therapy, photodamage to the anti-apoptotic protein Bcl-2 was observed. This led to an apoptotic outcome if that death pathway was available. Lysosomal photodamage ultimately resulted in activation of the pro-apoptotic protein Bid, also leading to apoptosis. Photodamage to the plasma membrane was associated with migration of sensitizers to the cytosol and procaspase photodamage, with apoptosis impaired. Where apoptosis was unavailable because of lack of necessary components of the program, an autophagic outcome has been observed. It is also clear that autophagy can occur along with apoptosis as a PDT response, and may play a role in immunologic responses to photodamaged tumor cells.

Keywords

Photodynamic; Localization; Apoptosis; Autophagy

Introduction

During the course of our studies into the mechanisms involved in loss of cell viability after photodamage, we examined a variety of pathways that lead to cell death in several cell lines. This report summarizes these studies, and fills in a few details not hitherto reported.

Photodynamic therapy (PDT) has a long history, dating in some reports to the prehistoric use of light for bleaching of fabrics. The modern era (ca. 1900) is generally considered to have begun with the studies by Raab on photosensitization of microorganisms, expanding into the clinical area with work by Schwartz and others in the 1960s, and reaching clinical significance when T.J. Dougherty at the Roswell Park Cancer Center began a series of studies in the early 1970s. This early history is summarized in Ref. [1].

Studies on death mechanisms were initially complicated by the relatively complex nature of the product hematoporphyrin derivative (HPD) and its successor, Photofrin. These both showed clinical efficacy and it was clear that both direct tumor kill and vascular shutdown were critical elements of the process. Determinants of long-term efficacy and of death pathways were far from clear [2]. The design of newer 'second generation' sensitizers with known sites of action (Fig. 1) has greatly expedited the discovery of phototoxic mechanisms.

Although many investigators have been involved in examining the route whereby direct tumor cell kill results from PDT, a substantial number of pertinent publications have come from Oleinick's laboratory at Case Western Reserve University. It would not be possible to prepare a summary of death pathway research without citing these references. Work of other groups

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is mainly cited in papers included in the references to this report. Otherwise, the bibliography would be longer than the text.

Apoptotic responses to PDT: the role of Bcl-2

Apoptosis is a process whereby cells undergo an orderly death by a process programmed into the genes. The program can be initiated by a variety of triggering mechanisms including both extracellular and intracellular signals. There are now many simple procedures for characterizing apoptosis that are readily available in any reasonably equipped laboratory. Apoptosis is expressed by the activation of proteases (caspases) that ultimately cleave DNA into fragments that are contained in vesicles with markers on their surfaces that prompt for engulfment by macrophages and related cell types. This process avoids the inflammatory effects of necrosis, a situation where cells burst open releasing DNA, lysosomal proteases and a variety of other products. Since the machinery for apoptosis is contained in most cell types, all that is required for initiating the process is a suitable signal.

In the context of PDT, Oleinick's group provided the first firm evidence that apoptosis could be initiated by photodamage [3]. In 1999, we reported that the anti-apoptotic protein Bcl-2 was a target for PDT, using aluminum phthalocyanine as the photosensitizing agent [4]. Bcl-2 overexpression led to stabilization of the pro-apoptotic protein Bax. After Bcl-2 photodamage, excess Bax was then available to initiate an interaction with the mitochondrial membrane, resulting in the release of cytochrome c: a trigger for apoptosis.

In the ensuing years, Oleinick's group demonstrated that only membrane-bound Bcl-2 was a target for photodamage, using the phthalocyanine sensitizer Pc 4 [5]. This agent appears to target both the endoplasmic reticulum (ER) and mitochondria. In the latter organelle, Pc 4 was also found to bind in the close vicinity of the lipid cardiolipin, a product unique to mitochondria; this may provide a basis for the resulting mitochondrial photodamage [6]. We discovered [7] that three other photosensitizing agents also targeted Bcl-2: the tin etiopurpurin termed SnET2, the porphycene CPO [9-capronyloxy-tetrakis(methoxyethyl) porphycene], and mTHPC (meta-tetrahydroxyphenyl) chlorin). This report also demonstrated that Bcl-2 photodamage did not result in loss of the mitochondrial membrane potential unless the temperature was raised above 15 °C. This result is consistent with a report by Pryde [8] showing that Bax did not create permeability channels in mitochondria at temperatures < 15 °C.

The bile acid ursodeoxycholic acid (UDCA) had previously been shown to offer protection from apoptotic stimuli [9]. In the context of PDT however, UDCA markedly promoted the apoptotic response to Bcl-2 photodamage [10]. This occurred in both L1210 mouse leukemia and 1c1c7 mouse hepatoma cells, using either SnET2 or CPO as the photosensitizing agent. The mechanism derived from a UDCA-induced conformational change in the Bcl-2 protein promoting affinity for the photosensitizer and thereby increasing Bcl-2 photodamage [11]. UDCA also enhanced binding to Bcl-2 by the small-molecule non-peptidic Bcl-2 antagonist HA14-1, initially described by Huang's group [12]. During the course of these studies, we examined the ability of PDT to enhance the level of reactive oxygen species (ROS) in cells leading to enhanced oxidation of the fluorescent probe dichlorofluorescein (DCFDA). Initiation of apoptosis by HA14-1 had the same effect [3], most likely as a result of ROS formation during the perturbation of mitochondrial processes during apoptosis.

A persisting question relating to PDT targets was the apparent resistance of the Bcl-2 analog Bcl-x_L to photodamage in L1210 murine leukemia cells in suspension culture [11]. In contrast, Oleinick's group had found that Bcl-x_L was as sensitive as Bcl-2 to photodamage in adhering cells [14]. This difference was eventually traced to a unique property of many suspension cell cultures, i.e., localization of Bcl-x_L in the cytosol [15]. Fractionation studies revealed that this

was also true for the mouse leukemia L1210 cell line, but not for adhering MCF-10A cells (Fig. 2), where Bcl-x_L was associated with non-cytosolic loci.

A potentially important issue involved the role of Ca²⁺ translocation in the apoptotic response to photodamage. The ER is a known repository of calcium ions [16], and it seemed possible that ER photodamage might release sufficient Ca²⁺ to result in interactions with the mitochondrial matrix that would lead to apoptosis. A study of the effect of ER photodamage on calcium fluxes [17] revealed several unexpected results.

(A) The reagent ruthenium red (RR), generally employed as an antagonist of Ca²⁺ uptake by mitochondria, could initiate release of calcium ions from the ER at a 30 μM concentration (in the dark). In this regard, RR was as effective as thapsigargin, a drug often used to evoke Ca²⁺ translocation [18]. (B) RR, perhaps because of the multiple oxidation states of ruthenium, was a potent ROS scavenger, and could protect cells from adverse effects of PDT. (C) The ruthenium complex Ru360, known to be a potent antagonist of Ca²⁺ uptake by mitochondria, did not protect cells from the pro-apoptotic effects of ER photodamage, nor did the cytosolic calcium chelator BAPTA. (D) Analysis of mitochondrial Ca²⁺ levels revealed that ER photodamage did not result in a significant Ca²⁺ influx. We concluded that release of Ca²⁺ from the ER after lethal photodamage was insufficient to cause a significant translocation to mitochondria and therefore plays no role in the apoptotic response to PDT targeted to the ER. Although Ru360 is considered to be the 'active' component of RR [19], in the study outlined above, we used a preparation of RR that contained no Ru360.

Effects of membrane photodamage

The plasma membrane [20] was identified as the site of localization of a monocationic porphyrin (MCP). A prior study had involved another drug: tin octaethyl-purpurin amadine (SnOPA), a cationic agent that localized in a variety of sites including the plasma membrane [21]. Treatment with an LD₉₀ PDT dose of SnOPA resulted in a very slow phase of DNA degradation. Unlike the very rapid activation of caspases and DNA fragmentation noted after photodamage by SnET2, with DNA 'ladders' observed within 60 min, SnOPA evoked prolonged DNA fragmentation with genomic DNA only reaching the 50 kbp degradation state after 60 min. DNA 'ladders' did not appear before 24 h after irradiation. We usually see this phase of DNA degradation within 60 min.

The explanation for these effects was provided by a 2002 study where we demonstrated the monocationic sensitizers were initially localized in the plasma membrane, but during subsequent irradiation migrate to the cytosol. Continued irradiation then resulted in photodamage to procaspases -3 and -9 [22], thereby preventing an apoptotic response. Addition of MCP to a CPO-PDT protocol also abolished the apoptotic PDT response observed with CPO alone [22]. These results may not necessarily be applicable to any photosensitizer that initially binds to the plasma membrane, but indicate that the absence of an apoptotic response can result from photodamage to critical elements of the apoptotic program.

Apoptosis after lysosomal photodamage

A different localization pattern was observed using two dicationic porphyrins bearing positively charged -N(CH₃)₃ groups on adjacent or opposite phenyl groups attached to the bridging carbons of a porphyrin structure [23]. Unlike MCP, these drugs localized to mitochondria and lysosomes, respectively. On the basis of photons absorbed/cells killed, the mitochondrial sensitizer was 5-fold more efficacious. Lysosomal photodamage did, however, lead to cell death. Studies on death pathways after lysosomal photodamage were carried out using the Nippon Petrochemicals product NPe6 (an analog of chlorin e6 containing 3 -COOH groups). This agent is now termed LS11 by the current supplier, Light Sciences Inc.

Lysosomal photodamage resulted in apoptotic cell death, but via different route. This involved release of lysosomal proteolytic enzymes into the cytosol, leading to caspase-3 activation and DNA fragmentation [24]. A more detailed study revealed further details of this process [25]. Release of lysosomal enzymes after PDT resulted in cleavage of the pro-apoptotic protein Bid to a truncated form termed tBid. The latter product can interact with mitochondria, resulting in release of cytochrome c, followed by a triggering of apoptosis via activation of caspases-9 and -3. Confirmatory evidence was provided by a test involving the drug BI-6C9, a specific inhibitor of the interaction between tBid and mitochondria [26]. Addition of BI-6C9 abolished the apoptotic response to lysosomal photodamage by NPe6 (Fig. 3).

Autophagic responses to photodamage

We often observed that the number of cells with an apoptotic morphology appeared to be substantially less than the corresponding loss of viability after photodamage to mitochondria and/or the ER. Our assumption was that more cells would ultimately become apoptotic with time, and that an LD₉₀ PDT dose would eventually lead to a 90% apoptotic cell population, but this was never clearly established. Another unanswered question was the ability of PDT directed toward ER and/or mitochondria to kill a cell population even when apoptosis was inhibited by caspase inhibitors [7] or where the cell line lacked caspase-3 or Bax [27,28], important elements of the program.

A potential answer to these questions was provided when we observed that autophagy accompanied apoptosis after ER photodamage to L1210 cells and to the bax-deficient DU145 prostatic tumor line [29]. Autophagy is a process whereby a portion of the cytosol, usually containing cellular organelles, is sequestered by a double membrane. The resulting vesicle then fuses with a lysosome, the contents are then digested and can be recycled during periods of starvation [30]. There is also evidence that autophagy can be a cell-death mode under appropriate circumstances [31]. An equilibrium between apoptosis and autophagy has been reported, with inhibition of one process leading to an enhanced expression of the other [32].

Treatment of L1210 cells with the ER-sensitizer CPO resulted in both an apoptotic response and formation of double-membraned vacuoles (Fig. 4). We also observed enhanced expression of a marker for autophagy: conversion of the microtubule-associated protein LC3-I to a product termed LC3-II that migrates more rapidly during gel electrophoresis [33]. These results are consistent with the proposal that autophagy is another response to ER photodamage, perhaps serving to eradicate cells not initially eliminated by apoptosis. A plausible explanation for the ability of PDT to elicit an autophagic response may lie in the finding that down-regulation of Bcl-2 can result in stimulation of autophagy by release (and hence activation) of the pro-autophagic protein Beclin from a Beclin:Bcl-2 complex [34]. Buytaert et al. have also reported an autophagic response after hypericin photodamage to HeLa cells, and considered this to be an alternative death pathway when apoptosis is blocked [35]. We currently prefer to consider autophagy as a simultaneous process, especially when PDT results in a loss of Bcl-2 function.

Autophagy may also represent an important part of the overall PDT response, since the process can result in class II presentation of antigens derived from cytosolic proteins [36]. The autophagic response to photodynamic therapy may therefore provide an explanation for the finding that treatment of tumor cells with low-dose PDT can yield anti-tumor vaccines [37].

Conclusions

A commonly reported feature of PDT is the ability of the procedure to lead to cancer control if enough drug and light can be brought to a neoplastic lesion. While only direct tumor cell has been considered here, vascular shutdown and immunologic phenomena are known to play an important role in the overall response to PDT [2]. The ability of PDT to eradicate neoplastic

cells regardless of their drug-resistance pattern, phase of the cell cycle, growth rate, and nutritional requirements is well-known. Based on studies reported here and from other laboratories, it appears that this broad-spectrum pattern of lethality is based, in part, on the ability of PDT to evoke multiple death pathways. Lockshin has observed that a cell will take any available pathway to death [38]. With PDT, there appear to be a multiplicity of pathways that can lead to death without extensive necrosis that could result in many adverse host responses.

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Fig. 1.
Localization patterns of an assortment of photosensitizing agents.

Fig. 2. Localization of Bcl-x_L in L1210 cells (suspension culture) vs. MCF 10A (adhering cells) as determined by western blots of cytosolic fractions vs. whole cell preparations.

Fig. 3.
Inhibition of apoptosis after lysosomal photodamage from NPe6 by the tBid antagonist BI-6C9.

Fig. 4.
Double-membrane structure of autophagosomes formed after photodamage to L1210 cells using the ER sensitizer CPO at an LD₉₀ PDT dose.